EVALUATION OF PREBIOTIC AND PROBIOTIC AS FUNCTIONAL FEED ADDITIVES ON PHYSIOLOGICAL AND IMMUNOLOGICAL PARAMETERS OF NILE TILAPIA, OREOCHROMIS NILOTICUS

by

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TO

MY PARENTS, FOR THEIR CONSTANT LOVE AND SUPPORT,

Elizabeth Ann Saillant and Kenneth Edward Saillant Sr.

&

MY FIANCÉE, MY BEST FRIEND, CONFIDANTE,

AND MOST ARDENT SUPPORTER,

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ABSTRACT

Author: Saillant, Kenneth, E. MS
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Title: Evaluation of Prebiotic and Probiotic as Functional Feed Additives on Physiological and Immunological Parameters of Nile Tilapia, *Oreochromis niloticus*
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Experiments were conducted to evaluate the ability of prebiotic and probiotic supplementation of commercial fish feed to improve the physiological, immunological, and growth responses of stressed Nile tilapia. To investigate these objectives, tilapia were divided in two major groups: control fish (fed regular commercial feed) and stressed fish (induced by dietary cortisol supplemented to regular commercial feed). Stressed fish were further divided into three sub-groups: stressed fish fed regular feed, stressed fish fed probiotic-supplemented feed, and stressed fish fed a mixture of prebiotic and probiotic supplemented feed. Fish were maintained and tested over an eight-week long experimental period. A variety of physiological, immunological, and growth parameters were measured over the course of the experimental period. These parameters include: serum cortisol, blood glucose, plasma protein, packed cell volume, hepato-somatic index (HSI), spleen-somatic index (SSI), lysozyme activity, feed conversion ratio (FCR), specific growth rate (SGR), protein efficiency ratio (PER), length gain, weight gain, length gain, and condition factor (K). The results of this study does not support the use of these specific prebiotic and probiotic as functional feed additives in Nile tilapia at the levels tested in this study. Further research is needed to determine which probiotic species are best suited for use in Nile tilapia and which prebiotic, when used in combination, will allow these probiotics to have maximum effect.

INTRODUCTION

The global population has been steadily increasing for some time, and it is projected to continue to do so for the foreseeable future. As of 2017, the world population had reached 7.55 billion people (United Nations, 2017). The world population was approximately 5.8 billion in 1997, and it increased by approximately 1.75 billion people from 1997 to 2017 (United Nations, 2017). This represents an over 30% increase in world population in just 20 years. While overall population growth rates have declined, world population growth is expected to continue through 2100. Based on current projections, the world population is expected to reach 8.551 billion by 2030, 9.772 billion by 2050, and 11.184 billion by 2100 (United Nations, 2017). However, these projections are based on assumptions formed from current trends that may or may not prove true.

One example of an assumption based on possible future trends is that the world population growth rate will continue to decline. The world population growth rate was 1.24% in 1997, and as of 2017, this growth rate had declined to 1.10% (United Nations, 2017). The assumption that the world growth rate will continue to slowly decline was made to produce these projections. However, this may or may not be the case. Further assumptions about projected decreases in fertility rates and increases to survival rates across all age groups were also made (United Nations, 2017). When all of the various assumptions are taken into account, the upper limit of the 95% confidence interval could see populations reach as high as 8.7 billion by 2030, 10.2 billion by 2050, and 13.2 billion by 2100 (United Nations, 2017). When these projections are looked at as a whole, it is clear that the world population is continuing to grow, and the world will likely see increases in population continue through 2100.

With the rising world population, the state of the world's food supply must be examined. Currently, there are issues of hunger affecting populations worldwide. There are several defined levels associated with hunger, and these levels are defined as the Food Insecurity Experience Scale (FAO et al., 2018). The lowest level of food insecurity on this scale is defined as those individuals who have food security or have only mild food insecurity. These people do not normally have issues with obtaining food, and they typically are able to obtain a regular and varied diet of quality food (FAO et al., 2018).These individuals did not have to worry about skipping any meals throughout the last year.

The next level of food insecurity is defined as those individuals who suffer from moderate food insecurity. Individuals suffering from moderate food insecurity often don't have the resources or finances to obtain a quality and varied diet (FAO et al., 2018). These individuals may have had to compromise on the quality or variety of foods that they consume, or they may have had to reduce the amount of food that they consume (FAO et al., 2018). It is likely that individuals suffering from moderate food insecurity have skipped meals or run out of food at times throughout the last year, and they tend to have an overall uncertainty about where their next meal will come from (FAO et al., 2018).

The highest level of food insecurity is defined as those individuals who suffer from severe food insecurity. Individuals suffering from severe food insecurity are the hardest hit, and they often run out of food and have no idea where their next meal will come from (FAO et al., 2018). These individuals have gone an entire day or more without food throughout the last year, and these occurrences usually occur at more than one point in time throughout the year (FAO et al., 2018). Like all individuals suffering from food insecurity, these individuals are especially affected by malnutrition and hunger throughout their lives.

With these definitions in mind, an overall survey of the current state of food insecurity and hunger in the world shows a truly staggering problem faced by the human race. As of 2017, approximately 10.2% of the world population is living with severe food insecurity, and this number has increased from 8.9% in 2014 (FAO et al., 2018). This represents an increase of 1.3% in just 3 years. At the current rate, there are an estimated 769.4 million people worldwide who are dealing with severe food insecurity (FAO et al., 2018). When population projections are taken into account, the number of people suffering from severe food insecurity in the future could be overwhelming. If current rates are not reduced, the number of individuals dealing with severe food insecurity could reach as high as approximately 887 million by 2030, 1.04 billion by 2050, and 1.35 billion by 2100 (FAO et al., 2018; United Nations, 2017). These numbers are especially concerning when the effects of food insecurity and hunger are examined.

With so many people dealing with food insecurity, the number of people affected by malnutrition worldwide is not surprising. As of 2017, approximately 10.9% of the world population is dealing with malnutrition (FAO et al., 2018). This represents approximately 821 million people, or almost one out of every nine individuals, worldwide (FAO et al., 2018). If the data on undernourished individuals and population projections are looked at together, there could be as many as 948 million people by 2030, 1.11 billion people by 2050, and 1.44 billion people by 2100 dealing with malnutrition (FAO et al., 2018; United Nations, 2017). While these numbers are of great concern, the overall rate of malnutrition does not tell the whole story.

Food insecurity and malnutrition can affect different segments of the population in different ways. The rates of food insecurity and malnutrition can vary widely. The rates of food insecurity can range from as low as 1.4% in Europe and North America to as high as 48.5% in parts of Africa in 2017, and the rates of malnutrition can range from as low as <2.5% in Europe

and North America to as high as 31.4% in parts of Africa in 2017 (FAO et al., 2018). The disparities in food insecurity and malnutrition do not end at differences between various regions of the world. Certain segments of the population can be more greatly affected than others.

About 32.8%, or approximately one out of every three, women of reproductive age suffers from anemia due to food insecurity and malnutrition, and the effects on children are equally concerning (FAO et al., 2018). Over 22%, or approximately 151 million, children under the age of five were affected by stunting in 2017, and 7.5%, or approximately 50 million, children under the age of five were affected by wasting (FAO et al., 2018). Stunting is defined as being at least 2 standard deviations below the median height for their age based on 2006 WHO Child Growth Standards, and wasting is defined as being at least 2 standard deviations below the median weight for their height based on 2006 WHO Child Growth Standards (FAO et al., 2018). Children that suffer from stunting and wasting are at increased risk of morbidity and mortality (FAO et al., 2018). All of these effects could be increased by climate change, and increases as high as 20% over current rates could occur by 2050 if nothing is done (World Food Programme, 2017). From the various effects of food insecurity and malnutrition shown here, it is clear that something must be done to address this continued and potentially growing problem.

Aquaculture presents a possible solution to the problems of food insecurity and malnutrition. Aquaculture can be defined as, “all forms of culture of aquatic animals and plants in fresh, brackish, and marine environments” (Pillay & Kutty, 2005). According to Lucas and Southgate (2011), “The definition of aquaculture is understood to mean the farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants. Farming implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding and protection from predators” (Lucas & Southgate, 2011). Aquaculture has a long and extensive

history throughout the world, and its origins can be traced as far back as ancient Egypt and China (Parker, 2005). Images depicting the raising of what are believed to be tilapia in ponds can be found as early as 2500 B.C. (Pillay & Kutty, 2005).

Aquaculture is also believed to have been practiced as early as 3500 B.C. in China (Parker, 2005). The earliest known work written on the commercial aquaculture production, *Classic of Fish Culture*, can be attributed to a Chinese politician named Fan Lei who lived around 500 B.C. (Parker, Pillay & Kutty, 2005). In this work, Fan Lei attributed his wealth to the growth and production of common carp (*Cyprinus carpio*) in ponds, and the production of various carp species through aquaculture has continued in China to this day (Pillay & Kutty, 2005). In order to fully understand the current state of aquaculture worldwide, the different types of aquaculture currently being utilized must be examined.

The production of aquatic organisms can be classified into two main categories: capture fisheries and aquaculture. Capture fisheries are still the most common production method, and as the name suggests, they represent the traditional practices of capturing wild fish and aquatic organisms from their natural environments (Pillay & Kutty, 2005). As of 2016, capture fisheries worldwide produced approximately 90.9 million tonnes, and it accounted for about 53 percent of total global production (FAO, 2018). Production levels for global fisheries production have remained relatively stable since the 1980's, but overall, global fisheries production has been slowly decreasing worldwide (FAO, 2018).

The decrease in production is related to increased regulation and management of natural capture fisheries (FAO, 2018; Parker, 2005; Pillay & Kutty, 2005). The risks to natural fisheries cannot be overstated. For example, the percentage of marine fisheries that were being sustainably utilized worldwide decreased from 90.0% in 1974 to 66.9% in 2015 (FAO, 2018). From this

data, it is easy to see that natural fisheries and ecosystems are constantly and increasingly threatened. Based on this fact, it is likely that the global trend in reduced capture fisheries production will continue into the future. As restrictions on fisheries continue to increase and overall production from capture fisheries is slowly declining, aquaculture has not suffered the same fate and is thriving.

Aquaculture represent the future of aquatic organism production, and they show the most promise in meeting global food demands. While capture fisheries have been stable or declined slightly since the 1980's, the production from aquaculture has nearly doubled over the same time period (FAO, 2018). As of 2016, aquaculture produced approximately 80.0 million tonnes, and it represented 47% of global production (FAO, 2018). Aquaculture production saw annual growth rates as high as 11.3% during the 1980's, and while the growth rate of aquaculture production has slowed slightly, it still averaged a 5.8% growth rate from 2000 to 2016 (FAO, 2018). If this growth rate continues, global aquaculture production will likely surpass capture fisheries production worldwide in the near future, and this production will occur without continuing to threaten natural populations and ecosystems.

In addition to having several advantages over capture fisheries, aquaculture has several advantages over traditional terrestrial farming techniques and livestock production as well. The growth rate of aquaculture production is higher than any other major food production method, and this continued growth will be crucial to fighting food insecurity and malnutrition worldwide and meeting the world's ever increasing demand for food (FAO, 2018). In addition to having a higher growth rate than other food production industries, the use of aquaculture to meet current and future food demands is a better option than either traditional farming or livestock production methods in terms of environmental impact as well. For example, if inland capture fisheries were

replaced by other food production methods, it would result in an increase of greenhouse emissions, but the increase in emissions would be vastly different depending on what replaced the inland capture fisheries (FAO. 2018). The increases in greenhouse emissions for aquaculture of salmon, livestock production of beef, and traditional farming of rice would be 22.3 million tonnes, 0.82 billion tonnes, and 9.3 billion tonnes, respectively (FAO, 2018). Based on these numbers, increased livestock production would result in greenhouse emissions that are 36 times higher than aquaculture of salmon, and traditional farming of rice would result in greenhouse emissions that are over 417 times higher than aquaculture of salmon (FAO, 2018). With climate change projected to increase food insecurity and malnutrition worldwide by as much as 20% worldwide, aquaculture offers a better option to meet food demands with a lower associated increase in greenhouse gases.

Aquaculture is also a better option in terms of production. One good measure of the production capacity of a species is the feed conversion ratio. The feed conversion ratio, in its simplest form, is the ratio of feed given needed to produce a similar increase in body weight. For example, a feed conversion ratio of 1.0 might refer to giving 1 kilogram of feed for 1 kilogram of body weight gained or refer to giving 1 gram of feed for 1 gram of body weight gained. The scale will depend on the species being examined. Aquatic species have an average feed conversion ratio of about 1.5, but other species have higher feed conversion ratios (Fry et al., 2018). Chickens have an average feed conversion ratio of approximately 2, pigs have an average feed conversion ratio of approximately 4, and beef cattle have an average feed conversion ratio of approximately 8 (Fry et al., 2018). This means that fish require about 25% less feed than chickens, about 63% less feed than pigs, and about 81% less feed than beef cattle in order to

produce the same amount of meat (Fry et al., 2018). Based on this information, it would appear that aquaculture represents the best option to meet current and future food demands.

While aquaculture shows great promise and potential, there are unique problems faced by aquaculture. One of the number one issues faced by aquaculture is the introduction of stress to the fish being cultured. The idea of stress and how organisms respond to it was first proposed by Hans Selye in 1950, and Selye defined stress as, "the sum of all the physiological responses by which an animal tries to maintain or reestablish a normal metabolism in the face of a physical or chemical force" (Selye, 1950; Wedemyer et al., 1990). Barton and Iwama (1991) stated that, "stress represents a response reaction by fish to a stimulus and this response may somehow alter the fish's homeostatic state" (Barton & Iwama, 1991). Based on this definition, stressors can be thought of as any stimulus that causes a disruption to the fish's homeostasis. Stressors can also be defined as a "force or challenge that elicits a compensatory physiological response" (Wedemyer et al., 1990). Once a fish encounters a stressor, it initiates a stress response, or the General Adaptation Syndrome, originally described by Selye (Selye, 1950; Wedemyer et al., 1990).

The stress response consists of three main stages. The first phase of the stress response is the alarm stage (Barton & Iwama, 1991; Selye, 1950; Wedemyer et al., 1990). During the alarm reaction, a stressor is felt by the fish, and it begins the stress response. The first thing to occur is the activation of the hypothalamus-pituitary-interrenal (HPI) axis, and upon activation, the HPI axis begins releasing catecholamines and corticosteroids (Barton & Iwama, 1991; Wedemyer et al., 1990). These catecholamines and corticosteroids can be generally thought of as "stress hormones" (Wedemyer et al., 1990). Once the stress hormones are released, it begins a cascade

of physiological changes in an attempt by the fish to maintain homeostasis (Barton & Iwama, 1991; Wedemyer et al., 1990). At this point, the fish enters the next stage of the stress response.

The second stage of the stress response is the resistance stage (Barton & Iwama, 1991; Wedemyer et al., 1990). During the resistance stage, the physiological changes begun during the alarm stage allow the fish to adapt to and compensate for the stressors encountered, and by doing so, the fish is able to maintain or return to homeostasis (Barton & Iwama, 1991; Wedemyer et al., 1990). The changes required to adapt or compensate for the stressor may have costs, in terms of energy and resources used, that could lead to reduced performance (Wedemyer et al., 1990). If the fish is successfully able to adapt to the stressor or if the stressor is removed, then the stress response would stop at this stage. However, if the fish is unable to adapt or the stressor persists, the fish will enter the third and final stage of the stress response.

The third and final stage of the stress response is the exhaustion stage (Barton & Iwama, 1991; Wedemyer et al., 1990). As the name suggests, the exhaustion stage occurs when the stressor persists for an extended period of time or when the stressor is too severe for the fish to be able to adapt to it (Barton & Iwama, 1991; Wedemyer et al., 1990). Upon entering the exhaustion stage, the physiological changes that have occurred as a result of the fish trying to adapt begin to be maladaptive, and at this point, negative physiological effects begin to occur (Barton & Iwama, 1991; Wedemyer et al., 1990). Once a fish enters the third stage of the stress response, it will no longer be able to effectively maintain homeostasis, and if the stressor persists, negative effects will continue to increase and move the fish further from its homeostatic state.

The consequences and physiological changes that occur during the stress response in fish can also be broken down to the different biological levels affected (Wedemyer et al., 1990).

These changes can be adaptive or maladaptive depending on how severe the stressor is, how long the stressor persists, and what stage of the General Adaptation Syndrome model the fish is in (Wedemyer et al., 1990). The first level of the biological response is called the primary response, and it involves the changes that occur in the endocrine system of the fish (Wedemyer et al., 1990). The primary response begins with the fish detecting the stressor, and this activates the HPI axis (Wedemyer et al., 1990). Once the HPI axis is activated, the hypothalamus will release corticotrophin releasing factor (CRF) which, in turn, activates the pituitary gland of the fish (Wedemyer et al., 1990). Once the pituitary gland receives stimulation from CRF, it begins to release the adrenocorticotrophic hormone (ACTH) into the blood stream, and ACTH travels from the pituitary gland through the circulatory system to the head kidney, or anterior kidney (Wedemyer et al., 1990). Once activated by ACTH, the interrenal cells begin to release cortisol into the blood stream (Wedemyer et al., 1990). In addition to the release of cortisol, epinephrine, also known as adrenaline, is released from the anterior portion of the kidney by the chromaffin tissue, and this release occurs due to sympathetic nervous system stimulation (Wedemyer et al., 1990). If the stressor persists or is too severe, the fish will not be able to adapt, and the primary responses will lead to changes at the next biological level.

The second biological level of the stress response is the secondary response. The secondary responses are changes that begin to occur in the blood chemistry and tissues of fish, and these secondary responses will continue to occur and intensify as long as the fish is under stress (Barton & Iwama, 1991; Wedemyer et al., 1990). The changes that occur as part of the secondary response begin with changes to blood chemistry, and these changes can include things like increases in blood glucose levels and clotting factors (Wedemyer et al., 1990). The changes to the blood chemistry will continue to increase and can result in changes like a reduction of

electrolytes in the blood, reduced numbers of circulating lymphocytes, and general osmoregulation problems (Barton & Iwama, 1991; Wedemyer et al., 1990). Eventually, if the fish is unable to adapt, tissue level changes will begin to occur, and the effects of the stress response will become more pronounced (Wedemyer et al., 1990). Some examples of tissue level changes that can occur are reduction in glycogen stores in the liver and lowered vitamin C levels in the interrenal cells (Wedemyer et al., 1990). If the stressor continues to persist and the fish is still unable to adapt, it will begin to lead to changes at the highest biological level.

The highest biological level of the stress response is the tertiary response. Tertiary responses occur on the organismal and population level, and they are the most dramatic and are often maladaptive (Wedemyer et al., 1990). These tertiary response likely occur once the fish reaches the exhaustion stage of the General Adaptation Syndrome and is no longer capable of adapting to the stressor, but they can also occur during the resistance stage (Wedemyer et al., 1990). There are many maladaptive changes that begin to occur once a fish reaches the tertiary response level. At this level, overall reductions in growth begin to occur, and these changes can lead to reduced fitness and reduced reproductive success for the fish (Wedemyer et al., 1990). In addition to the reductions in growth and reproduction, the fish's immune system also begins to suffer, and the overall immune response of the fish is reduced (Wedemyer et al., 1990). Taken in combination, the reductions to growth, fitness, and immune response can lead to increased disease susceptibility (Wedemyer et al., 1990). Ultimately, if nothing is done, the fish will eventually become diseased and die.

Although stress in aquaculture settings can be reduced through proper husbandry methods, it can never be completely eliminated, and eventually, the stress will lead to increased disease susceptibility for the fish being raised. In order to combat disease in aquaculture, current

treatments involve the use of chemicals and antibiotics, and while use of these treatments has been reduced and restricted in some areas, they continue to be commonly used in many parts of the world (Assefa & Abunna, 2018; Rodgers & Furones, 2009). The use of various chemicals and antibiotics represents a risk to consumers and to the environment. One major concern about the use of chemical and antibiotic treatments is the ability for bioaccumulation of these chemicals, antibiotics, and their residues to occur in fish (Rodgers & Furones, 2009). Bioaccumulation is defined as, "The net accumulation of a contaminant in (and in some occasional instances on) an organism from all sources including water, air, and solid phases of the environment. Solid phases include food sources" (Newman, 2015). The risks to consumers and the environment presented by bioaccumulation are not a trivial thing. However, there is another major concern associated with the use of antibiotic treatments in aquaculture.

The biggest concern about antibiotic use in aquaculture is the possible development of antibiotic resistant bacterial strains (Rico et al., 2014; Santos & Ramos, 2018). Antibiotic resistance occurs when bacteria are exposed to antibiotics at sub-therapeutic levels for extended periods of time, and the bacteria are able to evolve antibiotic resistance genes or plasmids (Santos & Ramos, 2018). These antibiotic resistant bacteria are then able to horizontally transfer there resistant genes and plasmids to other bacteria (Santos & Ramos, 2018). Santos and Ramos (2018) stated that, "It is now widely recognized that the passage of antimicrobial resistance genes and resistant bacteria from aquatic to terrestrial animal husbandry and to the human environment and vice versa can have detrimental effects on both human and animal health and on aquatic ecosystems" (Santos & Ramos, 2018). Aquaculture has been found to be a key cause of antibiotic contamination in areas where antibiotics are used (Rico et al., 2014). Based on this

information, a new and better method for dealing with stress and preventing disease in aquaculture is required.

One possible solution to the problems of bioaccumulation and antibiotic resistance that aquaculture faces is the use of nutraceuticals. The term nutraceutical is a portmanteau of nutrition and pharmaceutical, and nutraceuticals can be defined as, “substances that have physiological benefits or provide protection against chronic diseases” (Hamid et al., 2014). There are many different types of nutraceuticals such as phytochemicals and dietary supplements, and one important category is functional food (Hamid et al., 2014). Functional foods can be defined as, “any food or ingredient that has a positive impact on an individual’s health, physical performance, or state of mind” (Hardy, 2000). There are many different types of functional foods, and prebiotics and probiotics are two emerging types of feed additives that have shown promise for use in aquaculture (Gatlin & Peredo, 2012). With this in mind, this research study was developed.

Prebiotics are an important functional feed additive that has been gaining interest in aquaculture and beyond. Prebiotics are defined as a, “non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health” (Gibson et al., 2004). The prebiotics are not digestible by the host, but they are capable of being broken down by the gut microbiota (Gibson et al., 2004). In order for the prebiotic to be effective and to be classified as a prebiotic, it must be able to resist being broken down or absorbed by the host, it must be able to be broken down through fermentation by the gut microbiota, and it must be able to increase the growth and activity of beneficial bacteria in the gut that are linked to overall host health (Gibson et al., 2004).

Probiotics are another important category of functional feed additives that are growing in interest for use in aquaculture. Probiotics were originally defined by Fuller as, “A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance” (Fuller, 1989). A more modern definition is, “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO & WHO, 2006). Probiotics work by supporting and complementing the natural gut microbiota of the host, and they can accomplish this through a variety of means (Kechagia et al., 2013). While the beneficial effects of probiotic supplementation have been shown in previous research, the exact method that probiotics are able to produce these beneficial effects is still poorly understood (Kechagia et al., 2013). Probiotics can be administered on their own, but they are often combined with prebiotic supplementation. The joint use of prebiotics and probiotics together has previously been referred to as “conbiotics” or “symbiotics” by some researchers, but these terms have not been properly defined and are not universally used (Kechagia et al., 2013). With the interest in prebiotic and probiotic supplementation in aquaculture growing, this study was devised to examine their potential.

For this study, Nile tilapia (*Oreochromis niloticus*) were chosen as the species of interest to evaluate the use of prebiotics and probiotics as functional feed additives. In order to understand the decision to use Nile tilapia in this study, some background information on the species, and its importance in aquaculture, are required. Aquaculture of Nile tilapia can be traced as far back as 2500 B.C. in Egypt (Pillay & Kutty, 2005). Nile tilapia production began to increase worldwide in the 1960’s, and the production of Nile tilapia continued to spread through the 1980’s (Rakocy, 2005). Eventually, Nile tilapia were introduced for aquaculture production in China in 1978, and since that time, China has consistently accounted for the vast majority of

Nile tilapia production worldwide (Rakocy, 2005). In fact, Chinese production has accounted for more than half of the total global Nile tilapia production since 1992, and by 2003, Chinese producers accounted for almost 806,000 tonnes of Nile tilapia produced (Rakocy, 2005). One of the main reasons that Nile tilapia production has been so successful is due to the development in the 1970's of sex-reversal techniques that allow for all-male populations of Nile tilapia to be produced (Rakocy, 2005). Advances in nutrition and cultivation methods have continued to drive the expansion of Nile tilapia production worldwide since the 1980's (Rakocy, 2005). The continued growth of Nile tilapia production has led to it becoming one of the most widely and highly produced species in aquaculture. Due to its importance as an aquaculture species, Nile tilapia were chosen for this experiment.

For this study, the prebiotic inulin was chosen. Inulin is a type of fructose polymer, and they are similar to fructo-oligosaccharides (Hoseinifar et al., 2015). The main difference between inulin compounds and fructo-oligosaccharides is the degree of polymerization that is seen in the compounds (Hoseinifar et al., 2015). Compounds are considered to be inulin if the degree of polymerization is greater than 10, and compounds are considered to be fructo-oligosaccharides if the degree of polymerization is less than or equal to 10 (Niness, 1999). Inulin is most commonly derived from chicory root, and chicory root contains between 15% and 20% inulin (Niness, 1999). Inulin has been shown in previous research in fish to have a beneficial effect on the immune response when it is supplemented on its own (Bakke-McKellep et al., 2007; Cerezuela et al., 2012; Cerezuela et al., 2013a; Cerezuela et al., 2013b; Ibrahem et al., 2010; Mourino et al., 2012; Reyes-Becerril et al., 2014). The results of the previous research studies can be seen in Table 1. As a prebiotic, inulin should also be able to assist in the colonization of the probiotic

being tested and increase the beneficial effects associated with probiotic supplementation. For these reasons, inulin was chosen as the prebiotic of interest for this study.

For this study, a probiotic powder that contained a combination of probiotic species was also chosen. In total, the probiotic powder contained four species of lactic acid bacteria. The probiotic powder used contained the following species: *Enterococcus faecium*, *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Lactobacillus plantarum*. The probiotic powder was chosen because each of the probiotic species have been shown to cause positive effects in Nile tilapia and other fish species when supplemented individually (Al-Dohail et al., 2009; Aly et al., 2008; Bogut et al., 2000; Goncalves et al., 2011; Hamdan et al., 2016; Hernandez et al., 2010; Wang et al., 2008). The results of the previous research studies can be seen in Table 2. This study was focused on seeing if the supplementation of a combination of probiotic species could produce improved results over supplementation of the individual probiotic species on their own. For these reasons, this probiotic powder was chosen.

There were three main research objectives for the present study. The first research objective was to evaluate the ability of prebiotic and probiotic supplementation to improve the physiological responses of stressed Nile tilapia. The second research objective was to evaluate the ability of prebiotic and probiotic supplementation to improve the immune response of stressed Nile tilapia. The third research objective was to determine if the combination of prebiotic and probiotic supplementation was more effective than probiotic supplementation on its own in improving the physiological and immune responses of Nile tilapia. There were two hypotheses for this study as well. The first hypothesis was that both the probiotic supplemented group and the prebiotic and probiotic supplemented group would have improved physiological and immune responses in stressed Nile tilapia compared to the stressed and non-supplemented

Nile tilapia. The second hypothesis was that the combination of prebiotic and probiotic supplementation would be more effective at improving the physiological and immune responses of stressed Nile tilapia than probiotic supplementation on its own.

Table 1. Previous research on inulin. This table shows examples of previous research on the prebiotic, inulin, which was used in this experiment, and the results found from testing in Nile tilapia and other fish species.

Dosage (g/kg)	**Length of Supplementation**	**Fish Species**	**Results**	**References**
75	3 weeks	Atlantic salmon	Increased leukocyte infiltration	Bakke-McKellup et al., 2007
10	4 weeks	Gilthead sea bream	Increased intraepithelial leucocytes	Cerezuela et al., 2013a
10	4 weeks	Gilthead sea bream	Increased IL-8 production	Cerezuela et al., 2013b
10	2 weeks	Gilthead sea bream	Increased complement activity Increased IgM levels	Cerezuela et al., 2012
5	8 weeks	Nile tilapia	Increase lysozyme activity	Ibrahem et al., 2010
5	15 days	Hybrid surubim	Increased total immunoglobulin	Mourina et al., 2012
10	8 weeks	Leopard grouper	Increased lysozyme activity Increased IgM levels Increased myeloperoxidase activity	Reyes-Becerril et al., 2014

Table 2. Previous research on probiotic species. This table shows examples of previous research on the probiotic species used in this experiment, and the results found from testing in Nile tilapia and other fish species.

Probiotic Species	**Fish Species**	**Effects of Treatment**	**References**
Enterococcus faecium	Nile tilapia	Increased weight gain	Wang et al., 2008
	Sheatfish	Reduced harmful bacteria	Bogut et al., 2000
Lactobacillus acidophilus	Nile tilapia	Increased survival rate Increased growth	Aly et al., 2008
	African catfish	Increased growth	Al-Dohail et al., 2009
Lactobacillus casei / *Lactobacillus rhamnosus*	Nile tilapia	Increased growth performance	Goncalves et al., 2011
	Porthole livebearer	Increased body weight Increased specific growth rate	Hernandez et al., 2010
Lactobacillus plantarum	Nile tilapia	Increased growth rates Increased feed efficiency	Hamdan et al., 2016

MATERIALS AND METHODS

Acquisition of Materials

Fish Acquisition

200 Nile tilapia fingerlings were purchased from Troyer Fish Farms located in Geneva, Indiana. These fingerlings were distributed evenly among two identical systems containing two identical tanks per system. Each tank holds 200 gallons, and 50 fingerlings were placed in each tank. The fingerlings obtained from Troyer Fish Farms are hybrid all male Nile tilapia originally produced by AmeriCulture, Inc. located in Animas, New Mexico. The fingerlings had an average length of approximately 7 cm and an average weight of approximately 25 g when they were received. The fish were starved for two days upon receiving them in order to allow the handling and transport stress to dissipate, and they were maintained in the systems for a two week acclimation phase to allow them to fully adjust to the new environment before experimental conditions were applied. All of the fish were taken care of following the approved animal care protocol.

Prebiotic and Probiotic Acquisition

Prebiotic

The inulin prebiotic powder used for this research was obtained from Micro Ingredients located in Diamond Bar, California. A 1 kilogram package of powder was obtained for this experiment, and the powder is USDA Certified Organic. It is free of any added sugar, soy, dairy, yeast, gluten, corn, or other additives. The powder was stored in its original packaging in a cool and dry cabinet and was used before the expiration date.

Probiotic

The probiotic powder used in this experiment was obtained from the Tractor Supply Co. store located in Fort Wayne, Indiana. The probiotic powder used for this research is called Probios® Dispersible Powder manufactured by Vets Plus, Inc. located in Menomonie, Wisconsin. This product is created under license from Chr. Hansen, Inc. located in Hoersholm, Denmark. This product was purchased because it was lab certified to contain the four probiotic strains of interest for this research: *Enterococcus faecium*, *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Lactobacillus plantarum*. The powder was also certified to contain no less than 10 million colony forming units (CFUs) per gram of powder by the manufacturer. The powder was stored in its original packaging in a cool and dry cabinet and was used before the expiration date.

Maintenance of Fish

System Setup

Two identical university owned systems housed in room 142 in the Life Sciences Resource Center of Purdue University Fort Wayne in Fort Wayne, Indiana were used for this research. Each system consists of two identical round 200 gallon tanks that drain through a center stand pipe into a 400 gallon rectangular sump tank. (Figure 1) The sump tank contained approximately 1.5 cu. ft. of 1/2 inch lava rock obtained from Lowe's Home Improvement located in Fort Wayne, Indiana. The lava rock was used to maximize space for denitrifying bacteria to colonize in order to ensure complete and rapid denitrification of the water. The water was pumped from the sump tank and filtered using a sand filter to remove any particulate waste and to allow for further denitrification of the water.

After passing through the sand filter, water was pumped back into the two tanks housing the fish, and the water was vented into the tanks in such a way as to create a slow, circular flow around the tank. This flow helped to ensure that the water in the tanks housing the fish was thoroughly mixed and that water quality was even throughout the entire tank. Each tank was individually aerated by an aeration bar powered by a central air pump. The aeration, along with the other design features of the system, ensured that adequate oxygen levels were maintained for the fish and for proper denitrification of nitrogenous wastes in the system.

The water used in the system was obtained from a municipal water source. First, it was dechlorinated by passing through separate filter chambers filled with activated carbon, and second, by using Prime® dechlorinator produced by Seachem Laboratories located in Madison, Georgia at the recommended dosage of 5 mL per 50 gallons. These steps were taken to ensure no chlorine or chloramines entered the system circulation.

Room and Water Quality

Room Temperature and Humidity

The room where the systems were housed ranged between 25°C and 30°C throughout the acclimation phase and sampling period. The humidity in the room ranged from 24% to 59% throughout the acclimation phase and sampling period. The room temperature was maintained by the central ventilation system, and the humidity was not separately controlled. The room temperature and humidity were measured using a combination digital thermometer and hygrometer placed in the room at all times. The combination thermometer and hygrometer also measured the highest and lowest values for temperature and humidity, and these values were reset after being recorded each day. This ensured that the temperature and humidity were within

appropriate levels at all times even when the systems were not being physically monitored or tested (Table 3).

Photoperiod

The fish were exposed to a natural photoperiod of 12 hours of light and 12 hours of darkness. The photoperiod was set using a timer that turned the lights on at 8 a.m. each day and shut the lights off at 8 p.m. each evening. This photoperiod has been shown to not have any negative effects for tilapia culture and to be ideal for tilapia reproduction (El-Sayed, 2006).

Source Water Quality

The water used in the systems was obtained from a municipal water source supplied by Fort Wayne Water Utility and filtered at the Three Rivers Filtration Plant. The pH, ammonia, nitrite, and nitrate levels were tested from the source using an API Freshwater Master Test Kit produced by Mars Fishcare Inc. The pH was found to be 8.4. The ammonia level was found to be 0.5 parts per million. Nitrite was found to be at 0 parts per million. Nitrate was found to be at 10 parts per million (Table 4).

Water Temperature

The water temperature was maintained by heating and cooling the room that the systems were housed in. By maintaining the ambient room temperature at the correct level, the water temperature could be consistently regulated without the need for additional heaters. This removed the possibility of individual heaters failing and disrupting the experiment, and it ensured that all four tanks were kept at the same temperature throughout the acclimation phase and sampling period. The temperature was checked daily to ensure that it matched the room temperature. Water temperature was maintained between 25°C and 30°C, at the ideal

temperature for Nile tilapia, throughout the acclimation phase and sampling period (El-Sayed, 2006). Water temperature was measured daily using a standard thermometer and probe throughout the acclimation phase and sampling period (Table 5).

Dissolved Oxygen

Nile tilapia are very tolerant of a wide range of dissolved oxygen levels. Previous research has shown that tilapia can tolerate dissolved oxygen levels as low as 0.1 mg/L to 0.5 mg/L, and they can survive in water with dissolved oxygen levels as low as 0 mg/L for short periods of time if they are able to reach the surface of the water (El-Sayed, 2006). In addition to being able to tolerate very low dissolved oxygen levels, tilapia have also been shown to tolerate oxygen saturation levels as high as 400% (El-Sayed, 2006). For this research, the dissolved oxygen levels were measured daily during the acclimation period and weekly during the sampling period. The levels were measured both as percent saturation and as parts per million, or mg/L. A dissolved oxygen level of 79.9% to 91.5% saturation or 6.53 mg/L to 7.80 mg/L was maintained throughout the acclimation phase and sampling period. The dissolved oxygen was measured using a standard dissolved oxygen meter and probe produced by YSI Inc., and at a minimum, it was tested daily during the acclimation phase and weekly during the sampling period (Table 5).

pH

Nile tilapia are tolerant of a wide range of pH, and an ideal pH can range from 5.5 to 9.0 for Nile tilapia (Reboucas et al., 2016). The key to providing the correct pH for tilapia culture is to ensure that the pH does not change too much or too rapidly over a short period of time (El-Sayed, 2006). During the acclimation phase and sampling period, the pH was maintained

between 6.0 and 7.8. The pH was adjusted in the systems using sodium bicarbonate in the form of baking soda purchased from Walmart in Fort Wayne, Indiana. The pH was maintained and adjusted to ensure that no large or rapid changes in pH occurred throughout the acclimation phase and sampling period. pH was measured using an API Freshwater Master Test Kit produced by Mars Fishcare Inc., and at a minimum, it was tested daily during the acclimation phase and weekly during the sampling period (Table 5).

Salinity

Tilapia species are overall very halotolerant. Nile tilapia can tolerate a range of salinity from 0% up to 29%, and they reproduce in water with salinity ranges from 13.5% and 29% (El-Sayed, 2006). The salinity in both systems was maintained between 3.1 parts per thousand and 3.6 parts per thousand during the acclimation phase to help the fish adjust and to help minimize the risk of disease development due to handling and transportation stress. Then, it was allowed to slowly dissipate through water changes. This method allowed the salinity to slowly be reduced, and salinity slowly dropped from about 2.0 parts per thousand to 0.1 parts per thousand during the sampling period. At a minimum, the salinity was measured daily during the acclimation phase and weekly during the sampling period using a standard salinity meter and probe produced by YSI Inc. (Table 5).

Ammonia, Nitrite, and Nitrate

The ammonia, nitrite, and nitrate levels were tested for all systems throughout the acclimation phase and sampling period. Since ammonia and nitrite are extremely toxic to fish, there levels were kept at a minimum throughout the acclimation phase and sampling period. Nitrate is only toxic to fish at extremely high levels, but it was also maintained at relatively low

levels through frequent water changes. At a minimum, the ammonia, nitrite, and nitrate levels were tested daily during the acclimation phase and weekly during the sampling period. Testing was performed using an API Freshwater Master Test Kit produced by Mars Fishcare Inc.

Ammonia levels were maintained at 1 part per million or less throughout the entire acclimation phase and sampling period and were typically at 0 parts per million after the first week of the acclimation phase. Nitrite levels were maintained at 0.5 parts per million or less throughout the entire acclimation phase and sampling period and were consistently measured at 0 parts per million after the first week of the acclimation phase. Nitrate levels were maintained at 160 parts per million or less throughout the entire acclimation phase and sampling period, and normally ranged between 40 parts per million and 80 parts per million after the first week of the acclimation phase (Table 5).

Experimental Design

Experimental Groups

There were 4 experimental groups for this experiment based on the treatment applied to each group: Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic. Each tank was assigned to one experimental group. The first system housed the tanks for the Control Control and Stress Control experimental groups, and the second system housed the tanks for the Stress Probiotic and Stress Prebiotic and Probiotic experimental groups. The experimental groups were maintained throughout the sampling period, and fish were fed at 1.5% of body weight twice daily throughout the sampling period. Any excess food that was not immediately eaten by the fish was removed from each tank after feeding. Fish were starved for 24 hours before sampling periods (Table 6, Figure 2).

Feed Preparation

The fish were fed with commercial feed, Purina® Aquamax® Fingerling Starter 300, that was purchased from Troyer Fish Farms in Geneva, Indiana. The feed was 100% nutritionally complete, and the nutritional information can be seen in Table 7. This commercial feed was used to create the different treatments applied to the experimental groups. The Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic groups were all fed commercial feed that was supplemented with cortisol in the form of hydrocortisone powder at 100 mg/kg of feed (Barton et al., 1987). The hydrocortisone powder produced by ACROS Organics was obtained from Thermo Fisher Scientific located in Waltham, Massachusetts.

Hydrocortisone supplementation was accomplished by dissolving 100 mg of hydrocortisone in 500 mL of pure ethanol and slightly heating the mixture, while stirring, until fully dissolved. Once the hydrocortisone was fully dissolved, the ethanol mixture was placed in a spray bottle and evenly applied to 1 kilogram of feed. The feed was allowed to dry at room temperature overnight in order to allow for total evaporation of the ethanol. Cortisol supplementation was used to ensure that a stress condition was present in the fish during the sampling period, and this method for maintaining a stress condition has been previously evaluated (Barton et al., 1987).

The Stress Probiotic group's feed was additionally supplemented with 50 g/kg of probiotic powder (Cruz et al., 2012; Hai, 2015; Lin et al., 2004). 50 g of probiotic powder was combined with 950 g of commercial feed in a lidded container and mixed thoroughly until homogenized. Then, 50 mL of fish oil was added to the container to encapsulate the probiotic powder on the commercial feed, and the container was mixed thoroughly until all pellets were evenly coated (Abidi, 2003; Antony & Philip, 2008; Michael et al., 2014). The feed was then

spread out in trays and allowed to dry overnight at room temperature before being placed in containers and refrigerated until used (Table 8).

The Stress Prebiotic and Probiotic group's feed was created in the same way as the Stress Probiotic group's feed with the addition of inulin prebiotic powder. For the Stress Prebiotic and Probiotic group's feed, 50 g of probiotic powder and 5 g of prebiotic powder was combined with 945 g of commercial feed in a lidded container and mixed thoroughly until homogenized. Then, 50 mL of fish oil was added to the container to encapsulate the prebiotic and probiotic powder on the commercial feed, and the container was mixed thoroughly until all pellets were evenly coated. The feed was then spread out on trays and allowed to dry overnight at room temperature before being placed in containers and refrigerated until used (Table 8).

The Control Control group's feed was a sham-supplemented commercial feed. Both the ethanol and fish oil used to prepare the feed for the other treatment groups were applied in order to ensure that they did not affect the results of this experiment. The commercial feed was sprayed with ethanol and allowed to dry overnight at room temperature. 50 mL of fish oil was added to the feed and mixed thoroughly until the pellets were evenly coated. The feed was allowed to dry overnight on trays at room temperature, and then, it was placed in containers and refrigerated until used (Table 8).

Similarly, the Stress Control group's feed was sham-supplemented using the fish oil. After drying overnight from the cortisol supplementation, the Stress Control group's feed was combined with 50 mL of fish oil and mixed thoroughly until all pellets were evenly coated. The feed was then allowed to dry overnight at room temperature before being placed in containers and refrigerated until used (Table 8).

The pure ethanol used for feed preparation was obtained from the Department of Biology stock supply at Purdue University Fort Wayne. The fish oil used for feed preparation was obtained from Jedwards International Inc. located in Braintree, Massachusetts, and it is a refined, deodorized, purified, and molecularly distilled Omega-3 fish oil (18% EPA 12% DHA) derived from anchovy (*Engraulis ringens*) and sold as a food additive.

Sampling Periods

There were five separate sampling days that occurred throughout the sampling period. Each sampling consisted of 6 fish per treatment group, and sampling occurred on Day 0, Day 3, Week 2, Week 4, and Week 8. For the sampling on Day 0, only six fish were sampled from the Control Control experimental group to serve as the baseline values for this experiment. The fish were euthanized using tricaine methanesulfonate (Tricaine-S, MS-222) produced by Syndel USA located in Ferndale, Washington. 250 mg/L of MS-222 was mixed with warm water because warm water has been shown to increase the potency of MS-222 in fish (AVMA, 2013). For the Day 0 sampling, a lower dose of 200 mg/L was used, but this dosage was increased to reduce the time for deep anesthesia and cessation of opercular movement to occur. At a dosage of 250 mg/L, deep anesthesia and cessation of opercular movement occurs in less than 2 minutes, and fish are maintained in the solution for 10 minutes after cessation of opercular movement to ensure death has occurred (AVMA, 2013).

The physiological and immunological status of the fish was determined by measuring the following parameters on all sampling days except for Day 3: serum cortisol, blood glucose, plasma protein, packed cell volume, hepato-somatic index, spleen-somatic index, lysozyme activity, feed conversion ratio, specific growth rate, protein efficiency ratio, length gain, weight gain, and condition factor. On Day 3, the fish were not euthanized, and therefore, the hepato-

somatic index and spleen-somatic index could not be tested for Day 3. All other parameters were measured on Day 3 (Table 9).

Phlebotomy

Blood collection was accomplished using heparinized 1 mL BD syringes with 25 G needles produced by Becton, Dickinson and Compay located in Franklin Lakes, New Jersey. The needle was inserted posterior to the caudal fin on the ventral side until contact is made with the vertebral column. Then, the needle is retracted slightly, and the plunger is withdrawn to create suction. If done correctly, the syringe should fill with blood rather quickly. This method has been previously described for the collection of blood samples in fish (Perrott et al., 1991).

Experimental Parameters

Stress Response Parameters

Serum Cortisol

A portion of the blood collected from each fish was used to test the serum cortisol levels. The blood samples were placed in sterile Eppendorf tubes and centrifuged for 10 minutes at 5000 RPMs in order to separate the serum. After centrifuging, the serum was collected and placed in a sterile Eppendorf tube. The samples were stored at -80°C until the conclusion of the 8 week sampling period. Then, the samples were tested using a Cortisol ELISA Kit produced by Cayman Chemical using 96 well plates. The plates were read using the 96 well plate reader and analyzed using Cayman Chemical's recommended software to find the results of the serum cortisol assay.

Blood Glucose

Blood samples collected from each fish were used to determine the blood glucose level in circulation. Blood glucose levels are a common measure of stress in fish. Changes in blood glucose levels have been shown to occur due to a variety of stressors, and the resulting changes can lead to decreased metabolism and decreased immunity. A FreeStyle Freedom glucometer and matching test strips from Abbott Diabetes Care Ltd. located in Witney, United Kingdom were used to determine the blood glucose levels. A small drop of blood was placed on the test strip, and then, the blood glucose level was recorded. This method has been validated by previous research for analyzing blood glucose levels in fish (Wedemyer et al., 1990; Gensic et al., 2004).

Plasma Protein

In order to measure plasma protein, a small amount of plasma obtained from the hematocrit tubes used to measure packed cell volume was used. A standard refractometer produced by VEE GEE Scientific located in Kirkland, Washington was used to measure the plasma protein levels of each fish. The refractometer was calibrated using distilled water. After calibration, a drop or two of each plasma sample was applied to the refractometer, and the plasma protein level was measured and recorded to the nearest g/100mL. This method for measuring plasma protein has been validated by previous research (Gensic et al., 2004).

Packed Cell Volume / Hematocrit

A small portion of the blood collected from each fish was used to determine the packed cell volume, or hematocrit. Blood was inserted into Fisherbrand® Blue-Tip Plain Micro-Hematocrit Capillary Tubes produced by Fisher Scientific located in Pittsburgh, Pennsylvania.

After filling each capillary tube 2/3 to 3/4 of the way full, the opposite end was sealed using Surgipath Critocaps produced by Leica Biosystems Richmond Inc. located in Richmond, Illinois. The capped hematocrit tubes were then placed in a micro-hematocrit centrifuge and centrifuged at 10,000 RPMs for 5 minutes. By centrifuging, the plasma was separated from the cellular portion of the blood sample. The packed cell volume was then measured using a Micro-Hematocrit Capillary Tube Reader from Leica Biosystems. This method for measuring packed cell volume in fish has been validated by previous research (Siwicki et al., 1994; Wedemyer et al., 1990).

Hepato-Somatic Index

After measuring for weight and length and obtaining a blood sample, each fish was carefully dissected in order to obtain the liver. The livers of healthy Nile tilapia are typically a light, pinkish-peach color, and it is located in the most anterior portion of the coelomic cavity. After removing the liver, the organ was immediately weighed using an AG204 DeltaRange analytical scale from Mettler-Toledo, LLC located in Columbus, Ohio. The weight of the liver was recorded out to the ten-thousandths of a gram. The weight of the liver was used along with the overall somatic weight from each corresponding fish in order to determine the hepato-somatic index (HSI). The hepato-somatic index is an important measure of the condition of the fish because the liver is important in production of bile, hormone metabolism, and general detoxification of the blood. The hepato-somatic index can be a good indicator of stress, and its use has been validated by previous research (Barton et al., 2002; Goede & Barton, 1990). The hepato-somatic index was calculated using the following equation:

$$HSI = \frac{liver\ weight\ (g)}{body\ weight\ (g)} * 100$$

Spleen-Somatic Index

During dissection of the fish, the spleen was also carefully removed. The spleen of healthy Nile tilapia are typically a very dark red color that appears almost black, and it is located in the middle of the coelomic cavity among the intestines. After removal of the spleen from each fish, the spleen was immediately weighed using an AG204 DeltaRange analytical scale from Mettler-Toledo, LLC located in Columbus, Ohio. The weight of the spleen was recorded out to the ten-thousandths of a gram. The weight of the spleen was used along with the overall somatic weight from each corresponding fish in order to determine the spleen-somatic index (SSI). The spleen serves an important role in the immune response and blood filtration, and it is the site of blood storage and blood-cell production (Garcia-Aibiado et al., 2004; Goede & Barton, 1990; Ruane et al., 2000). Therefore, the spleen-somatic index can serve as an important indicator of overall fish health, and it has been shown to contract under acute stress (Pearon & Stevens, 1991). The spleen-somatic index was calculated using the following equation:

$$SSI = \frac{spleen\ weight\ (g)}{body\ weight\ (g)} * 100$$

Lysozyme Activity

A portion of the blood collected from each fish was used to test the lysozyme activity. The blood samples were placed in sterile Eppendorf tubes and centrifuged for 10 minutes at 5000 RPMs in order to separate the serum. After centrifuging, the serum was collected and placed in a sterile Eppendorf tube. A suspension of *Micrococcus lysodeikticus* at a concentration of 0.2 mg/mL in 0.05M (pH = 6.2) sodium phosphate buffer solution was created for the lysozyme assay. This suspension was vortexed, and then, 1 mL of the suspension was placed into a sterile Eppendorf tube. Next, 50 µL of serum was added to the Eppendorf tube and vortexed to

thoroughly mix. This solution was then placed in a cuvette, and the absorbance at 530 nm was measured using Spectronic 601 spectrophotometer from Milton Roy Company. The spectrophotometer was calibrated using uninoculated sodium phosphate buffer. The absorbance was measured at 1 minute after mixing and at 5 minutes after mixing. These measurements were used to calculate the lysozyme activity (LA) using the following equation:

$$LA = \frac{Absorbance\ Final - Absorbance\ Intial}{Total\ Time\ Elapsed\ (minutes)}$$

A unit of lysozyme activity is equal to the amount of the sample that causes a decrease in absorbance of 0.001/minute. This method for evaluating lysozyme activity in fish has been validated by previous research (Bonga, 1997; Ellis, 1990; Rawling et al., 2009).

Whole Body Response Parameters

Feed Conversion Ratio

The feed conversion ratio (FCR) was calculated in order to determine how well the fish were utilizing the feed given in order to put on body weight. This metric is a good indicator of overall fish health and metabolism, and it has been shown to be reduced under stress conditions (El-Sayed, 2006). The following equation was used to calculate the feed conversion ratio (Adeoye et al., 2016):

$$FCR = \frac{Feed\ Given\ (g)\ Per\ Fish}{Average\ Weight_{final}(g) - Average\ Weight_{initial}(g)}$$

Specific Growth Rate

The specific growth rate (SGR) was calculated in order to determine the percent growth per day of the fish. Like feed conversion ratio, the specific growth rate is a very good indicator of overall fish health and metabolism, and it has also been shown to be reduced under stress

conditions (El-Sayed, 2006). The following equation was used to calculate the specific growth rate (Adeoye et al., 2016):

$$SGR = \frac{\ln(Average\ Weight_{final}\ (g)) - \ln(Average\ Weight_{initial}(g))}{Time\ (Days)} * 100$$

Protein Efficiency Ratio

The protein efficiency ratio (PER), sometimes also called protein energy retention, was calculated for each fish to determine how well each fish was able to convert the protein they were being fed into energy and how much each fish was able to retain that energy (Weirich et al., 2001). This metric takes into account the weight gain of the fish compared to the amount of protein each fish was fed. The protein efficiency ratio for each fish was calculated using the following equation (Adeoye et al., 2016; Weirich et al., 2010):

$$PER = \frac{Average\ Weight_{final}\ (g) - Average\ Weight_{initial}\ (g)}{Feed\ Protein\ Intake\ (g)}$$

Length Gain

Similar to the overall weight gain, the overall length gain of the fish was measured to determine how much length the fish were able to gain over the course of the experiment. The length of each fish was measured using a ruler, and measurements were made of the maximum total length to the nearest tenth of a centimeter. The maximum total length is measured from the tip of the mouth to the tip of the tail when the tail fin is pinched closed. The same equation used for measuring overall weight gain was adapted to determine the overall length gain (Khan et al., 2018). The overall length gain was determined using the following equation:

$$\text{Length Gain} = \text{Average Length}_{\text{final}}\ (\text{cm}) - \text{Average Length}_{\text{initial}}\ (\text{cm})$$

Weight Gain

The overall weight gain of the fish was measured to determine how well the fish were able gain weight over the course of the experiment. The weight of each fish was measured on each sampling day using a Scout Pro digital scale from Ohaus Corporation. The weight was recorded to the tenth of a gram. The overall weight gain is one of the most important factors for producers and is a large part of determining the market value of the fish. The weight gain was calculated using the following equation (Khan et al., 2018):

$$\text{Weight Gain} = \text{Average Weight}_{\text{final}}\ (g) - \text{Average Weight}_{\text{intial}}\ (g)$$

Condition Factor

The condition factor (K) for each fish was determined. The condition factor takes into account both the length and weight of the fish in one metric, and it is used as an estimate of the overall growth and condition of the fish. It served as an indicator of overall fish health (Ibrahim et al., 2000). The length and weight measurements taken of each fish were used to calculate the condition factor using the following equation (Adeoye et al., 2016):

$$K = \frac{Weight\ (g) * 100}{(Length\ (cm))^3}$$

Graphical and Statistical Data Analysis

Data obtained in this experiment was analyzed using SigmaPlot 14 Scientific Graphing & Statistical Analysis Software from Systat Software, Inc. located in San Jose, California. A one way analysis of variance (ANOVA) test was used to determine statistical significance ($P<0.05$). All data that was found to be statistically significant ($P<0.05$) was further analyzed by Holm-Sidak method for comparison of multiple means. All graphs were prepared using SigmaPlot 14, and additional labels were added using Microsoft PowerPoint. All graphs and written data that

were statistically analyzed within this document are presented as the means ± standard error of the means (SEM). In cases where statistical analysis was not possible, data values are presented as the mean.

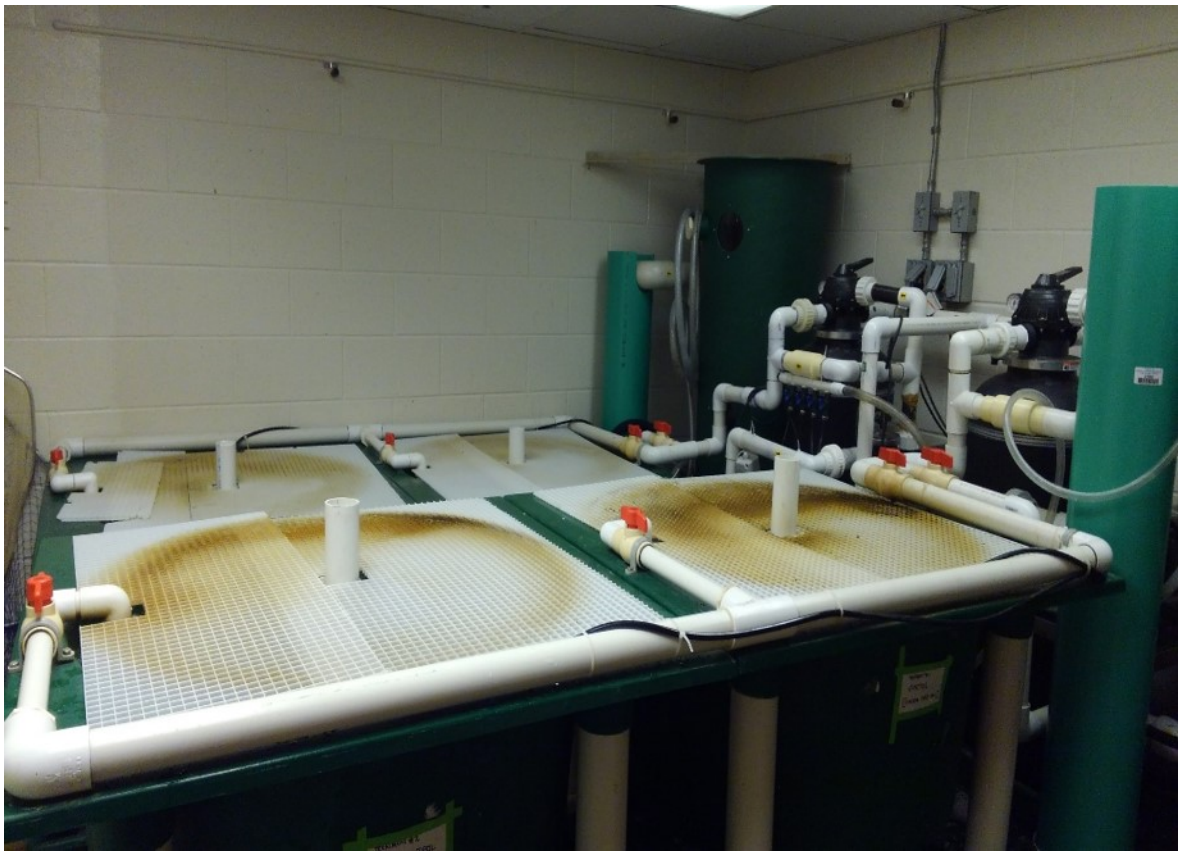

Figure 1. Recirculating aquaculture systems. This picture shows the two identical recirculating aquaculture systems used for this experiment. Both systems consist of two identical 200 gallon tanks that drain into a 400 gallon sump located beneath the tanks. Each system is filtered by its own sand filter that can be seen in the picture. The water is then returned to the tanks to complete the system.

Table 3. Room temperature and humidity. This table shows the room temperature and humidity recorded for the room housing both systems used in this experiment. The highest and lowest values recorded are shown for the entire acclimation phase and sampling period.

	Low	High
Room Temperature (°C)	25	30
Room Humidity (%)	24	59

Table 4. Source water quality. This table shows the source water quality parameters measured from the municipal water source supplied by Fort Wayne Water Utility and filtered at the Three Rivers Filtration Plant that was used for this experiment.

pH	8.4
Ammonia (parts per million)	0.5
Nitrite (parts per million)	0
Nitrate (parts per million)	10

Table 5. Water quality for both systems. This table shows the water quality parameters for both systems throughout the acclimation phase and sampling period. The highest and lowest values measured for each parameter throughout the entire acclimation phase and sampling period are shown.

	Low	High
Water Temperature (°C)	25	30
Dissolved Oxygen (% Saturation)	79.9	91.5
Dissolved Oxygen (mg/L)	6.53	7.80
pH	6.0	7.8
Salinity (parts per thousand)	0.1	3.6
Ammonia (parts per million)	0.0	1.0
Nitrite (parts per million)	0.0	0.5
Nitrate (parts per million)	0	160

Table 6. Experimental groups. This table shows the experimental groups used for this experiment and the tanks they were assigned to. The overall tank setup showing which tanks belong to each system can be seen in Figure 2.

Tank Number	Experimental Group
1	Control Control
2	Stress Control
3	Stress Probiotic
4	Stress Prebiotic and Probiotic

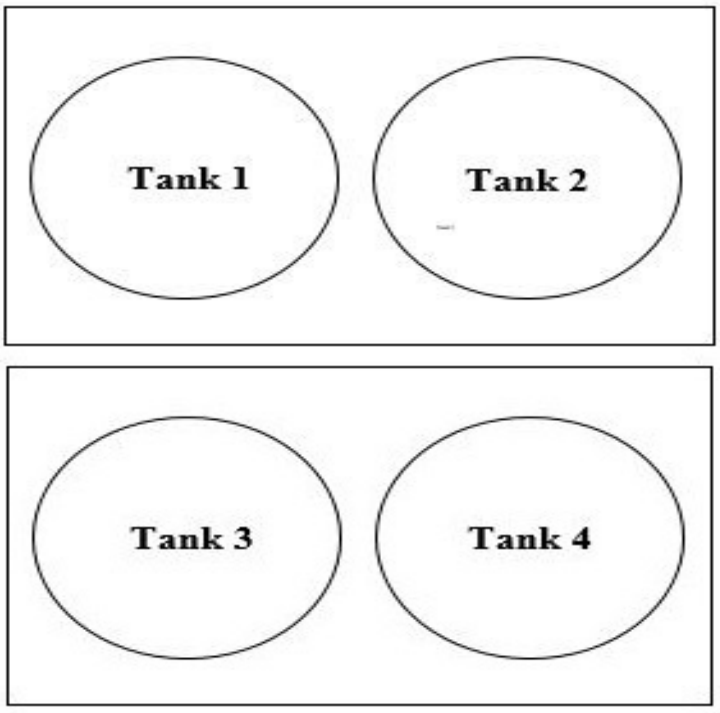

Figure 2. Diagram of tank numbering. This diagram shows how the tanks were numbered and which tanks belong to each system. Each rectangular box represents a separate system, and each circle represents a separate tank.

Table 7. Commercial feed nutritional information. This table shows the nutritional information for the Purina® Aquamax® Fingerling Starter 300 commercial feed that was used for this experiment. This information was obtained from the labeling on the package. These are the nutritional values for the non-supplemented feed.

Nutrient	Minimum / Maximum	Amount (%)
Crude Protein	Minimum	50.00
Crude Fat	Minimum	16.00
Crude Fiber	Maximum	3.00
Calcium (Ca)	Minimum	2.35
Calcium (Ca)	Maximum	2.85
Phosphorous (P)	Minimum	1.30
Sodium (Na)	Maximum	0.60

Table 8. Feed preparation. This table shows the feed preparation for each experimental group. The amounts shown are what was used to produce 1 kilogram of prepared food.

Experimental Group	Commercial Feed (g)	Hydrocortisone (mg)	Prebiotic (g)	Probiotic (g)	Fish Oil (mL)	Ethanol Applied (mL)
Control Control	1000	0	0	0	50	500
Stress Control	1000	200	0	0	50	500
Stress Probiotic	950	200	0	50	50	500
Stress Prebiotic and Probiotic	945	200	5	50	50	500

Table 9. Sampling periods, sample size, and experimental parameters. This table shows the sampling periods for this experiment, the sample size used for each sampling period, and the experimental parameters measured for each sampling period. * = These parameters were not measured on Day 3 because no fish were euthanized and no dissection occurred for this sampling period.

Sampling Periods	Day 0 and Day 3 Week 2, Week 4, and Week 8
Sample Size For Each Sampling Period	6 Fish Per Experimental Group
Stress Response Parameters	Serum Cortisol Blood Glucose Plasma Protein Packed Cell Volume / Hematocrit (PCV) Hepato-Somatic Index (HSI)* Spleen-Somatic Index (SSI)* Lysozyme Activity
Whole Body Response Parameters	Feed Conversion Ratio (FCR) Specific Growth Rate (SGR) Protein Efficiency Ratio (PER) Length Gain Weight Gain Condition Factor (K)

RESULTS

Stress Response Parameters

Serum Cortisol

There were statistically significant differences ($P<0.05$) found for the serum cortisol values between the experimental groups within each sampling period. Statistically significant differences ($P<0.05$) were found between the Control Control experimental group and the Stress Probiotic and Stress Prebiotic and Probiotic experimental groups within the sampling period for Week 2, Week 4, and Week 8. Statistically significant differences ($P<0.05$) were found between the Control Control experimental group and the Stress Control experimental group within the sampling period for Week 4 and Week 8. There were no statistically significant differences ($P<0.05$) found between the Control Control experimental group and the Stress Control experimental group within the sampling period at Week 2, and there were no statistically significant differences ($P<0.05$) found between the Control Control experimental group and any of the stressed groups within the sampling period for Day 0 or Day 3. There were no statistically significant differences ($P<0.05$) found between the stressed experimental groups within the sampling period for any of the sampling periods.

There were also statistically significant differences ($P<0.05$) found between the baseline serum cortisol values on Day 0 and the serum cortisol values for the experimental groups throughout the experiment. The serum cortisol values for the Control Control experimental group at Day 0 were used as the control for this comparison. Statistically significant differences ($P<0.05$) were found between the baseline serum cortisol values on Day 0 and the serum cortisol values for the Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental

groups for Day 3, Week 2, Week 4, and Week 8. Statistically significant differences ($P<0.05$) were found between the baseline serum cortisol values on Day 0 and the serum cortisol values for the Control Control experimental group for Week 8. There were no statistically significant differences ($P<0.05$) found between the baseline serum cortisol values on Day 0 and serum cortisol values for any of the stressed experimental groups for Day 0, and there were no statistically significant differences ($P<0.05$) found between the baseline serum cortisol values on Day 0 and the serum cortisol values for the Control Control experimental group for Day 3, Week 2, and Week 4.

The mean ± SEM baseline serum cortisol (pg/mL) value on Day 0 was 138.150±28.415. The mean ± SEM serum cortisol (pg/mL) values for the Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups from Day 3 were 84.400±25.598, 69.617±16.188, 41.800±14.709, and 70.867±16.756, respectively. The mean ± SEM serum cortisol (pg/mL) values for the Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups from Week 2 were 79.583±20.877, 41.700±8.665, 14.200±3.910, 23.083±4.104, respectively. The mean ± SEM serum cortisol (pg/mL) values for the Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups from Week 4 were 84.750±21.454, 18.383±5.539, 11.017±5.997, and 8.583±2.153, respectively. The mean ± SEM serum cortisol (pg/mL) values for the Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups from Week 8 were 24.367±4.676, 5.450±1.683, 7.683±2.369, and 7.933±2.659, respectively (Figure 3).

Blood Glucose

There were no statistically significant differences ($P<0.05$) found for the blood glucose values between the experimental groups within each sampling period. There were statistically significant differences ($P<0.05$) found between the baseline blood glucose values on Day 0 and blood glucose values for the experimental groups. The blood glucose values for the Control Control experimental group at Day 0 were used as the control for this comparison. Statistically significant differences ($P<0.05$) were found between the baseline blood glucose values on Day 0 and the blood glucose values for the Control Control and Stress Probiotic experimental groups for Week 2. Statistically significant differences ($P<0.05$) were found between the baseline blood glucose values on Day 0 and the blood glucose values for the Stress Prebiotic and Probiotic experimental group for Week 4 and Week 8.

There were no statistically significant differences ($P<0.05$) found between the baseline blood glucose values on Day 0 and the blood glucose values for any of the experimental groups for Day 0 and Day 3. There were no statistically significant differences ($P<0.05$) found between the baseline blood glucose values on Day 0 and the blood glucose values for the Stress Control experimental group for Week 2, Week 4, and Week 8, and there were no statistically significant differences ($P<0.05$) found between the baseline blood glucose values on Day 0 and the blood glucose values for the Control Control and Stress Probiotic experimental groups for Week 4 and Week 8. There were also no statistically significant differences found between the baseline blood glucose values on Day 0 and the Stress Prebiotic and Probiotic experimental group for Week 2.

The mean ± SEM baseline blood glucose (mg/dL) value on Day 0 was 42.500±4.595. The mean ± SEM blood glucose (mg/dL) values for the Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups from Day 3 were

38.333±3.809, 39.333±1.256, 35.667±2.108, and 35.333±1.687, respectively. The mean ± SEM blood glucose (mg/dL) values for the Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups from Week 2 were 79.500±5.852, 67.500±7.442, 77.000±9.842, and 72.333±9.831, respectively. The mean ± SEM blood glucose (mg/dL) values for the Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups from Week 4 were 49.000±2.852, 57.000±10.835, 75.667±20.803, and 81.333±7.719, respectively. The mean ± SEM blood glucose (mg/dL) values for the Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups from Week 8 were 66.167±3.291, 63.167±7.277, 60.667±8.011, and 89.667±10.262, respectively (Figure 4).

Plasma Protein

There were statistically significant differences ($P<0.05$) found for the plasma protein values from the terminal sampling at Week 8. The plasma protein values for the Control Control experimental group were found to be statistically significantly different ($P<0.05$) from the Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups. Statistically significant differences ($P<0.05$) were also found between the Stress Control experimental group and the Stress Probiotic and Stress Prebiotic and Probiotic experimental groups. There were no statistically significant differences ($P<0.05$) found between the Stress Probiotic experimental group and Stress Prebiotic and Probiotic experimental group.

The mean ± SEM plasma protein (g/mL) values for the Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups from the terminal sampling at Week 8 were 4.83±0.0615, 7.467±0.349, 6.400±0.253, and 6.533±0.169, respectively. The percentage compared to the control value for each experimental group was

calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. The percentage compared to Control Control for the Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups were 155%, 132%, and 135%, respectively (Figure 5).

Packed Cell Volume / Hematocrit

There were no statistically significant differences ($P<0.05$) found for the packed cell volume, or hematocrit, values for the terminal sampling at Week 8. The mean ± SEM packed cell volume (%) values for the Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups from the terminal sampling at Week 8 were 34.333±1.256, 39.000±1.571, 35.000±0.730, and 38.000±1.862, respectively. The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. The percentage compared to Control Control for the Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups were 114%, 102%, and 111%, respectively (Figure 6).

Hepato-Somatic Index

There were statistically significant differences ($P<0.05$) found for the hepato-somatic index values from the terminal sampling at Week 8. The hepato-somatic index values for the Control Control experimental group were found to be statistically significantly different ($P<0.05$) from the Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups. There were no statistically significant differences ($P<0.05$) found between the Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups. The mean ± SEM hepato-somatic index values for the Control Control, Stress Control, Stress Probiotic, and Stress

Prebiotic and Probiotic experimental groups from the terminal sampling at Week 8 were 1.615±0.0656, 3.289±0.272, 3.291±0.432, and 3.033±0.191, respectively. The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. The percentage compared to Control Control for the Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups were 204%, 204%, and 188%, respectively (Figure 7).

Spleen-Somatic Index

There were no statistically significant differences ($P<0.05$) found for the spleen-somatic index values for the terminal sampling at Week 8. The mean ± SEM spleen-somatic index values for the Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups from the terminal sampling at Week 8 were 0.233±0.0435, 0.316±0.0653, 0.282±0.0486, and 0.196±0.00743, respectively. The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. The percentage compared to Control Control for the Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups were 136%, 121%, and 84%, respectively (Figure 8).

Lysozyme Activity

There were no statistically significant differences ($P<0.05$) found for the lysozyme activity values for the terminal sampling at Week 8. The mean ± SEM lysozyme activity ($abs._{530nm}$/minute) values for the Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups from the terminal sampling at Week 8 were

0.00625±0.000194, 0.00779±0.000684, 0.00708±0.000715, and 0.00692±0.000380, respectively. The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. The percentage compared to Control Control for the Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups were 125%, 113%, and 111%, respectively (Figure 9).

Whole Body Response Parameters

Feed Conversion Ratio

The mean feed conversion ratio values for the Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups from the terminal sampling at Week 8 were 1.148, 1.439, 1.809, and 1.548, respectively. The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. The percentage compared to Control Control for the Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups were 125%, 158%, and 135%, respectively (Figure 10).

Specific Growth Rate

The mean specific growth rate (% growth/day) values for the Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups from the terminal sampling at Week 8 were 2.543, 2.058, 1.917, and 1.915, respectively. The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean.

The percentage compared to Control Control for the Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups were 81%, 75%, and 75%, respectively (Figure 11).

Protein Efficiency Ratio

The mean protein efficiency ratio values for the Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups from the terminal sampling at Week 8 were 2.104, 1.627, 1.465, and 1.456, respectively. The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. The percentage compared to Control Control for the Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups were 77%, 70%, and 69%, respectively (Figure 12).

Length Gain

The mean length gain (cm) values for the Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups from the terminal sampling at Week 8 were 7.133, 6.683, 6.183, and 6.350, respectively. The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. The percentage compared to Control Control for the Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups were 94%, 87%, and 89%, respectively (Figure 14).

Weight Gain

The mean weight gain (g) values for the Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups from the terminal sampling at Week 8 were 120.150, 81.250, 74.567, and 71.950, respectively. The percentage compared to the control

value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. The percentage compared to Control Control for the Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups were 68%, 62%, and 60%, respectively (Figure 15).

Condition Factor

There were statistically significant differences ($P<0.05$) found for the condition factor values from the terminal sampling at Week 8. The condition factor for the Control Control experimental group was found to be statistically significantly different ($P<0.05$) from the Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups. There were no statistically significant differences ($P<0.05$) found between the Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups. The mean ± SEM condition factor values for the Control Control, Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups from the terminal sampling at Week 8 were 2.102±0.0726, 1.711±0.0329, 1.726±0.0402, and 1.671±0.0515, respectively. The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. The percentage compared to Control Control for the Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic experimental groups were 81%, 82%, and 79%, respectively (Figure 16).

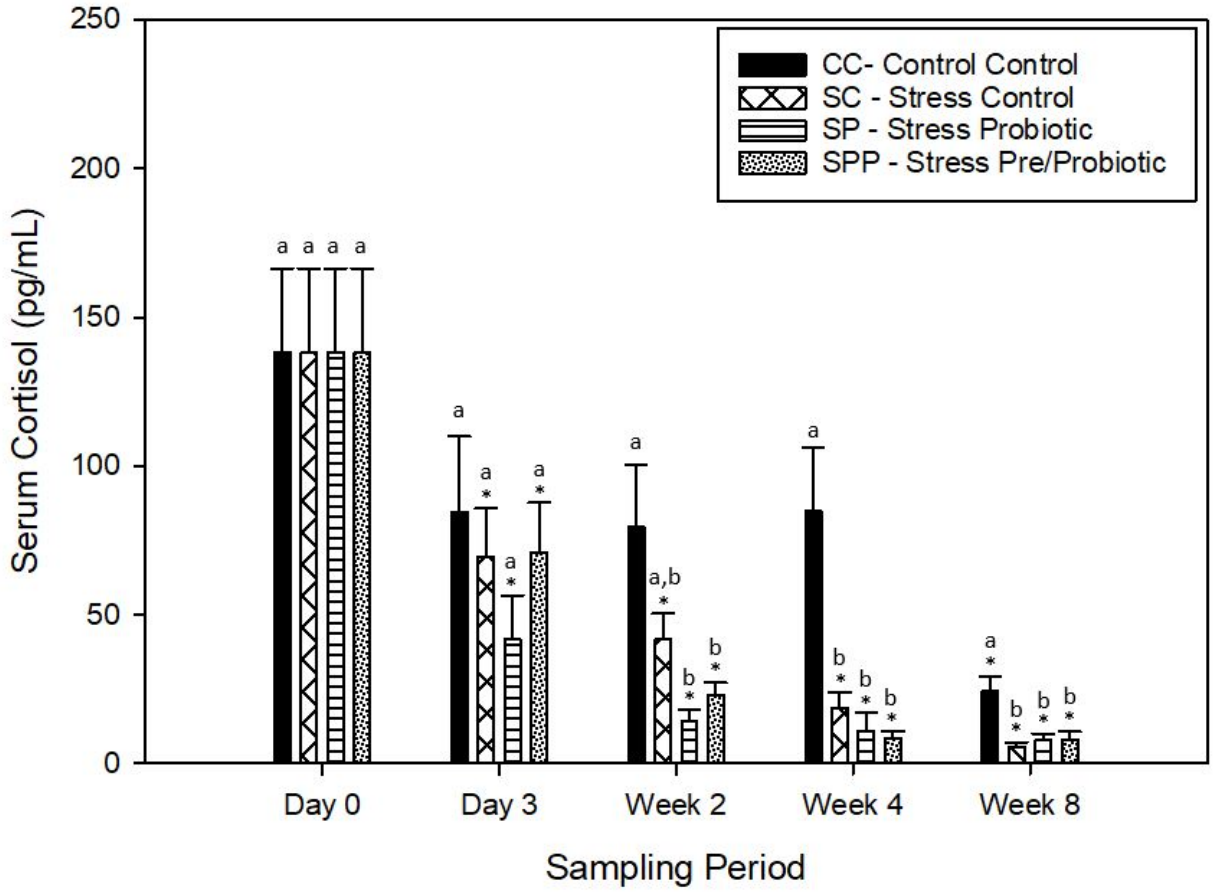

Figure 3. Serum cortisol (pg/mL) for all sampling periods. This graph shows the serum cortisol levels for each experimental group for each sampling period. The lowercase letters indicate statistically significant differences between groups ($P<0.05$) within each sampling period. Statistical differences between the individual treatments for each sampling period and the baseline levels on Day 0 are marked with a * ($P<0.05$). All data is shown as the mean ± standard error of the mean (SEM).

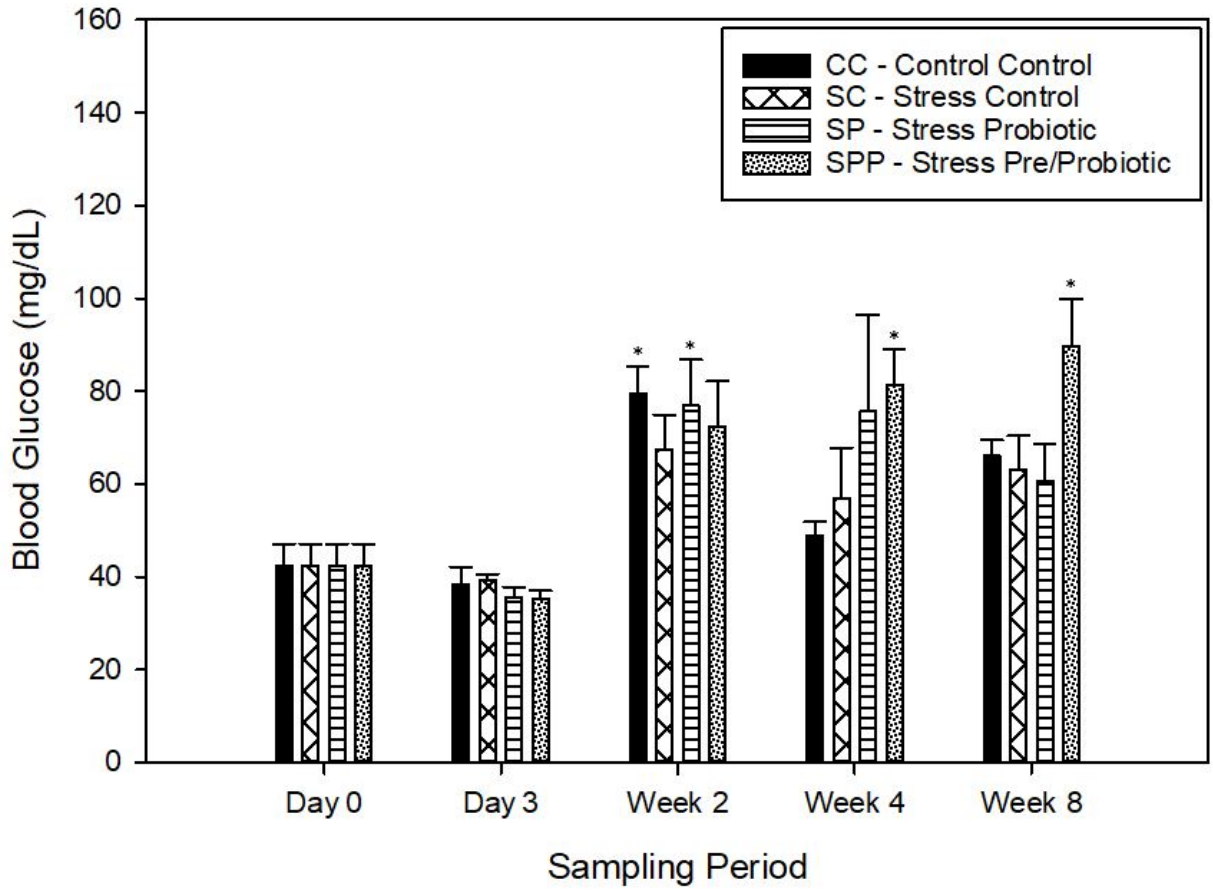

Figure 4. Blood glucose (mg/dL) for all sampling periods. This graph shows the blood glucose levels for each experimental group from each sampling period. There were no statistical differences found between groups within each sampling period. Statistical differences between the individual treatments for each sampling period and the baseline levels on Day 0 are marked with a * ($P<0.05$). All data is shown as the mean ± standard error of the mean (SEM).

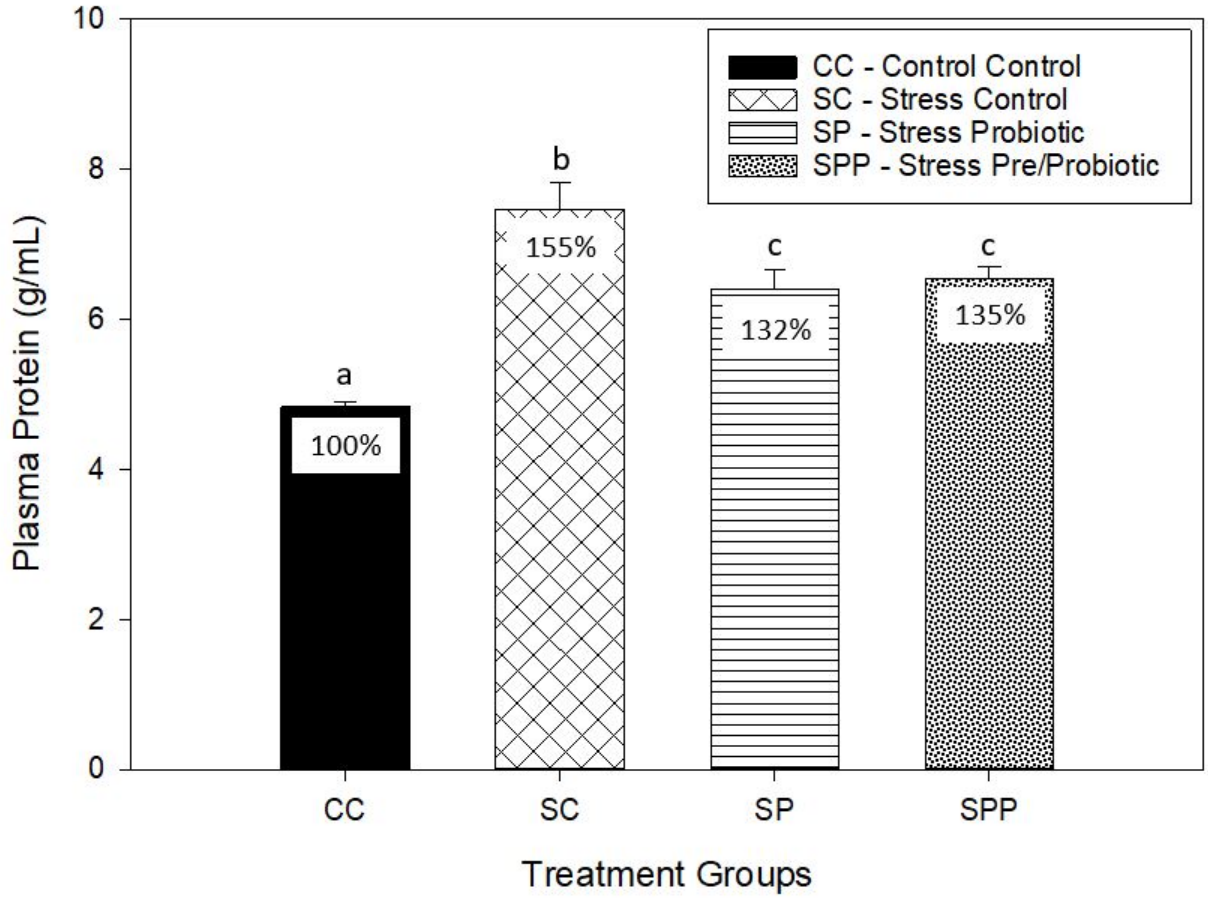

Figure 5. Plasma protein (g/mL) for terminal sampling. This graph shows the plasma protein levels for each experimental group from the terminal sampling period at Week 8. The lowercase letters indicate statistically significant differences between groups ($P<0.05$). All data is shown as the mean ± standard error of the mean (SEM). The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. Values are shown in boxes on each experimental group's bar.

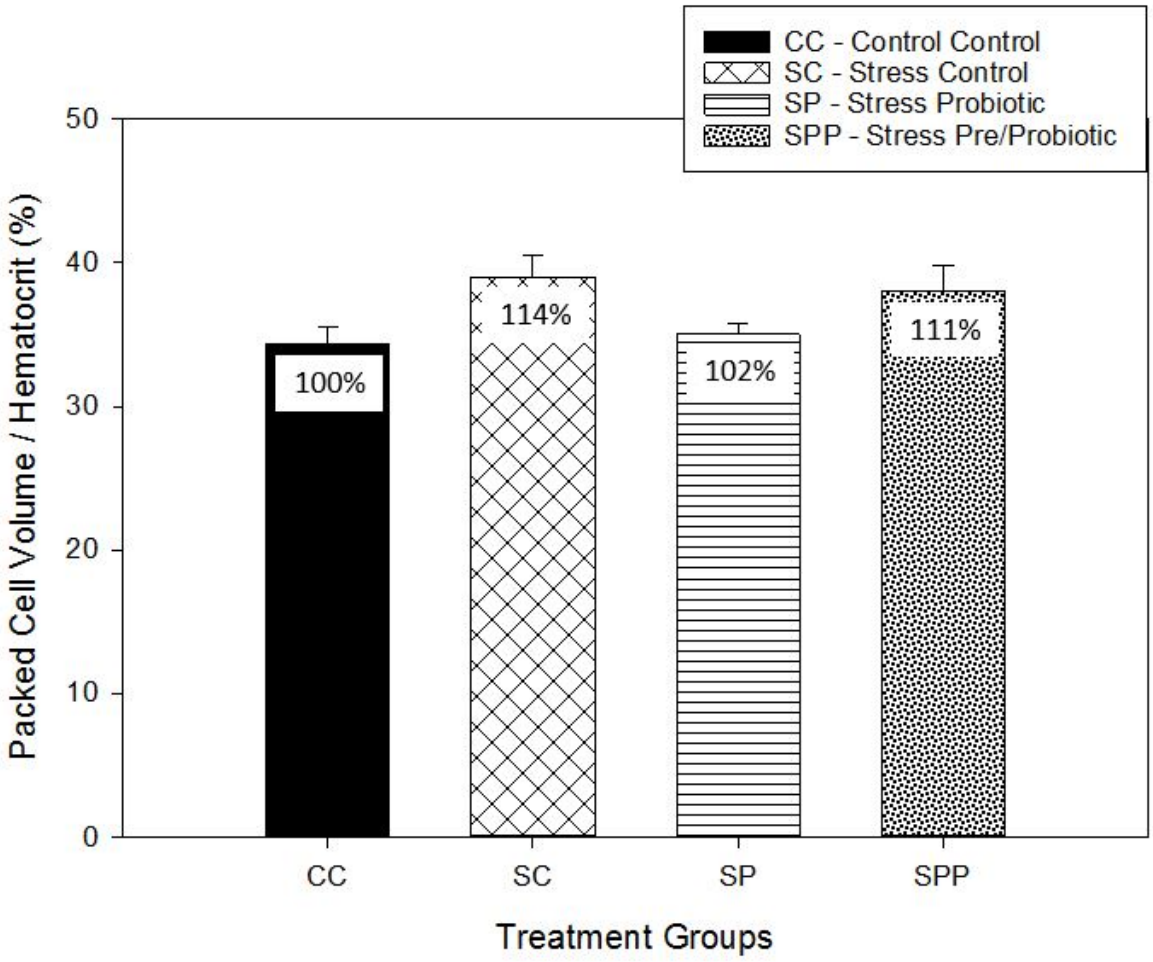

Figure 6. Packed cell volume / hematocrit (%) for terminal sampling. This graph shows the packed cell volume, or hematocrit, values for each experimental group from the terminal sampling period at Week 8. There were no statistically significant differences ($P<0.05$) found between the experimental groups. All data is shown as the mean ± standard error of the mean (SEM). The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. Values are shown in boxes on each experimental group's bar.

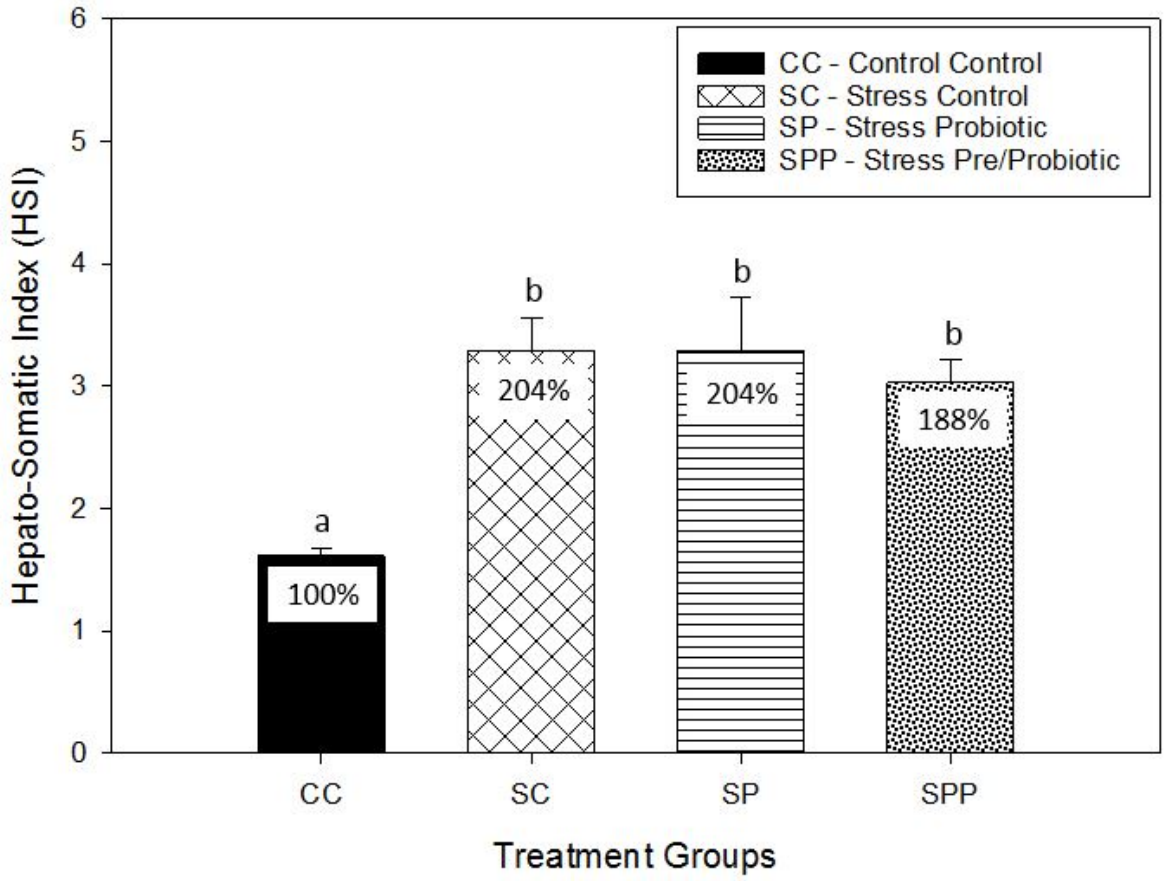

Figure 7. Hepato-somatic index (HSI) for terminal sampling. This graphs shows the hepato-somatic index values for each experimental group from the terminal sampling period at Week 8. The lowercase letters indicate statistically significant differences between groups ($P<0.05$). All data is shown as the mean ± standard error of the mean (SEM). The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. Values are shown in boxes on each experimental group's bar.

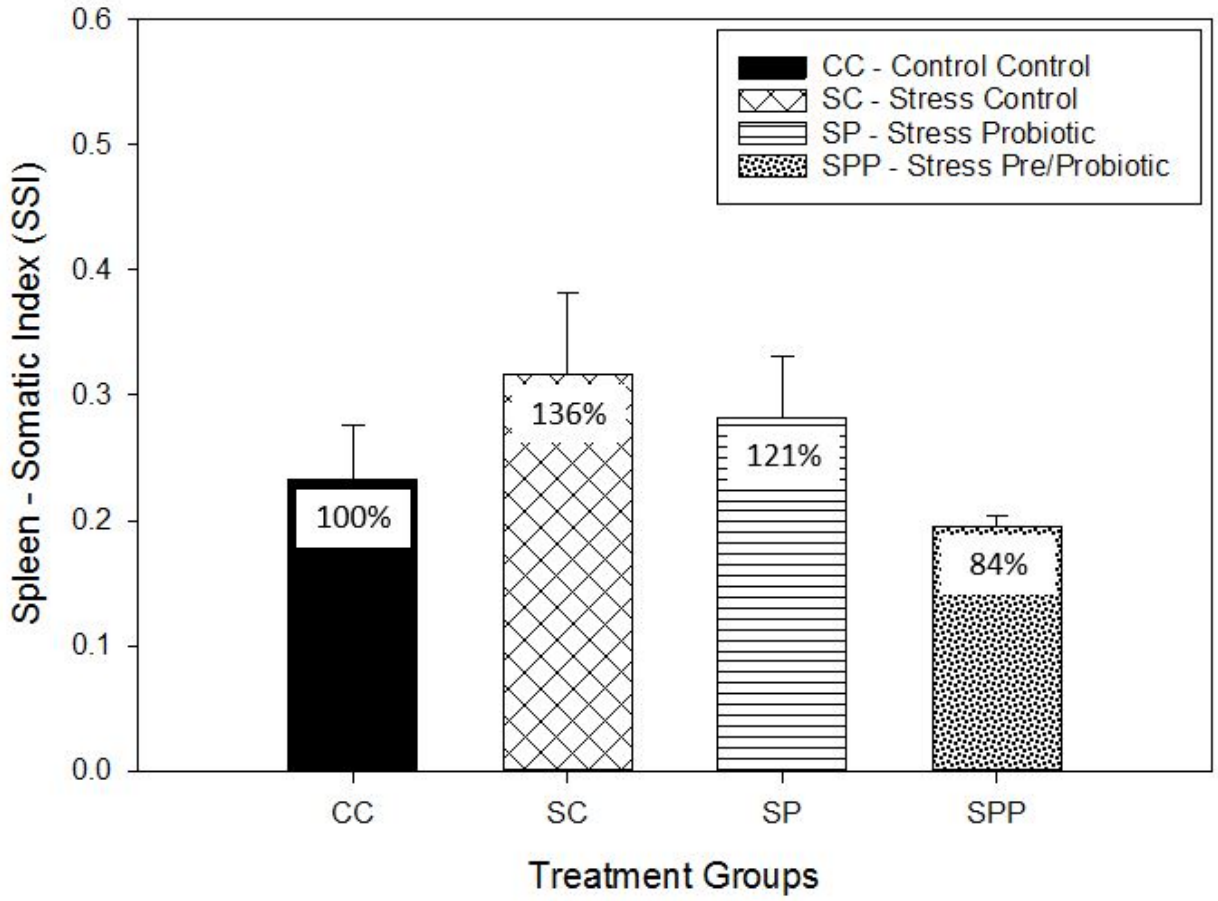

Figure 8. Spleen-somatic index (SSI) for terminal sampling. This graph shows the spleen-somatic index values for each experimental group from the terminal sampling period at Week 8. There were no statistically significant differences ($P<0.05$) found between the experimental groups. All data is shown as the mean ± standard error of the mean (SEM). The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. Values are shown in boxes on each experimental group's bar.

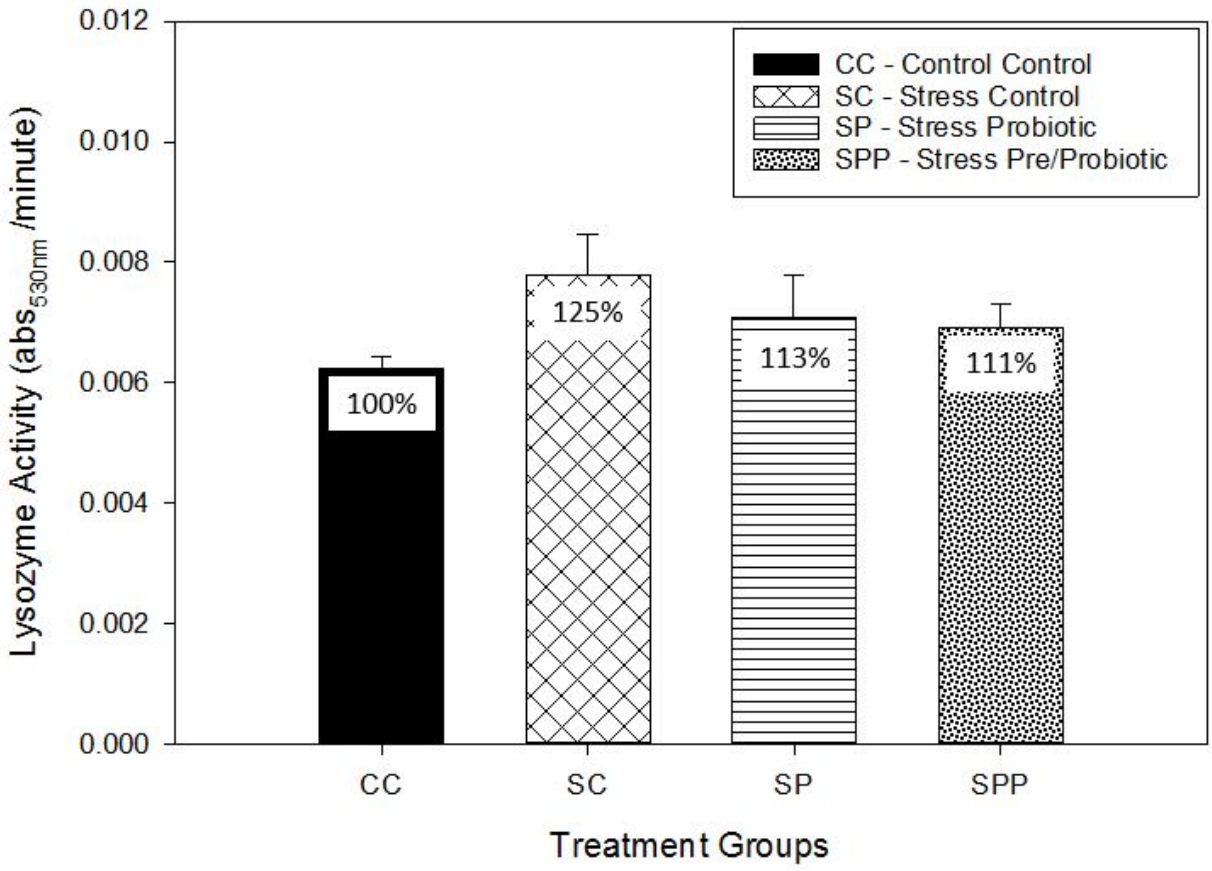

Figure 9. Lysozyme activity (abs.$_{530nm}$/minute) for terminal sampling. This graph shows the lysozyme activity values for each experimental group from the terminal sampling period at Week 8. There were no statistically significant differences ($P<0.05$) found between the experimental groups. All data is shown as the mean ± standard error of the mean (SEM). The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. Values are shown in boxes on each experimental group's bar.

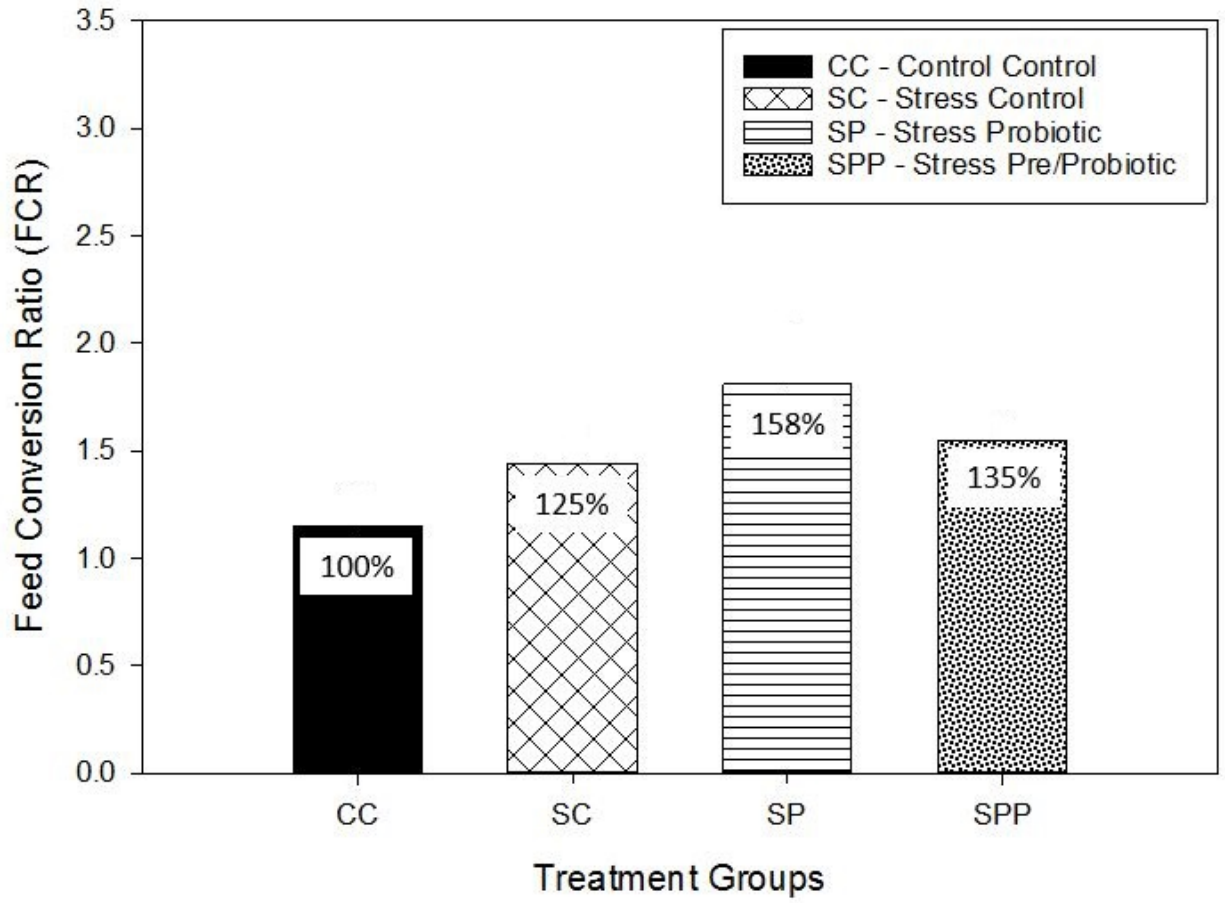

Figure 10. Feed conversion ratio (FCR) for terminal sampling. This graph shows the feed conversion ratio values calculated for each experimental group from the terminal sampling period at Week 8. All data is shown as the mean. The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. Values are shown in boxes on each experimental group's bar.

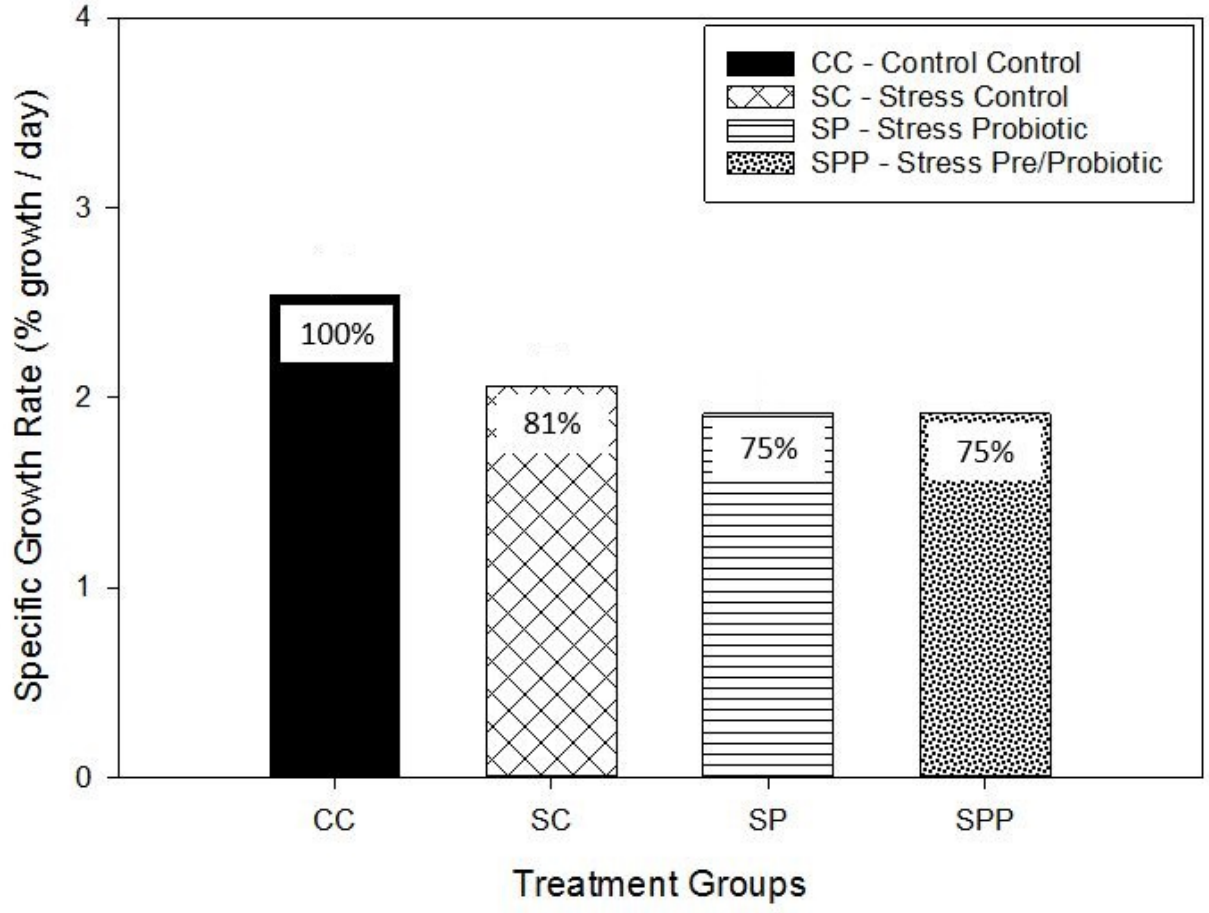

Figure 11. Specific growth rate (% growth/day) for terminal sampling. This graph shows the specific growth rate values calculated for each experimental group from the terminal sampling at Week 8. All data is shown as the mean. The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. Values are shown in boxes on each experimental group's bar.

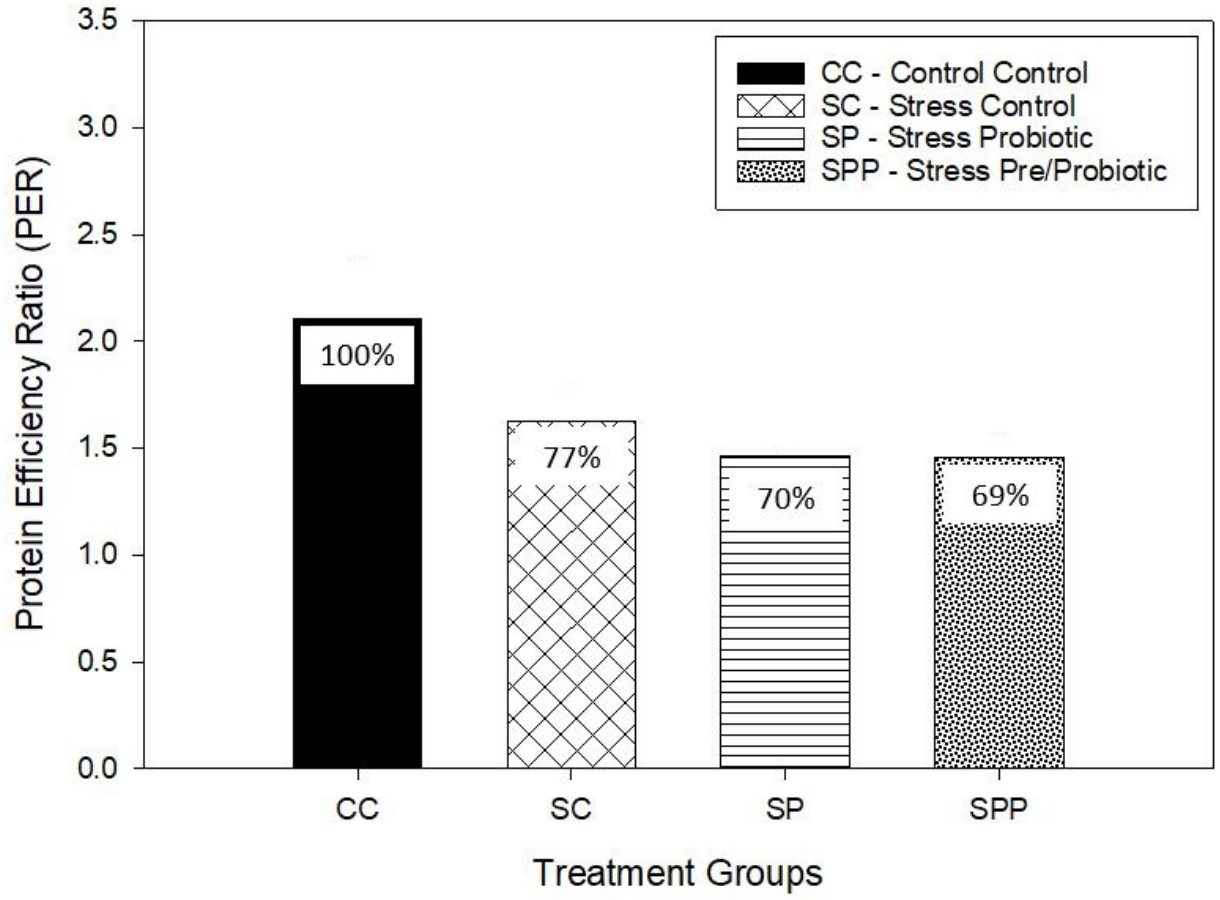

Figure 12. Protein efficiency ratio (PER) for terminal sampling. This graph shows the protein efficiency ratio values calculated for each experimental group from the terminal sampling at Week 8. All data is shown as the mean. The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. Values are shown in boxes on each experimental group's bar.

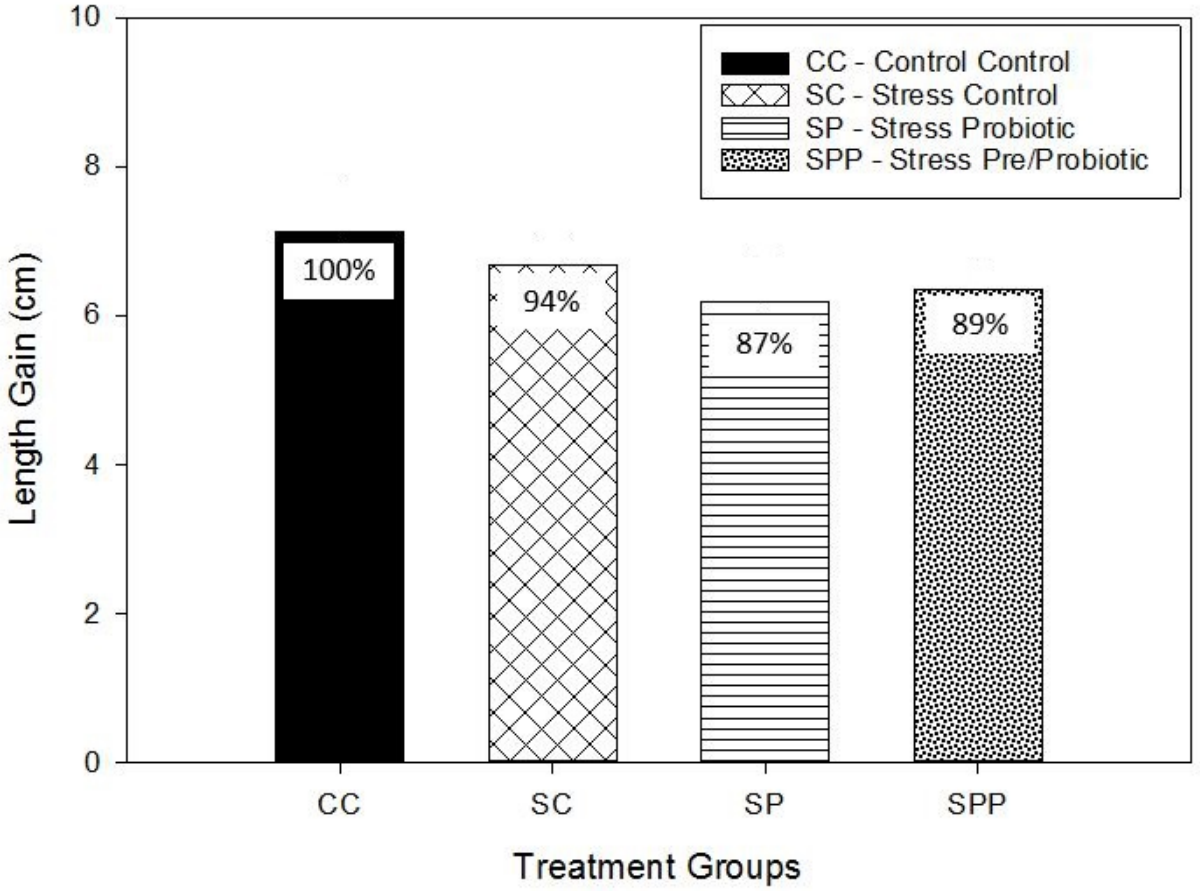

Figure 13. Length gain (cm) for terminal sampling. This graph shows the overall length gain values calculated for each experimental group for the entire experimental period. All data is shown as the mean. The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. Values are shown in boxes on each experimental group's bar.

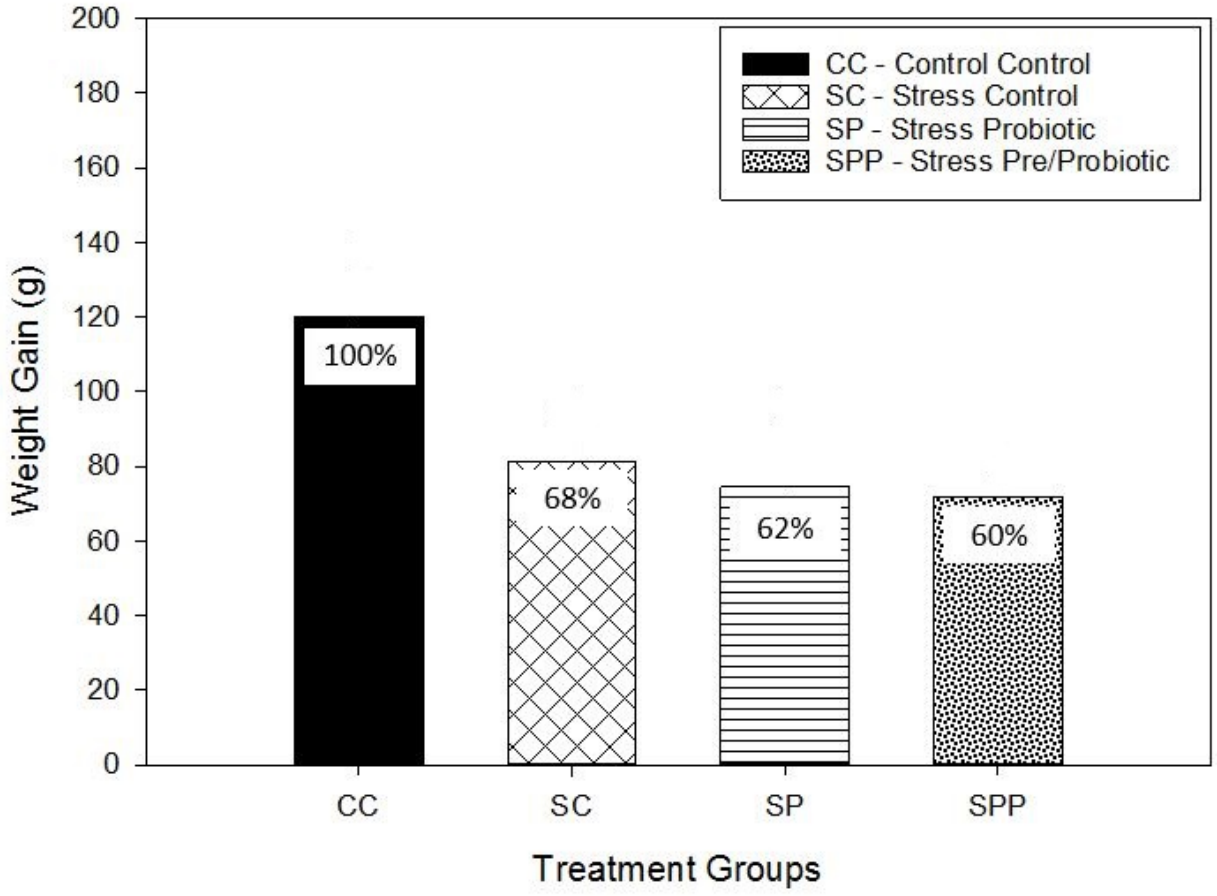

Figure 14. Weight gain (g) for terminal sampling. This graphs shows the overall weight gain values calculated for each experimental group for the entire experimental period. All data is shown as the mean. The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. Values are shown in boxes on each experimental group's bar.

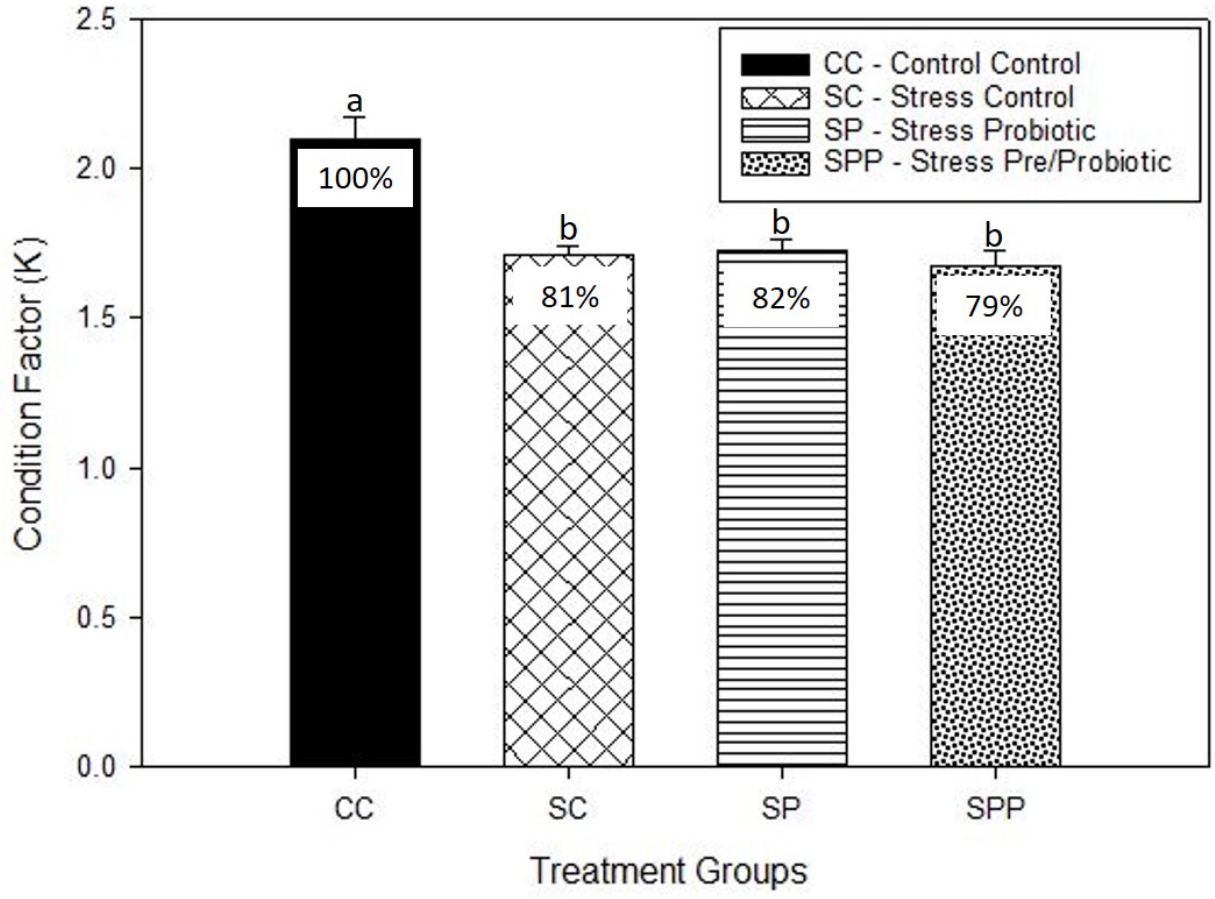

Figure 15. Condition factor (K) for terminal sampling. This graph shows the condition factor values calculated for each experimental group from the terminal sampling period at Week 8. The lowercase letters indicate statistically significant differences between groups ($P<0.05$). All data is shown as the mean ± standard error of the mean (SEM). The percentage compared to the control value for each experimental group was calculated by considering the Control Control mean value as 100% and dividing each other mean by the Control Control mean. Values are shown in boxes on each experimental group's bar.

DISCUSSION

The main purpose of this study was to evaluate the ability of prebiotic and probiotic supplementation in feed to improve the physiological and immune responses in stressed Nile tilapia (*Oreochromis niloticus*). Many physiological and immunological parameters were used to measure the results of this experiment, and these parameters have been validated by previous research to be effective measures of fish health and the stress response in fish (Adeoye et al., 2016; Barton, 2002; Barton & Iwama, 1991; Barton et al., 1987; Barton et al., 2002; Bonga, 1997; Ellis, 1990; El-Sayed, 2006; Garcia-Aibiado et al., 2004; Gensic et al., 2004; Goeded & Barton, 1990; Ibrahim et al., 2000; Khan et al., 2018; Pearson & Stevens, 1991; Rawling et al., 2009; Ruane et al., 2000; Siwicki et al., 1994; Wedemyer et al., 1990; Weirich et al., 2010). The range of parameters was chosen in order to see the effects of stress across all three biological levels of stress: primary responses, secondary responses, and tertiary responses.

In this study, the probiotic supplemented diet and the prebiotic and probiotic supplemented diet did not produce very many statistically significant results. The few statistically significant differences found were almost always between the Control Control group and the stressed groups. These results were not expected based on the previous research on prebiotic and probiotic supplementation that was found during the literature review performed before undertaking this study.

Neither of the two hypotheses were supported based on the results found in this study. The probiotic supplemented group and the prebiotic and probiotic supplemented group did not have improved physiological and immune responses compared to the stressed and non-supplemented Nile tilapia. Also, the prebiotic and probiotic supplemented group did not have better physiological and immune responses compared to the probiotic supplemented group.

While there were not a lot of statistically significant differences found, there was a trend visible based on the means of the different experimental groups. However, this trend also did not support either of the two hypotheses.

Many of the parameters actually showed the opposite effect than was expected based on the literature review. One surprising result and trend was seen in the results of the serum cortisol assay. For this assay, the serum cortisol values for the Control Control experimental group at Day 0 were used as the baseline serum cortisol levels for comparison. The Stress Control, Stress Probiotic, and Stress Prebiotic and Probiotic groups were found to be significantly different from the baseline cortisol values on Day 0 beginning on the sampling period on Day 3 and, continuing throughout the rest of the experiment, on Week 2, Week 4, and Week 8. The Control Control experimental group was also found to be statistically significantly different from the baseline serum cortisol values on Day 0 at Week 8.

In addition to the statistically significant differences found between the experimental groups and the baseline serum cortisol values from Day 0, there were also statistically significant differences found between the experimental groups within each sampling period beginning on Week 2 and, continuing throughout the rest of the experiment, on Week 4 and Week 8. All of the statistically significant differences that were found between the experimental groups within each sampling period occurred between the Control Control experimental group and the stressed experimental groups. The stressed experimental groups showed consistently lower levels than the Control Control experimental groups within each sampling period, and statistically significant differences began appearing on Week 2 and, continued throughout the rest of the experiment, on Week 4 and Week 8.

The trend seen for the serum cortisol levels was unexpected. All of the stressed groups showed lower mean cortisol levels than the Control Control group throughout the experiment, and there levels showed a steady reduction in serum cortisol levels throughout the experiment. While this result was unexpected, it is not without precedent. Previous research has shown that high levels of chronic stress can sometimes result in compensation by the fish, and this compensation results in the HPI axis being exhausted and shutting down (Barton, 2002; Barton et al., 1987; Madaro et al., 2015). With the shutdown of the HPI axis, the levels of cortisol circulating in the blood were almost completely reliant on the consistent supplementation provided through the feed twice a day. It has been shown in previous research that the serum cortisol levels of fish fed cortisol supplemented feed peaked 3 hours post-feeding and dropped to nearly undetectable levels by 24 hours post-feeding (Barton et al., 1987). Since fish were starved for 24 hours before sampling, the serum cortisol levels of the stressed groups appear to be following this trend as well. Also, the elevated serum cortisol levels seen on Day 0 are likely due to the amount of MS-222 used on Day 0 not being sufficient to cause deep anesthesia within less than two minutes. For this reason, the amount of MS-222 used for all subsequent sampling periods was increased. Since it took slightly longer than two minutes for deep anesthesia to occur on Day 0, the serum cortisol levels found for that sampling period and used for the baseline serum cortisol values were likely slightly elevated as a result.

There were very few statistically significant differences found between the baseline blood glucose values on Day 0 and the blood glucose values of the experimental groups throughout the experiment. For this assay, the blood glucose values from the Control Control experimental group at Day 0 were used as the baseline blood glucose values for comparison. The blood glucose values for the Control Control experimental group and the Stress Probiotic experimental

group for Week 2 were found to be statistically significantly different from the baseline blood glucose values on Day 0. The blood glucose values for the Stress Prebiotic and Probiotic experimental groups for Week 4 and Week 8 were also found to be statistically significantly different from the baseline blood glucose values on Day 0.

There were no statistically significant differences in blood glucose values found between the experimental groups within each sampling period. While no statistically significant differences were found, the blood glucose levels measured in the stressed groups varied much more than the Control Control experimental group, and the stressed groups showed elevated mean values on Week 4 compared to the Control Control group. Again, this result is consistent with the previous research found about chronic stress and cortisol supplementation (Barton et al., 1987; Madaro et al., 2015). When fish suffer from chronic stress or chronic cortisol supplementation, their blood glucose levels follow the same pattern seen in the serum cortisol levels. The levels rise rapidly and peak at 3 hours after the fish are fed or encounter the stressor (Barton et al., 1987). However, after 24 hours, the fish that suffer from chronic stress or chronic cortisol supplementation show blood glucose levels that are not statistically different from the control groups (Barton et al., 1987).

The results for the rest of the stress response and whole body response parameters measured were only shown for the terminal sampling at Week 8. Only the terminal sampling was shown because there were no statistically significant differences seen for any of the parameters for the other sampling periods. Except for serum cortisol and blood glucose, the only statistically significant differences found for all of the parameters occurred at the terminal sampling at Week 8.

The plasma protein levels for the Control Control experimental group were found to be statistically significantly different from the plasma protein levels for the stressed groups at Week 8. The Stress Control experimental group was also shown to be statistically significantly different from the Stress Probiotic and Stress Prebiotic and Probiotic experimental groups at Week 8. The stressed groups showed elevated plasma protein levels, compared to the Control Control experimental group, which is consistent with previous research that has shown diuresis that occurs as a result of the stress response can cause the blood to become concentrated and lead to elevated plasma protein levels (Wedemyer et al., 1990). The mean values for the Stress Probiotic group and Stress Prebiotic and Probiotic group were not as elevated as the Stress Control group, and these differences were found to be statistically significantly different. It is possible that this reduction in plasma protein levels was due to the supplemented diets, and the supplemented diets were able to help the fish combat the diuresis and overproduction of protein that was likely occurring.

For the hepato-somatic index, the Control Control group was found to be statistically significantly different from the stressed groups, but there were no statistically significant differences found among the stressed groups. All of the stressed groups showed hepato-somatic index values that were nearly twice as high as the Control Control experimental group, and this is opposite of what would normally be expected in stressed individuals (Barton et al., 2002). However, this result is consistent with previous research that has shown that the liver can become hypertrophic and hyperplastic under certain conditions, and these changes to the liver are especially common in the presence of pollutants or toxins (Barton et al., 2002; Goede & Barton, 1990). It is possible that the changes seen in the liver could be in response to the cortisol supplementation, and it could be an attempt by the fish to detoxify the cortisol present.

The stressed groups showed higher feed conversion ratio values than the Control Control group. Surprisingly, the Stress Probiotic group and Stress Prebiotic and Probiotic group had higher feed conversion ratio values than the Stress Control group. This result is the opposite of what was expected, but it cannot necessarily be linked with the supplemented diets.

The means of the stressed groups showed reduced specific growth rate values compared to the Control Control group. Interestingly, the Stress Probiotic group and Stress Prebiotic and Probiotic group showed lower means than the Stress control group. These results again were the opposite of what was expected based on previous research (Goncalves et al., 2011; Hamdan et al., 2016; Hernandez et al., 2010).

All three stressed groups had reduced weight gain, and the Stress Probiotic and Stress Prebiotic and Probiotic group showed lower mean values than the Stress Control group. Again, this result is not what was expected based on previous research (Al-Dohail et al., 2009; Aly et al., 2008; Wang et al., 2008).

A statistically significant difference was also found for the condition factor between the Control Control and the stressed groups, but there was almost no differences between the three stressed groups. The values for the protein efficiency ratio showed a similar trend as the condition factor. The means for all three stressed groups were almost the same, but all three were reduced compared to the mean of the Control Control group.

There were no statistically significant differences found for the packed cell volume, spleen-somatic index, or lysozyme activity parameters between all four experimental groups. There were also no obviously discernable trends seen from the data, and specific growth rate and length gain were found to be reduced for all three stressed groups. These results were not expected, and previous research suggested that the Stress Probiotic group and the Stress Prebiotic

and Probiotic group should have shown increased lysozyme activity, specific growth rate, and length gain compared to the Stress Control group (Goncalves et al., 2011; Hamdan et al., 2016; Hernandez et al., 2010; Ibrahem et al., 2010; Reyes-Becerril et al., 2014).

There are many possible factors that could have caused the supplemented diets to not show positive results. The first possible factor was that the level of probiotic supplementation used was not sufficient to produce a response. Based on the definition of probiotics put forth by the Food and Agricultural Organization of the United Nations and the World Health Organization, probiotics must be supplied "in adequate amounts" in order for their health benefits to be realized (FAO & WHO, 2006). If the amount of probiotics supplied was too low, then the benefits found in previous studies would not be realized (Al-Dohail et al., 2009; Aly et al., 2008; Bogut et al., 2000; Goncalves et al., 2011; Hamdan et al., 2016; Hernandez et al., 2010; Wang et al., 2008). It is also possible that the benefits seen in previous studies could be realized if the probiotic supplementation was introduced earlier in development (Chandran et al., 2017). It also possible that the combination of the four probiotic species was disruptive to the gut microbiome of the fish, and instead of being beneficial, it was detrimental.

Another possible factor is that the level of inulin provided may not have been sufficient to elicit a positive response, or that the ratio of prebiotic to probiotic was not sufficient to assist the establishment of the probiotic species in the gut. Also, if the probiotic species used were not able to utilize the inulin effectively, then they could fail to function together as a conbiotic or symbiotic (Kechagia et al., 2013). In addition to the level inulin level being too low, it is equally likely that the inulin level was too high as previous studies have shown that inulin can be detrimental in some situations where the dosage is too high (Hoseinifar et al., 2015).

In addition to the levels of prebiotic and probiotic used, it is possible that the method used to encapsulate the prebiotic and probiotic could be a problem. The fish oil used to encapsulate the prebiotic and probiotic to the feed was chosen because fish meal and fish oil are a common additive to feed used in aquaculture (FAO, 2018). While the fish oil may have been successful in adhering the prebiotic and probiotic to the feed, it is possible that it may have somehow hindered the ability of the probiotic to become established in the gut of the fish. Any of these possibilities could have contributed to the ineffectiveness of the prebiotic and probiotic supplemented feeds to produce improved physiological and immunological responses in stressed Nile tilapia.

CONCLUSION

The world population is continuing to grow, and it is projected to continue into the foreseeable future. Even at the current population level, millions of people around the world are suffering from food insecurity and malnutrition. The current food problem is likely to only continue to increase as the effects of climate change become more pronounced. With global populations projected to continue rising through 2100, something must be done to address the growing demand for food worldwide.

Aquaculture is currently the most rapidly growing sector of food production worldwide. It has many great advantages over traditional farming and livestock production methods including less feed requirements and fewer greenhouse emissions. With the growth of world production from capture fisheries virtually stagnant and restrictions on their use continuing to increase, aquaculture offers the best possible solution to meet the world's current and future food demands. Unfortunately, the aquaculture industry faces challenges of its own.

The main challenge faced by the aquaculture industry is the stress caused by raising aquatic organisms in non-natural settings. The fish raised are introduced to stressors they would not normally encounter in their natural habitat. The stress response induced by these stressors leads to reduced growth, reduced reproductive success and fitness, and a decreased immune response. All of these factors contribute to an overall increase in disease susceptibility.

Currently, chemical treatments and antibiotics are used to combat disease, but these methods have inherent problems, like bioaccumulation and antibiotic resistance, that they are associated with. New methods for treating and preventing disease in aquaculture are required. One possible alternative is the use of nutraceuticals, or functional feed additives, to reduce stress

and improve the physiological and immunological responses of stressed fish. In the end, nutraceutical research must continue if a better path forward is to be found. Prebiotics and probiotics represent one possible option, as functional food additives, going forward. However, more work must be done if the benefits of these nutraceuticals are to be realized.

One possible direction for future research is to begin to study the gut microbiome of the most important and widely cultured aquaculture species. Without knowing what the natural makeup of the gut microbiome is for these species, it will be very difficult, if not impossible, to find a suitable prebiotic and probiotic combination that will provide health benefits to the host. In addition to studying the gut microbiome, different production methods need to be developed to ensure the viability of the probiotic species being used and to try to reduce the cost of their use even further. Also, future research aimed at finding the prebiotic and probiotic combinations that work best as symbiotics needs to be done.

If the relationships between prebiotics and different probiotic species can be determined, then the benefits of probiotic supplementation can be maximized. All of these advances could drive the use of prebiotics and probiotics as functional feed additives forward. If successful, the use of prebiotics, probiotics, and other nutraceuticals could lead to a total end of chemical treatments and antibiotic use in aquaculture. This result would be the best possible outcome for the environment, for the consumer, for aquaculture, and for the world as a whole.

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