Evaluating the Efficacy of a Low-Dose Garlic Compound (Allicin) Against Infection with *Aeromonas salmonicida* in Rainbow Trout

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

Kate E. Breyer, D.V.M.

August, 2013

© 2013 Kate E. Breyer

ABSTRACT

Previous studies in several fish species have shown efficacy of a low-dose garlic compound, allicin, against several pathogens. In this study, young rainbow trout (*Oncorhynchus mykiss*) were fed diets containing 0%, 0.5%, 1.0% and 2.0% allicin and then challenged with a modified LD_{50} dose of *Aeromonas salmonicida* and monitored for 28 days. There were significant increases in survivability in the 0.5% and 1.0% groups and a significant reduction in survival at the 2.0% dose. A target animal safety was performed using the target dose of 0.5% allicin at 0x, 1x, 3x, and 5x the target dose for 3x the duration (6 weeks) of the original study. There were no statistically significant differences in erythrocyte parameters or average leukocyte counts; however, there was a significant decrease in the level of circulating monocytes in the high dose (2.5%) group. This correlated to an increased level of pigment-containing macrophage centers within the renal tissue as allicin dosing increased, denoting a potential inflammatory effect. On serum chemistry, glutamate dehydrogenase (GDH) was elevated, most likely as a result of increased cellular metabolism and increased hepatocyte turnover due to allicin dosing. Initial studies indicate that feeding 0.5% allicin improves survivability when challenged with *A. salmonicida* and appears safe; however, higher levels may cause negative effects on health.

BIOGRAPHICAL SKETCH

Kate Breyer was born in Voorhees, New Jersey. She received a Bachelor of Science in Biological Sciences in 2005 from Rowan University (Glassboro, NJ). She received a Doctorate of Veterinary Medicine from the University of Illinois, College of Veterinary Medicine, Champaign-Urbana (Urbana, IL) in 2010. Following veterinary school, she completed a Laboratory Animal Medicine Residency at Cornell University within the Department of Biomedical Sciences. During the residency, she was accepted into the Cornell Graduate School in the Field of Comparative Biomedical Sciences from which she will be receiving a Master of Science in August 2013.

This is dedicated to Dr. Paul Bowser for his mentorship, friendship, and unwavering faith in me.

ACKNOWLEDGMENTS

I would like to thank my committee members, Dr. Paul Bowser and Dr. Kirk Maurer, for their excellent support, mentorship, and guidance throughout this project. To everyone in the Bowser lab, especially Dr. Rod Getchell, Dr. Emily Cornwell, and Greg Wooster: without your help and technical support, this project would not have been possible. I can't thank you enough for your continuous patience and assistance. For my collaborators, Dr. George Ketola and Phyllis Randall (USGS, Tunison Laboratory of Aquatic Science), thank you for your expertise and support. I really enjoyed working with you and your facility. To Dr. Rocco Cipriano, thank you for providing a virulent strain of *A. salmonicida* for use in my study. To the USGS Tunison Laboratory of Aquatic Science and the NYSDEC Bath Fish Hatchery, thank you for your donation of rainbow trout. To the New York State Animal Health Diagnostic Center, especially Dr. Heather Priest, thank you for all of your assistance processing and interpreting samples. Additionally, I would like to thank the Department of Biomedical Sciences and my co-workers in the Center for Animal Resources and Education (CARE) for their support. Lastly, I'd like to thank my funding source, the National Research Support Project No. 7 (NSRP-7), Minor Use Animal Drug Program. Your support for minor animal species and minor drug uses is essential to improving the health of our aquaculture animals.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE:

INTRODUCTION

Overview

Aquaculture is an important economic asset for many countries, with an estimated $1 billion annual production in the United States alone and over $100 billion of production worldwide. Although the U.S. is a relatively small producer of aquaculture products, it is one of the largest seafood importers worldwide, with a trade deficit of over $10.4 billion annually (NOAA 2013). As aquaculture grows, it is important to emphasize aquatic animal welfare as it has broad impacts on stress response and resistance to disease, which in turn has serious consequences on industry sustainability (Ashley 2007).

Due to high product demand, high operating costs, and limited environmental resources, aquaculture facilities often hold a large number of animals within a small space, resulting in the need for emphasis on improved health management to reduce stress and associated morbidity/mortality. Stressors within the aquaculture setting include physical stressors (temperature fluctuations, water quality changes, sounds, lights, and dissolved gasses), biological stressors (high density, spawning season, pathogenic and nonpathogenic microorganisms, and internal and external parasites), chemical stressors (poor water quality, pollution, improper diet composition, and metabolic waste products) and procedural stressors (disease treatments, handling, and shipping). All of these factors have the ability to influence the health of the animals as well as their susceptibility to disease, which is why it is essential to ensure appropriate animal welfare and health management (Francis-Floyd 2012).

It is well known that diet has a strong effect on health and stress tolerance; therefore, it is essential to feed adequate quantities of diets meeting all nutritional requirements for proper growth and health (Trichet 2010). Information on required nutrients in aquatic species is limited, though basic data is available to assure minimum requirements are met in diet formulations for the majority of common species; however, data concerning bioavailability and metabolism of these nutrients is often lacking (Oliva-Teles 2012). Dietary factors, including essential and non-essential nutrients, have been shown to have specific effects on the immune response when provided at pharmacological levels (Trichet 2010). Additionally, constituents other than essential nutrients, such as probiotics, prebiotics, and immunostimulants, have been considered in aquatic nutrition to improve feed efficiency/growth, health, stress tolerance, and resistance to disease (Oliva-Teles 2012).

While it is generally possible for disease management to be accomplished using pharmaceutical drugs, this method has a high consequence in terms of environmental contamination and drug resistance. Additionally, aquaculture animals are meant for human consumption; therefore, only FDA-labeled drugs can be used and withdrawal periods must be specified and closely observed.

Currently in the United States, there are only three FDA-labeled antibiotics for usage in aquaculture. These drugs are florfenicol (Aquaflor®), oxytetracycline (Terramycin®-200 for Fish), and sulfadimethoxine/ormetoprim (Romet®-30), which are labeled for specific culture-positive pathogens within specific aquatic species (FDA 2013). There is a large concern that the usage of antibiotics within aquatic systems will have significant impacts on the environment, ecosystem, and other species. Historically, antibiotic overuse in aquaculture, agriculture, and human medicine have allowed for the development of multi-drug resistant bacteria, resistance

genes, and drug residues found within the environment. Since antibiotic resistance is a major concern, prevention measures, including risk management, environmental management, and alternative treatment measures, including vaccination, have become much more predominant in aquaculture, with antibiotic usage recommended as a last resort (FAO/WHO/OIE 2006).

Dietary Supplementation and Allicin

In an attempt to find alternative methods to pharmaceutical drugs for the prevention and treatment of disease in aquatic organisms, dietary supplements have been suggested for use in aquaculture. In order for a dietary supplement to be used, it must be effective, low-cost, and palatable to the fish.

The usage of therapeutic or medicinal dietary supplements (e.g. plants, minerals, oils) in humans has been practiced for thousands of years in many cultures. Ancient civilizations, including Egyptian, Greek, Indian, Roman, Viking, and Chinese, utilized garlic for the treatment of varied health conditions as early as 3,000 B.C. (Block 1985; Rivlin 2001). Garlic is a proven prophylactic and therapeutic in humans and agricultural species, leading to interest in its use in aquaculture. It is easily obtained, inexpensive, and acts against a broad spectrum of pathogens. The efficacy depends on the formulation, dose, and route of administration and overdosing may have negative impacts; therefore, it is important to optimize the dosing for each species prior to usage (Lee and Gao 2012).

Garlic (*Allium sativum*) belongs to the *Liliaceae* family and is cultivated across the world for its fleshy bulbs (cloves). The main biological effects of garlic are attributed to its characteristic organosulfur compounds. The principle active substance in garlic is allicin (diallyl

thiosulfate), which is responsible for its pungent smell and its therapeutic properties. Allicin is activated by crushing or cutting the garlic cloves (Block 1985).

Garlic has a wide spectrum of activity, including antibacterial (Cavallito and Bailey 1944), antiviral (Weber, Andersen et al. 1992), antiprotozoal (Ankri, Miron et al. 1997), and antifungal (Gupta and Porter 2001). In terms of antibacterial properties, garlic has effects on both gram-negative and gram-positive bacteria as well as effects on many drug resistant strains of bacteria, such as methicillin-resistant *Staphylococcus aureus* (Cutler and Wilson 2004) and *Helicobacter pylori* (Jonkers, van den Broek et al. 1999).

Though the exact mechanisms of allicin are not fully understood, it is generally accepted that allicin facilitates the function of phagocytic cells and increases their bactericidal activities; additionally, it stimulates natural killer cells, complement, lysozyme, and antibody responses (Talpur and Ikhwanuddin 2012). The up-regulation of these immunological functions are associated with increased protection against infectious disease (Lee and Gao 2012). Allicin can also function directly on pathogens, by completely inhibiting RNA synthesis as well as partially inhibiting DNA and protein synthesis (Feldberg, Chang et al. 1988). Functional activities of garlic include free radical scavenging activities, immune up regulation, anti-infectious and anti-cancer properties, and cardiovascular disease treatment (Colic and Savic 2000; Harris, Cottrell et al. 2001; Khanum, Anilakumar et al. 2004; Borek 2006; Herman-Antosiewicz, Powolny et al. 2007; Singh, Vinjamury et al. 2007).

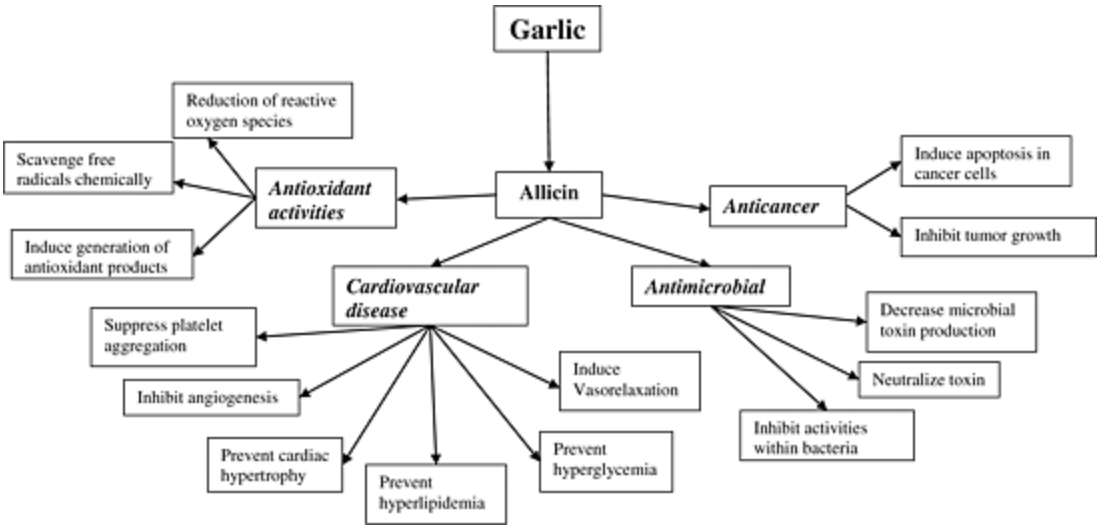


Figure 1: Summary of the therapeutic effects of allicin (Chan, Yuen et al. 2013)

Herbs initially add flavor to foods and thereby influence eating patterns, the secretion of digestive fluids, and total feed intake (Lee and Gao 2012). Allicin has a strong stimulatory effect on olfaction in many aquatic animals, inducing fish to eat and subsequently increasing feed intake. Additionally, allicin can enhance immune-competence, improve gastric motility, and modulate secretion of enzymes to improve digestion and nutrient absorption (Lee and Gao 2012). Several studies have documented the effect of allicin as a growth promoter in multiple species, including carp and tilapia, after long-term dosing of at least three months (Fo, Han et al. 1990; Zeng, Ren et al. 1996; Jia, Hu et al. 1997; Hu 1999; Jia 1999).

However, results of several other studies have demonstrated that garlic is not optimal for all fish species and can be very dose-dependent. Hatching rates of Manila clam (*Ruditapes philippinarum*) decreased with increasing concentrations of allicin (Yang, Zuo et al. 2010), growth rates declined in the red belly pacu (*Colossoma barchypomum*) with increasing concentrations of allicin (Xiang and Liu 2002), and the rice field eel (*Monopterus albus*) died

after being fed allicin. (Huang, He et al. 2001). This again highlights the fact that while garlic has many health benefits, it is important to optimize the dosing prior to usage in any species.

Several recent studies have demonstrated the ability of dietary supplements, including garlic, to upregulate immunity and increase resistance to disease in multiple fish species. One study determined that diets supplemented with dried rosemary (*Rosmarinus officinalis*) significantly reduced mortality in tilapia (*Oreochromis spp*) infected with *Streptococcus agalactiae* (Zilberg, Tal et al. 2010). Another study determined that Nile tilapia (*Oreochromis niloticus*) fed either Echinacea (*Ecinacea purpurea*) or garlic showed significant disease resistance when challenged with *Aeromonas hydrophila* as well as a significant increase in survival rate, leucocyte count, and body weight (Aly and Mohamed 2010). In Asian sea bass (*Lates calcirifer*) challenged with *Vibrio harveyi* infection, garlic supplementation at 10g/kg feed significantly improved survival. Additionally, there was a significant increase in growth, weight gain, and feed conversion in garlic fed groups as well as enhanced erythrocytes, leucocytes, hematocrit, hemoglobin, phagocytic activity, respiratory burst, lysozyme, anti-protease, and bactericidal activities (Talpur and Ikhwanuddin 2012).

This thesis expands upon studies performed by Drs. Nya and Austin, in which rainbow trout (*Oncorynchus mykiss*) were fed allicin at varied concentrations (0%, 0.5%, and 1%), challenged with the bacteria *Aeromonas hydrophila*, and monitored for morbidity and mortality. Mortality was reduced from 80% in the controls to 8% (0.5% dose) and 0% (1.0% dose). The Nya and Austin studies concluded that allicin was highly inhibitory against *A. hydrophila* and did not affect body weight, length, or weight gain. Additionally, fish fed allicin had higher quantity of erythrocytes and lymphocytes, with increased serum protein, serum lysosome activity, and serum bactericidal activity (Nya and Austin 2009; Nya, Dawood et al. 2010; Nya and Austin

2011). Though allicin is generally an unstable compound, this study also found that an allicin supplemented diet provided protection against *A. hydrophila* up to 28 days, though there was a steady reduction in the level of protection as time passed (Nya and Austin 2011). Our study followed the materials and methods from the Nya and Austin 2009 study, including feed preparation and feed duration, but a different common salmonid pathogen, *Aeromonas salmonicida*, was used for the infection challenge.

In work performed by our collaborator, Dr. George Ketola, multiple essential oils were used in-vitro to determine minimum inhibitory concentration against *A. salmonicida*. Dr. Ketola determined that allicin was inhibitive against growth of *A. salmonicida* on culture at 10% and 20% emulsions, demonstrating that allicin has direct effects on bacterial metabolism (Ketola, Starliper et al. 2012).

Aeromonas salmonicida

Furunculosis, a disease caused by *Aeromonas salmonicida*, can have significant economic impacts on fish, especially in the intensive aquaculture setting. Typically this bacterium affects salmonids, though atypical strains can infect many warm water, cold water, freshwater, and marine species (Bullock, Cipriano et al. 1983).

A. salmonicida is a gram-negative, non-motile rod, which grows readily on tryptic soy agar or brain-heart infusion agar and forms a brown diffusible pigment, usually within 72 hours at room temperature. Virulence factors include the production of extracellular products, such as hemolysins, proteases, and leucocidin, which are associated with the majority of the pathology associated with clinical signs (Cipriano 1983). Additionally, it was discovered that virulent strains have an extra-membrane protein called the "A-layer," which enhances bacterial cell

hydrophobicity, cell-to-cell aggregation, and cell-to-tissue adhesion (Udey and Fryer 1978). One method to determine whether a strain is virulent is culture on Coomassie Brilliant Blue agar (CBB) (Markwardt, Gocha et al. 1989). CBB is a protein specific dye, incorporated into common growth media, which can differentiate between A-layer positive and A-layer negative strains. For A-layer positive strains, the dye is incorporated into the colony growth, presenting as blue colonies, whereas A-layer negative strains do not incorporate the dye and will present as white colonies (Cipriano and Bertolini 1988).

Transmission occurs through contact with infected fish and contaminated water. Individuals that survive epizootic disease usually become carriers and serve as the prime reservoir for infection (Cipriano 1983). Although *A. salmonicida* typically does not survive in environments without fish, some mutated strains are able to survive in bodies of water, on equipment, and in tissues from dead fish for 6-32 days (McCarthy 1977).

In peracute infections, fingerlings turn dark in color and die. Clinical signs are generally not observed with the exception of slight exophthalmos. Acute infections occur in sub-adult and adult fish, with signs of darkened coloration, anorexia, and hemorrhage at the base of the fins. Internal hemorrhage also occurs within the viscera, abdominal walls, and heart. Other gross findings include splenomegaly, petechial hemorrhage, and sloughed epithelium, mucus, and blood within the gastrointestinal tract. In chronic furunculosis, usually in older fish, "furuncles" may be present on the flanks as well as visceral congestion and peritonitis. Furuncles in fish are described as areas of necrotic tissue, fluid exudate, and macrophages, differing from true furuncles of homeotherms (Klontz, Yasutake et al. 1966; McCarthy and Roberts 1980; Ellis, Hastings et al. 1981; Bullock, Cipriano et al. 1983).

In experimentally infected fish injected intramuscularly, a focus of infection develops at the site of injection, resulting in myofibrillar necrosis, vascular necrosis, and hemorrhage. Severe leukopenia occurs within 72 hours with resulting septicemia. In natural infections, foci of bacteria within minimal to no inflammation occur in the kidneys, spleen and myocardium, leading to hematopoietic necrosis and myocardial and renal tubular degeneration. In chronically infected fish, heart and spleen are the most commonly affected organs (Klontz, Yasutake et al. 1966; McCarthy and Roberts 1980; Ellis, Hastings et al. 1981; Bullock, Cipriano et al. 1983).

Study Goals

The goal of this study was to determine the efficacy and safety of allicin, when added to the diets of rainbow trout, as a management tool to reduce or eliminate the risk posed by the bacterial pathogen *Aeromonas salmonicida*. The study was based on the aforementioned in-vitro work by Ketola, 2012 as well as in-vivo work by Nya and Austin, 2011.

Experimental Plan

The work represented within this thesis was performed as three separate experiments. First, a direct infection trial was performed to determine the efficacy of allicin in animals infected intraceolomically with a modified LD_{50} (mLD_{50}) dose of *A. salmonicida*. Second, a target animal safety trial was performed to assess the safety of the compound at varied incremental doses based on the anticipated optimal dose. According to FDA guidelines, "the aim of target animal safety studies is to provide information on the safety of an investigational veterinary pharmaceutical product in the intended species under the proposed conditions of use. Furthermore, adverse effects associated with overdoses and increased duration of administration of the product should be identified" (FDA 2009). Although allicin is a dietary supplement and

not a pharmaceutical product, the target animal safety trial was performed to assess safety using the same standards as a pharmaceutical product. Third, a waterborne infection trial was attempted in order to mimic a more natural route of infection with *A. salmonicida* to determine if fish fed allicin would be more resistant to bacterial contaminated water and infected fish.

Hypotheses

For the infection trials, the null hypothesis was that rainbow trout fed allicin at varying concentrations will not have improved survivability when challenged with *A. salmonicida* as compared to rainbow trout fed standard trout feed. Additionally, there will be no difference in systemic quantities of bacteria based on the concentration of allicin fed. There were three alternate hypotheses: 1) Rainbow trout fed allicin will have increased survivability when challenged with *A. salmonicida* as compared to rainbow trout fed standard trout feed; 2) Rainbow trout fed the highest dose (2% allicin) will have the best survivability when challenged with *A. salmonicida;* and 3) Rainbow trout fed allicin will have lower quantities of systemic *A. salmonicida* post-infection.

For the target animal safety trial, the null hypothesis was that rainbow trout fed allicin at 0x (0%), 1x (0.5%), 3x (1.5%), and 5x (2.5%) the target concentration (0.5%) for 3x duration (6 weeks) of the previous studies will not have effects on growth, erythrocyte and leukocyte panels, serum chemistry parameters, or gills, liver, kidney, and spleen (i.e., the compound is safe at all test doses). The alternate hypothesis was that there is an effect on growth, erythrocyte and leukocyte panels, serum chemistry parameters, or gills, liver, kidney, and spleen based on the allicin dosing (i.e., the compound is detrimental/toxic at some doses).

CHAPTER TWO:

MATERIALS AND METHODS

Animal Husbandry

All protocols were approved by the Cornell University Institutional Animal Care and Use Committee and the Tunison Laboratory of Aquatic Science, United States Geological Survey (USGS).

For all infection studies, young rainbow trout (*Oncorhynchus mykiss*), approximately 4-6cm total length (TL), were obtained from the USGS Tunison Laboratory of Aquatic Science (Cortland, NY) and the NYSDEC Bath Fish Hatchery (Bath, NY). Fish were maintained in the Aquatic Animal Health Program Research Facility, College of Veterinary Medicine, Cornell University, a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. The experimental holding system consisted of multiple 75.7L glass aquaria placed in 719.2L Min-O-Cool (Frigid Units Inc., Toledo, OH) tanks individually supplied with de-chlorinated flowing water (15°C) and aeration. Each larger tank held up to five 75.7L aquaria, with 14-20 fish per aquaria.

For the target animal safety study, yearling rainbow trout, approximately 8-12cm TL, were maintained by the USGS Tunison Laboratory of Aquatic Science in outdoor concrete raceways (dimensions 1.5 x 5.8 x 0.5m; flow rate 5-7L/min) with water sourced from a local spring at ambient temperature. This facility is managed in accordance with the American Fisheries Society Fish Health Section Blue Book (AFS 2012).

Establishment of a modified LD_{50} dose of *Aeromonas salmonicida*

The modified LD_{50} dose of *A. salmonicida* was determined in five groups of fish. Each group contained ten yearling rainbow trout (approximately 6cm TL). All fish were injected with a 0.1mL intraceolomic (IC) injection containing either sterile saline or *A. salmonicida* at 10^3 CFU/0.1mL, 10^4 CFU/0.1mL, 10^5 CFU/0.1mL, or 10^6 CFU/0.1mL. Fish were held for 28 days and were monitored twice daily for morbidity or mortality. Any fish exhibiting abnormal behavior or signs of illness/distress were euthanized immediately with an overdose of tricaine methanesulfonate (Tricaine-S, Western Chemical, Ferndale, WA) buffered with an equal weight of sodium bicarbonate (Sigma, St. Louis, MO). Post mortem analysis included length and weight, a complete necropsy, and aseptic kidney culture on Brain Heart Infusion (BHI) agar (Becton Dickinson, Sparks, MD).

Diet Preparation and Feeding

A standard trout diet was used for the basis of all feed (Ziegler Feed – Slow Sinking 2.5mm Finfish, Ziegler Bros., Inc., Gardener, PA). Allicin as Allimed® liquid was obtained (Allicin International, East Sussex, TN) and feed was coated evenly depending on allicin treatment dosage: 0.5% (5mg Allimed®/1kg feed), 1.0% (10mg Allimed®/1kg feed) and 2.0% (20mg Allimed®/1kg feed).

For fourteen days prior to infection, fish were fed test diets depending on group (control 0%, 0.5% allicin, 1.0% allicin, 2.0% allicin) at 1% body weight per day. Feed was withheld for one day post-infection. For the remaining time on study, fish were fed control diet at 1% body weight per day (Nya and Austin 2011).

Feeding Trial by Direct Infection

Study groups included uninfected negative controls, untreated infected positive controls (0% allicin dose), 0.5% allicin, 1.0% allicin, and 2.0% allicin. Each treatment group contained 20 fish, housed within 75.7L aquaria, with four replicate tanks per group (80 fish total per treatment). Fish were randomized into study groups and tank assignments using a random number generator (random.org) and were fed their respective treatment diets for fourteen days prior to the beginning of the infection trial. On day 0, all fish in the negative control group received a 0.1 mL IC injection of sterile 0.85 % saline and were housed in a specific pathogen free room (free of *A. salmonicida*). All fish in the remaining study groups received a 0.1 mL IC injection containing a mLD_{50} dose of *A. salmonicida* (as determined by previous studies). Fish were monitored twice daily for morbidity and mortality. Any fish exhibiting abnormal behavior or signs of illness/distress were euthanized immediately with an overdose of tricaine methanesulfonate as previously described. After 28 days, all fish were euthanized for analysis, including length and weight, complete necropsy, and aseptic kidney culture on BHI agar (Nya and Austin 2009; Nya, Dawood et al. 2010; Nya and Austin 2011). Additionally, kidney tissue was collected for quantitative PCR (qPCR) to determine the quantity of *A. salmonicida* within each fish.

Target Animal Safety Trial

This study was performed in outdoor raceways at the Tunison Laboratory of Aquatic Science under USGS protocols. The target dose was 0.5% allicin (as determined by previous infection studies). Fish were fed test diets at 1% body weight per day containing 0% (0x), 1x 0.5% (1x), 1.5% (3x), and 2.5% (5x) allicin for 6 weeks (3 times the length of the initial trials).

Each group contained between 23 and 27 fish. At the termination of the study, all fish were euthanized with an overdose of tricaine methanesulfonate as previously described. A blood sample was immediately collected from the ventral tail vein using a vacutainer collection system (Becton Dickinson) for a blood smear and clinical chemistry panel. Gills, kidney, liver, and spleen were collected for histopathology.

Feeding Trial by Waterborne Exposure

Study groups included uninfected negative controls, untreated positive infected controls, 0.5% allicin, 1.0% allicin, and 2.0% allicin. Each group contained 14 fish, housed within 75.7L aquaria, with four replicate tanks per group (n=280). Fish were randomized within study groups using a random number generator (random.org) and were fed their respective test diets for fourteen days prior to the beginning of the infection trial. On day 0, four fish in the negative control group received a 0.1 mL IP injection of sterile saline and the remaining ten fish were not inoculated. All negative control fish were housed in a specific pathogen free room (free of *A. salmonicida*). For the remaining study groups, four fish per tank received a 0.1 mL IC injection containing a mLD_{50} dose of *A. salmonicida* (as determined by previous studies) and the remaining ten fish per tank were not inoculated. Fish were monitored twice daily for morbidity and mortality. Any fish exhibiting abnormal behavior or signs of illness/distress were euthanized immediately with an overdose of tricaine methanesulfonate as previously described. After 38 days, all fish were euthanized. Analysis included length and weight, a complete necropsy, aseptic kidney culture on BHI agar, and quantitative PCR.

Blood Smear

Blood smears were stained with Wrights stain by the New York State Animal Health Diagnostic Center (AHDC), Clinical Pathology (Ithaca, NY). Erythrocytes were evaluated for morphology. A leukocyte estimate was performed by counting the number of white blood cells per 10 high powered fields (HPF, 100X magnification), using the formula WBC estimate = Average # WBC/HPF x 3,500. A leukocyte differential was performed by counting 100 white cells on 100x, noting the specific type of cell (lymphocyte, monocyte, and neutrophil) as a percentage of the total number observed.

Serum Chemistry Panel

Blood chemistry panels were performed by the AHDC, Clinical Pathology (Ithaca, NY). Approximately 1mL of serum per fish was submitted in a lithium heparinized tube (Becton Dickinson) for processing.

Histopathology

Tissues for histopathology were fixed in 10% neutral buffered formalin (Sigma, St. Louis, MO) for at least 24 hours. Gills were further processed by placing them in 14% sodium EDTA (Sigma) for 7 days for decalcification (Luna 1968). All tissue cassettes were submitted to the AHDC Histology Laboratory for block preparation and slide processing with hematoxylin and eosin staining using using AHDC protocols. For density assessment of pigment-containing macrophage centers within the kidneys, a scoring system of 1-5 was created (1=no pigment-containing macrophage centers to 5=heavy pigment-containing macrophage centers) and slides were scored by a blinded observer to determine trends.

Bacterial Preparation

Aliquots of *A. salmonicida* strain 3.177 (obtained from a clinical salmonid case, courtesy Dr. Rocco Cipriano, USGS, Leetown, WV) were held at -80^{o}C in 10% glycerol (Amresco, Solon, OH). Prior to each study, an aliquot was thawed and plated onto BHI agar (Becton Dickinson) and allowed to grow at room temperature for 72 hours. Additionally, an aliquot was plated onto Comassie Brilliant Blue (CBB) agar (Brilliant Blue R 250, Sigma-Aldrich) to confirm virulence as indicated by the presence of the A-layer. After 72 hours of growth, approximately 20 colonies were suspended in BHI broth (Becton Dickinson). The suspension was allowed to grow at room temperature with gentle agitation until reaching a spectrophotometer (Spectronic 21, Bausch and Lomb, Rochester, NY) reading of 0.06 OD_{525}, which correlated to a $1x10^{8}$ CFU/mL reading according to previous growth curve studies performed within the laboratory with this strain of bacteria. Serial 10-fold dilutions were performed with sterile saline to reach the pre-determined mLD_{50} dose.

Bacterial Culture

The posterior kidney was swabbed using aseptic technique and streaked onto BHI agar (Becton Dickinson). Plates were held at room temperature for 7 to 21 days. Plates containing a brown-diffusible pigment were considered positive for *A. salmonicida.*

DNA Extraction and Quantitative PCR

A portion of posterior kidney was removed and placed in a sterile bead-beater tube (Biospec Products) containing a 1.3 mm chrome steel bead. HMEM-10 (200 uL) was added to the samples and then the samples were homogenized using a Minibeater-16 (BioSpec Products) for 1 min. Samples were then centrifuged at 8000 x g for 2 min prior to DNA

extraction. Extraction of DNA was performed using a MagMax magnetic bead extraction system and the MagMax-96 viral RNA isolation kit (Life Technologies, Carlsbad, California) using the protocols described in the kit and the manufacturer-supplied extraction program AM1836_DW_50_V2. Eluted DNA was immediately aliquoted into sterile microcentrifuge tubes following extraction and frozen at -80 °C until use in the qPCR assay.

The qPCR assay used in this study was developed from the *Aeromonas salmonicida* S-layer protein gene *vap*A (Gustafson, Thomas et al. 1992). The primers and FAM-TAMRA probe were created with the help of Primer Express Version 3 (Applied Biosystems) and synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa). Primer sequences were VapA-For 5'-CCC GTA AAG CAC TGT CTG TTA CC–3' and VapA-Rev 5'-GCA ACA TCA GCA GGC TTC AG–3'. The VapA probe sequence was 5'-/56-FAM/TGC CAA GCG GTG GTG CAG TGA /36-TAMSp/-3'. A 23bp fragment was amplified during the assay. Assays were run on an ABI 7500 detector, a linear regression was calculated from five DNA standards ranging from 1.5×10^2 to 1.5×10^6, and all plates were run for 42 cycles. DNA quality and quantity was measured using a Quawell Q3000 UV spectrophotometer (San Jose, California). All qPCR copy numbers are reported per 50ng total DNA.

Statistical Analyses

Survival data was analyzed with a Mantel-Cox log rank test. Parametric data were analyzed utilizing Chi-square analysis or one-way analysis of variance using Dunnett method followed by Tukey's post-test or *t* test. Nonparametric data were analyzed by the Wilcoxon test followed by Dunn's post-test. *P* values of ≤ 0.05 were considered statistically significant. All analyses were made with GraphPad Prism® 5.02 (GraphPad Software Inc., San Diego, CA), JMP® 10 (SAS Institute, Inc., Cary, NC), or Statistix® 8 (Analytical Software, Tallahassee, FL).

CHAPTER THREE:

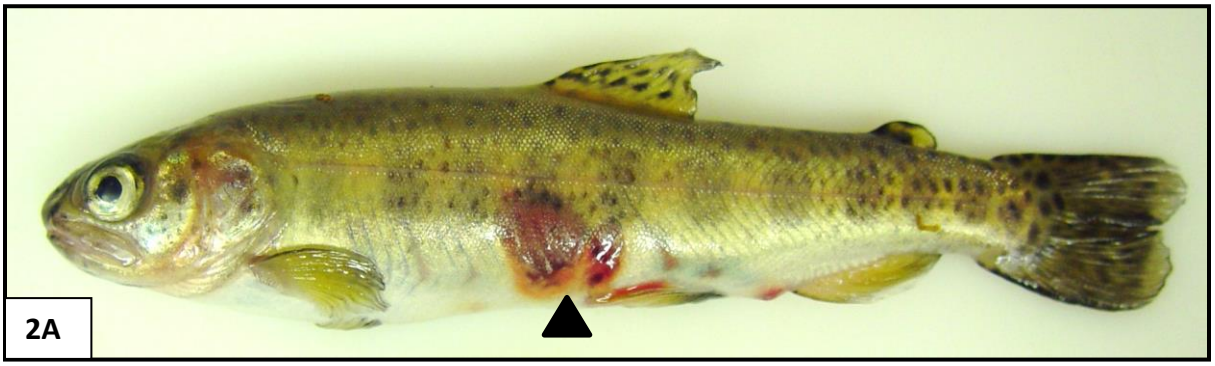
RESULTS

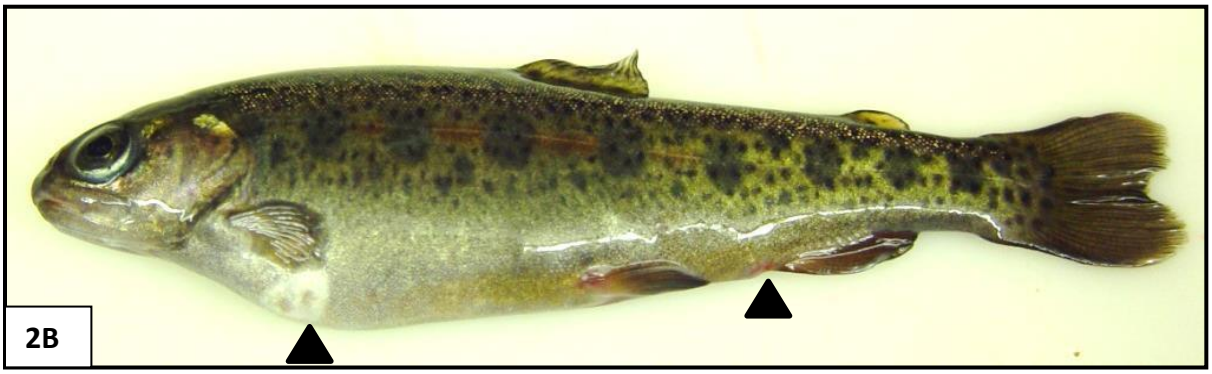
Modified LD_{50} Establishment

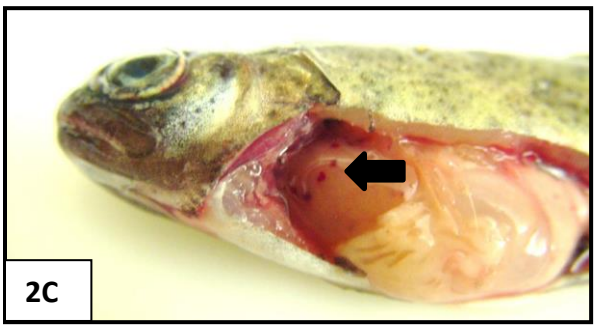
For fish from the USGS Tunison Laboratory of Aquatic Science, the mLD_{50} dose of *A. salmonicida* was 10^3 CFU/fish. For fish from the NYSDEC Bath Hatchery, the mLD_{50} dose was 10^5 CFU/fish (Figures 2A-2C show typical gross lesions). Survival curve data is not shown.

Figures 2A-2C: Gross lesions associated with *A. salmonicida* infection

A) Severe focal myofibrillar necrosis at injection site, hemorrhage at base of caudal fins and vent, B) abdominal swelling, hemorrhage at base of caudal fins and from vent, C) multifocal hepatic petechiation.







Feeding Trial by Direct Infection

Two trials were performed. For the 1st direct infection feeding trial, the mLD_{50} dose was not reached and the number of positive controls was reduced to half of the originally intended animals (20 rather than 40 fish), so beneficial effects were likely not evident. There was no significant difference in survival between the positive control group (0%) and the 0.5% and 1% groups (p-values 0.317 and 0.718, respectively). However, there were significant detrimental differences in survival between the 2.0% group and the 0.5% and 1% groups (p-values 0.003 and 0.028, respectively) (Figure 3), with the 2% having significantly more mortality than the other groups.

Additionally, there was no significant difference in the quantity of bacteria detected from positive samples in any group nor was there any difference in the proportion of fish detected as positive on qPCR based on treatment group (Figures 4 and 5).

Figure 3: Trial 1 Survival

LD_{50} dose of *A. salmonicida* was not reached. No significant difference was noted between the positive control (0%) and the 0.5% and 1% groups. A significant detrimental effect was noted at 2% as compared to the 0.5% and 1% groups.

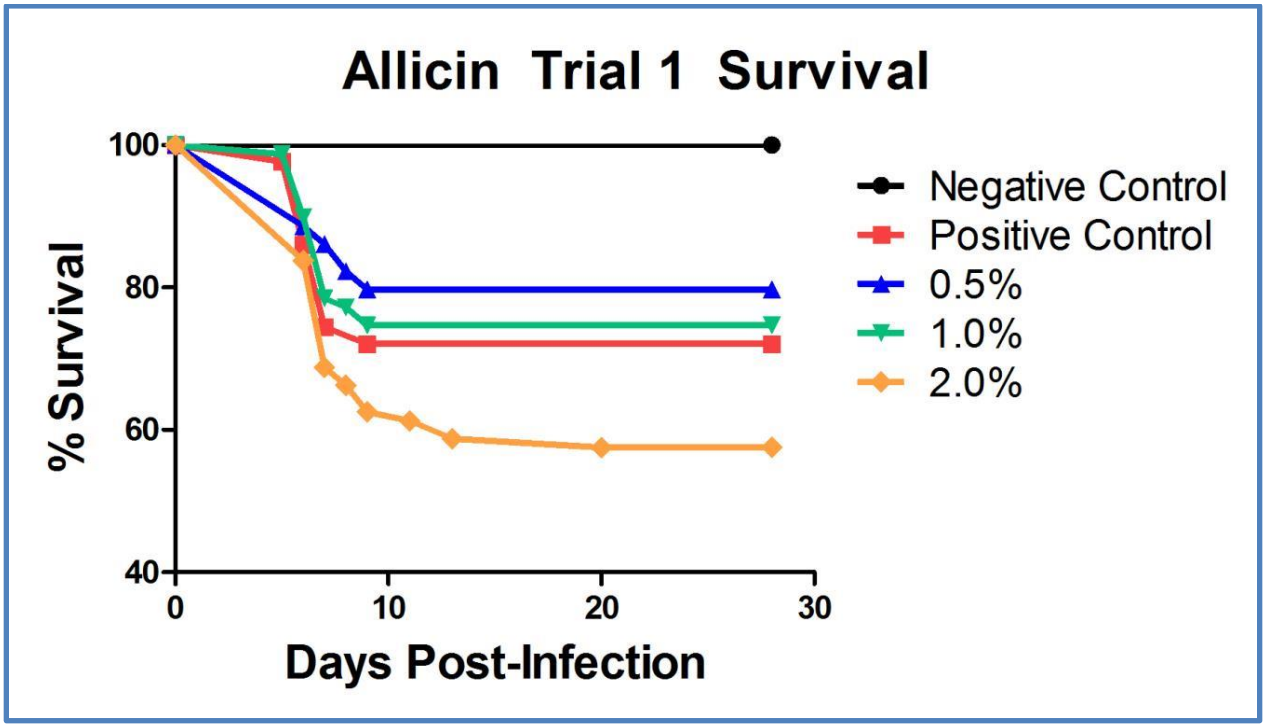


Figure 4: Trial 1 Systemic Bacterial Levels

No significant difference in *A. salmonicida* copy number per ng DNA within renal tissue for any treatment group.

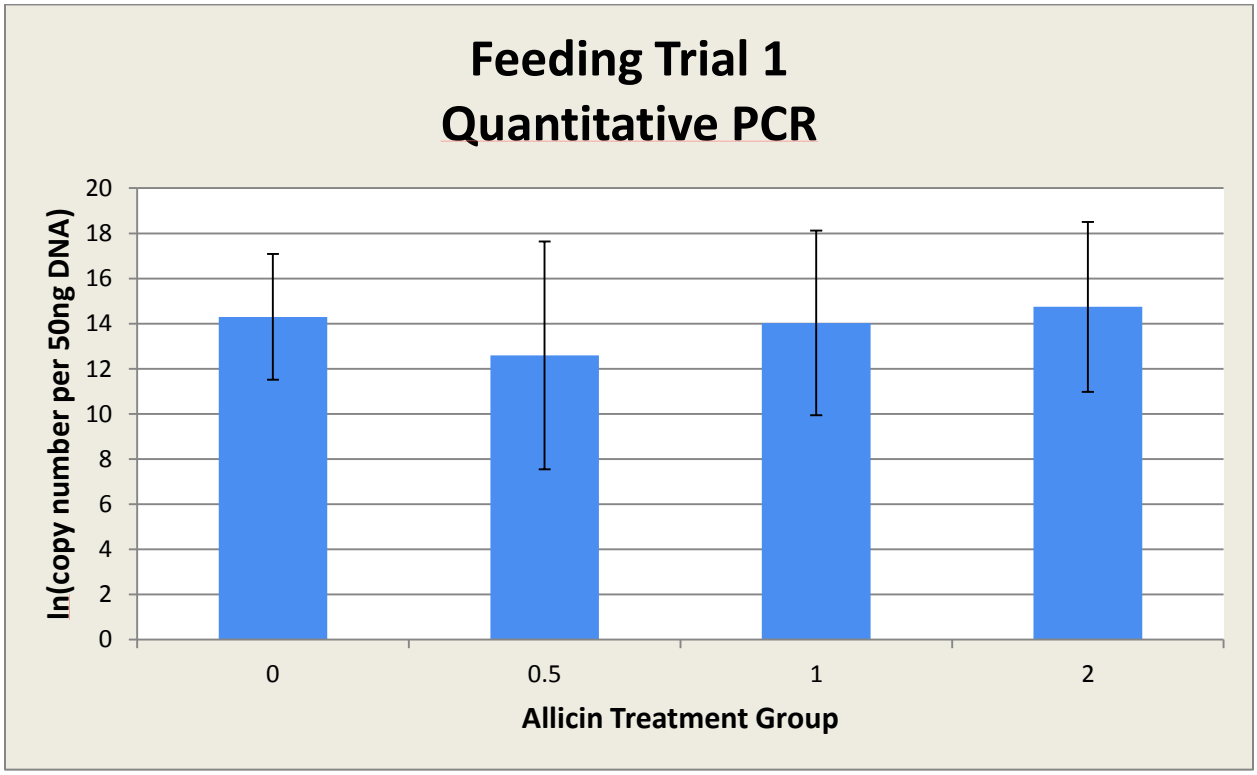
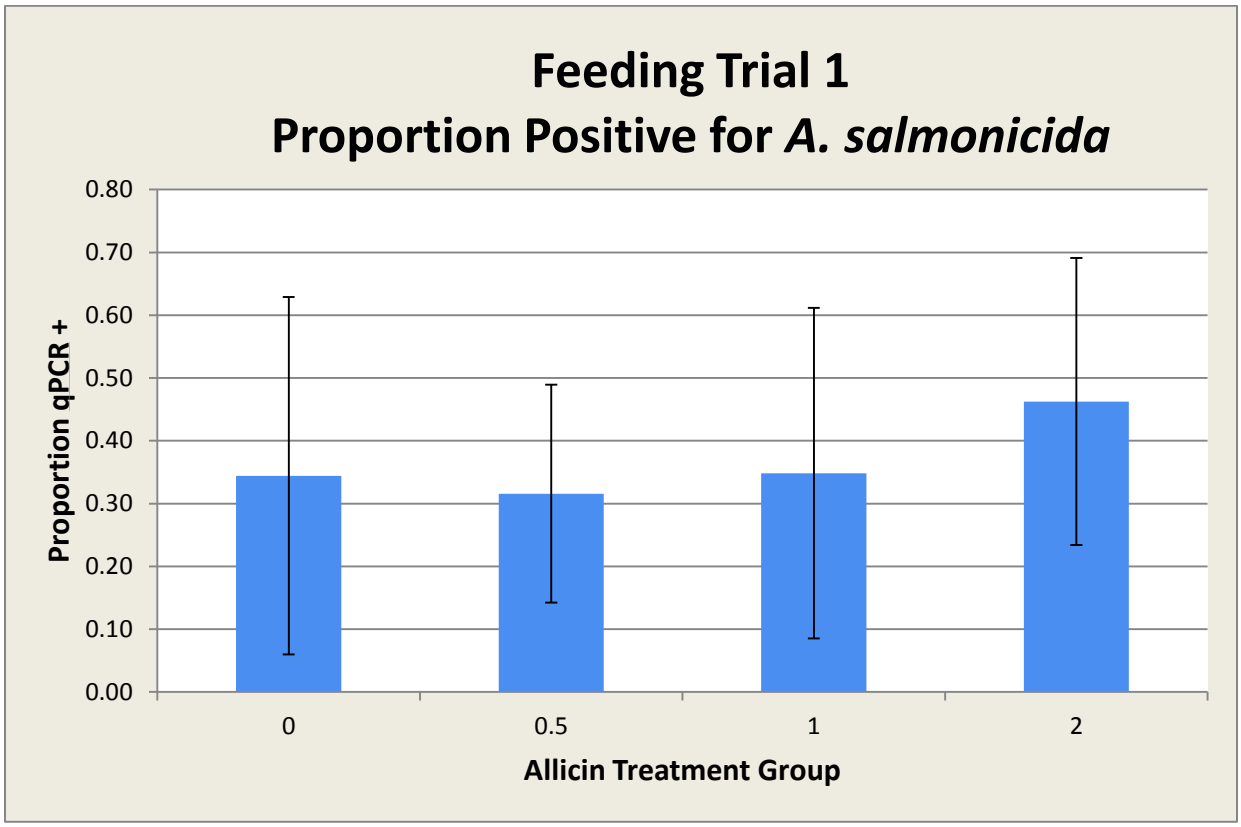


Figure 5: Trial 1 Proportion Infected

No significant difference in proportion of animals infected with *A. salmonicida* (positive by qPCR) for any treatment group.



For the 2nd direct infection feeding trial, there was a significant difference in survival between the positive control and the 0.5% and 1% groups (p-values 0.006 and 0.005, respectively), with these treatment groups promoting significant protection. Additionally, there were significant differences in survival between the 2% group and the 0.5% and 1% groups (p-values 0.002 and 0.018, respectively) (Figure 6).

Additionally, there was no significant difference in the quantity of bacteria detected from positive samples in any group; however, there was a significant difference in the proportion of positive samples detected per treatment group, with the 0.5% and 1% treatment groups having significantly less (p-values 0.023 and 0.008, respectively) infected animals compared to the 2% treatment group (Figures 7 and 8).

Figure 6: Trial 2 Survival

There was a significant difference ($p<0.05$) in survival between the 0.5% and 1% groups as compared to the positive control and 2% groups, with the 0.5% and 1% having improved survival.

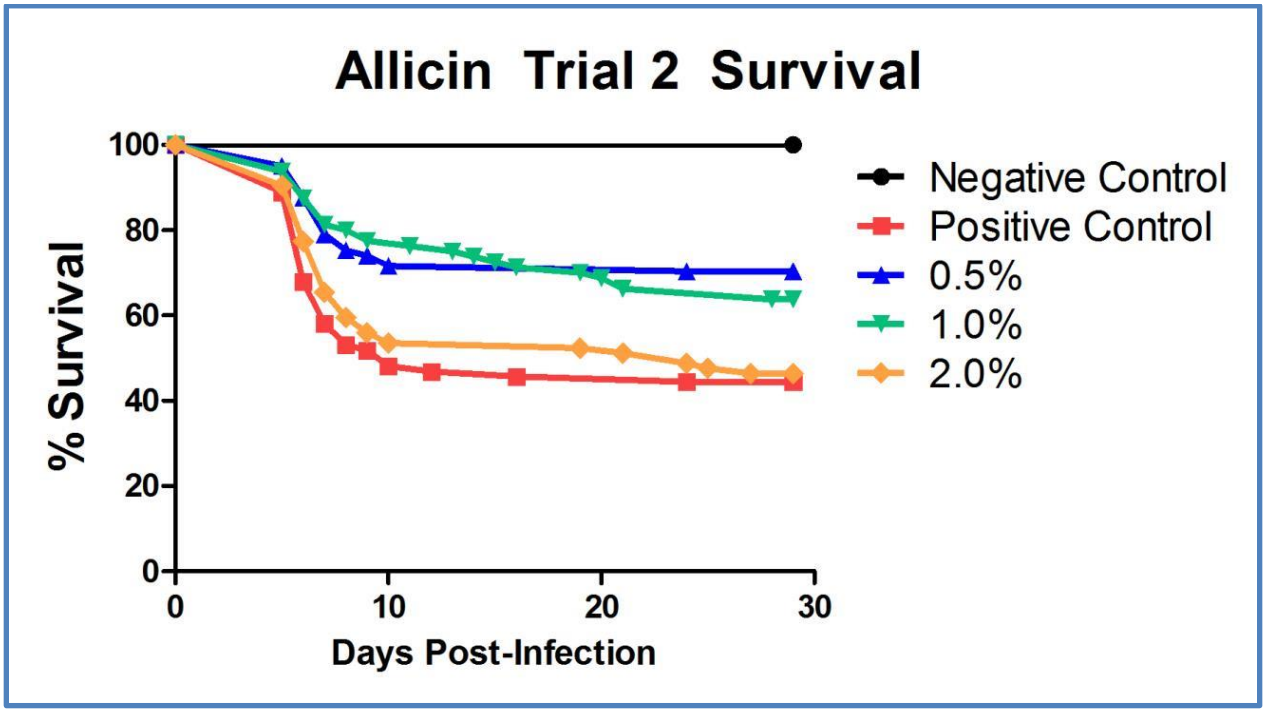


Figure 7: Trial 2 Systemic Bacterial Levels

No significant difference in *A. salmonicida* copy number per ng DNA within renal tissue for any treatment group.

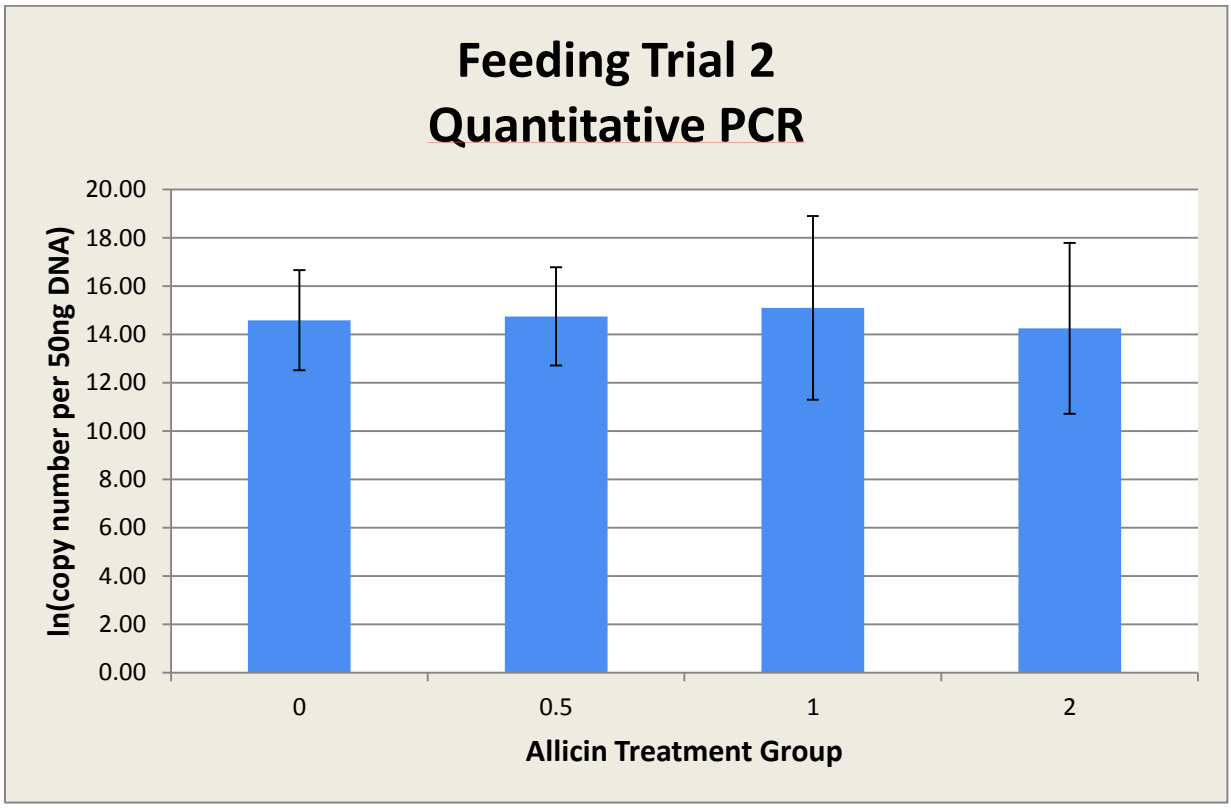
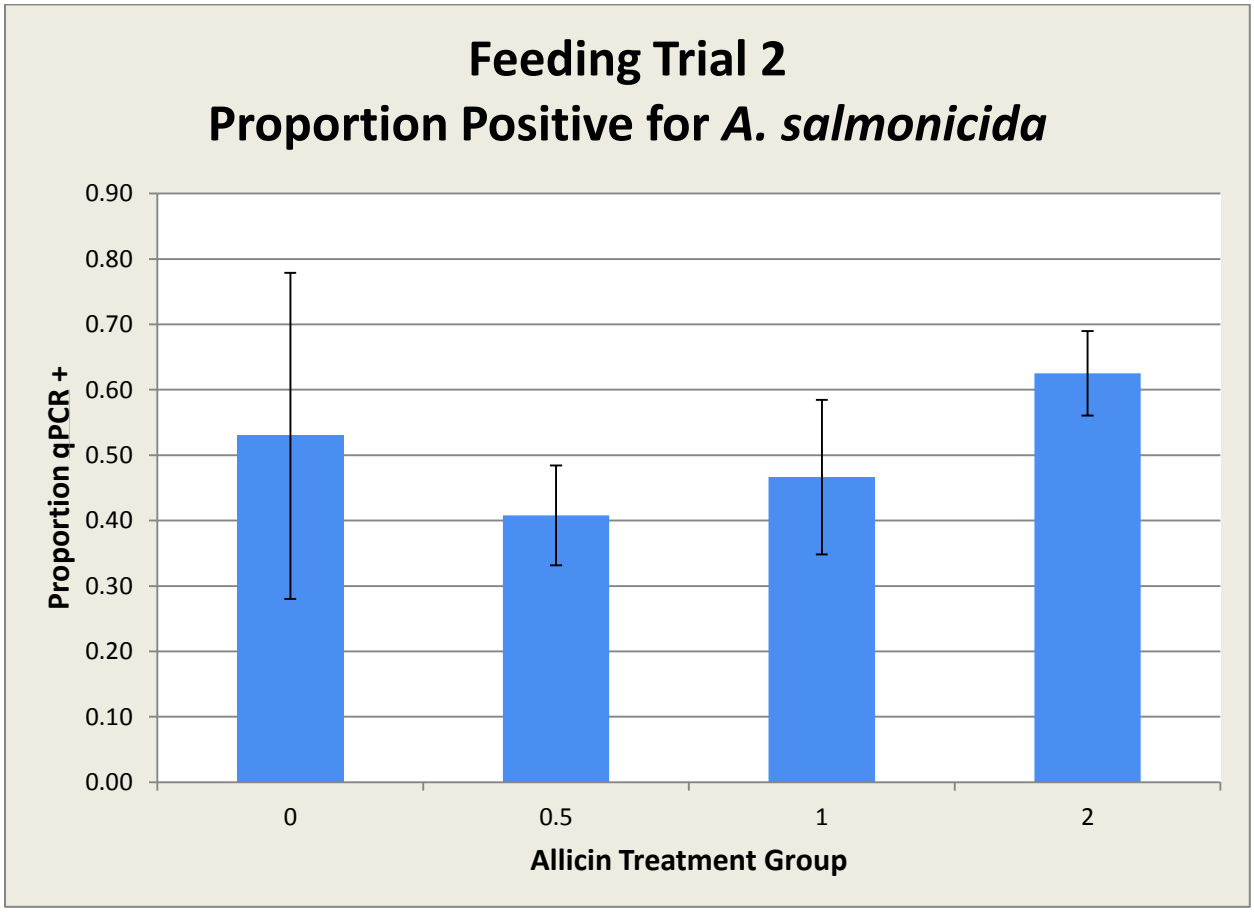


Figure 8: Trial 2 Proportion Infected

The 0.5% and 1% treatment groups have significantly ($p<0.05$) less proportion of the group infected (positive qPCR) than the 2% groups.



Target Animal Safety Trial

Length/Weight: There were no significant differences in average length or weight between treatment groups at the endpoint of the study (Table 1).

Blood Smear: Blood smears for the 0% allicin (0x) and the 2.5% allicin (5x) groups were evaluated. For both groups, red blood cell morphology was normal and polychromasia was marked. There was no significant difference in estimated white blood cell counts (cells/mL) for either group. Both groups had a very wide range of white cell counts (0%: 1,750-15,050 cells/mL, 2.5%: 3,150-11,900 cells/mL). For the white blood cell differential, there was a statistically significant difference in the percentage of lymphocytes and monocytes (p-values 0.02 and 0.003, respectively) with the 2.5% allicin group having a higher percentage of lymphocytes and a lower percentage of monocytes than the control group (Table 2).

Non-Mammalian Serum Chemistry Panel: There was no statistically significant difference between groups for the following serum chemistry parameters: sodium, potassium, chloride, uric acid, calcium, phosphate, total protein, glucose, AST, and lipemia. There was a statistically significant increase in the GDH values between the 0% (control) allicin group and the 0.5% (1X), 1.5% (3X), and 2.5% (5X) allicin groups for GDH (p-values <0.0001, <0.0001, 0.004 respectively), with all treatment groups elevated as compared to the control group (Table 3).

Histology: Gill, liver, and spleen tissue were normal from all groups. In the kidneys, there was a significant increase in the amount of pigment-containing macrophage centers between groups as the allicin dose increased (Table 4, Figures 9A and 9B).

Table 1: Target Animal Safety Average Lengths and Weights

Values are presented as mean ± SD. No significant ($P \leq 0.05$) differences between groups.

Allicin Dose	0% (0x)	0.5% (1x)	1.5% (3x)	2.5% (5x)
Weight (g)	131.8 ± 48.7	229.0 ± 20.7	225.0 ± 20.0	231.6 ± 22.6
Length (mm)	227.6 ± 23.1	126.5 ± 38.3	121.8 ± 36.0	133.0 ± 41.9

Table 2: Target Animal Safety Blood Smear Analysis

Values in a row with superscripts are significantly different from the control ($P \leq 0.005$).

Blood Smear		
Parameter	Allicin Dose	
	0x (0%)	5x (2.5%)
RBC Morphology	Normal	Normal
Polychromasia	Marked	Marked
Lymphocytes (%)	78	85 [a]
Monocytes (%)	15	8 [b]
Avg. Estimated WBC Count (cells/ml)	6,431	6,559

Table 3: Target Animal Safety Serum Chemistry Profiles

Values are presented as mean ± SD. Values in a row with different superscripts are significantly different ($P \leq 0.005$).

	Allicin Dose			
Serum Chemistry Parameters	0% (0x)	0.5% (1x)	1.5% (3x)	2.5% (5x)
Sodium (mEq/L)	160.5 ± 3.5	160.5 ± 3.2	160.8 ± 3.1	163.0 ± 3.7
Potassium (mEq/L)	1.1 ± 0.2	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3
Chloride (mEq/L)	122 ± 3.6	125.4 ± 4.4	124.6 ± 3.1	126.2 ± 3.8
Uric Acid (mg/dL)	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.2
Calcium (mg/dL)	14.7 ± 1.1	15.2 ± 1.4	14.2 ± 1.3	15.5 ± 1.6
Phosphate (mg/dL)	22.7 ± 2.3	24.0 ± 2.2	24.1 ± 2.8	24.2 ± 3.0
Total Protein (g/dL)	4.4 ± 0.5	4.7 ± 0.6	4.5 ± 0.6	4.6 ± 0.5
Glucose (mg/dL)	53.7 ± 33.9	42.0 ± 20.8	44.5 ± 21.7	48.4 ± 26.3
AST (U/L)	1016.7 ± 578.4	1218.3 ± 968.0	1230.0 ± 638.2	925.7 ± 297.0
GDH (U/L)	245.3 ± 83.4	368.8^{a} ± 104.2	445.7^{b} ± 169.3	328.0^{c} ± 97.1

Table 4: Target Animal Safety Renal Pigment-Containing Macrophage Center Scoring

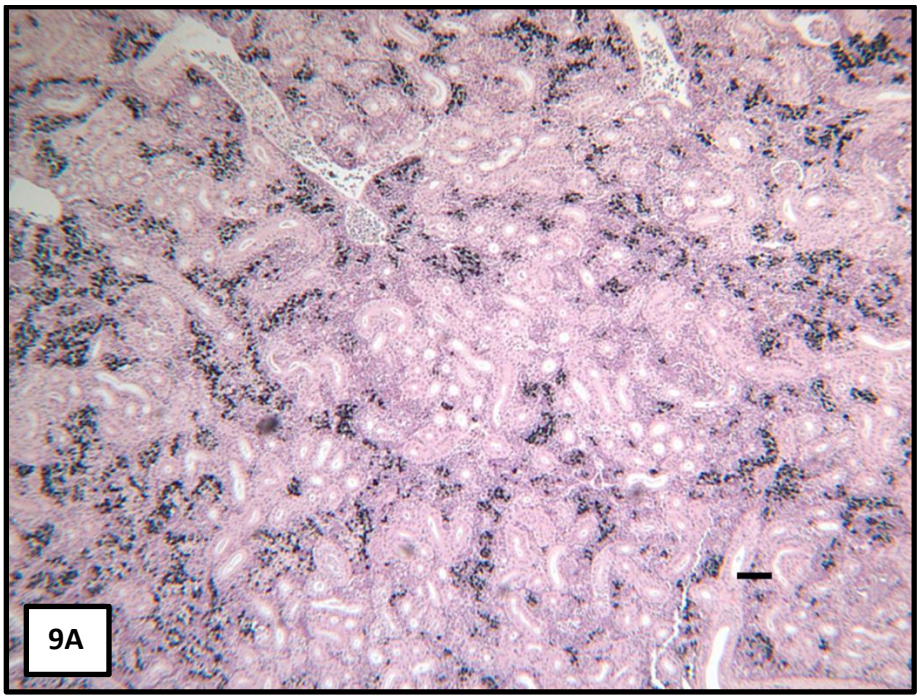
Density of pigmentation determined by a scoring system: 1 = no pigment-containing macrophage centers to 5 = heavy pigment-containing macrophage centers. Normal baseline score for control animals was 3, with trends indicating an increase in density as allicin dosage increased, denoting an inflammatory response.

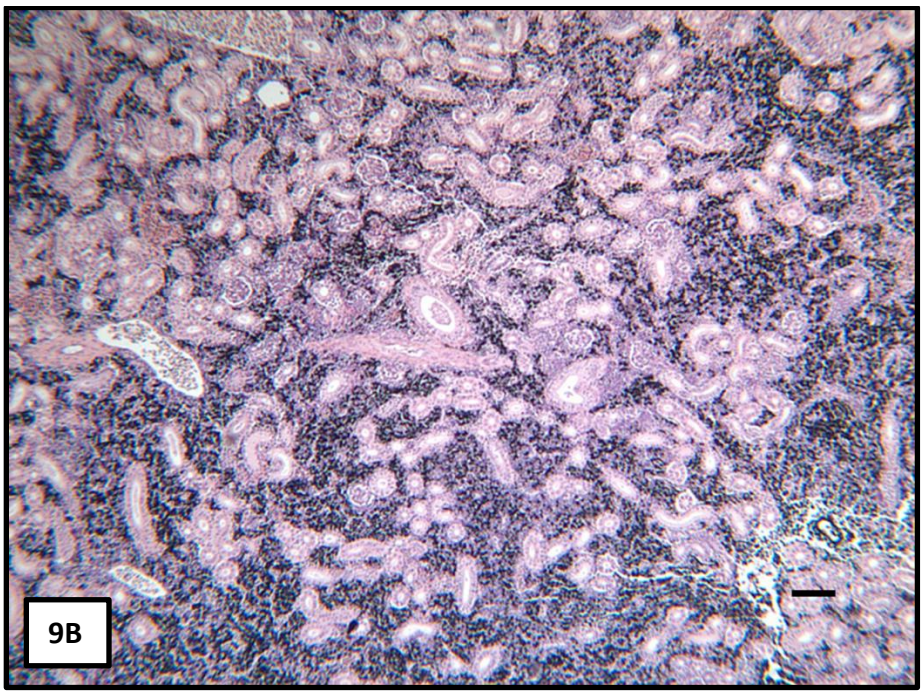
Renal Pigment-Containing Macrophage Center Scoring

Allicin Treatment Group	Average Score
0x (0%)	3
1x (0.5%)	3.1
3x (1.5%)	3.1
5x (2.5%)	4.2

Figures 9A and 9B: Target Animal Safety Renal Histopathology

Scale Bar = 100 micrometers.
A) Renal tissue from the control group (0% allicin) with a normal level of pigment-containing macrophage centers. Score = 3 and B) renal tissue from the 5x (2.5% allicin) showing severe interstitial infiltration with pigment-containing macrophage centers. Score = 5.





Feeding Trial by Waterborne Exposure

An initial trial was performed using the initially determined modified LD_{50} dose of 10^3 CFU/fish. After 38 days, there were no animals that showed any clinical evidence of infection and all animals cultured negative for the bacterium. Since these fish were from a different source, a second mLD_{50} was performed to verify the dose, in which the dose was determined to be 10^5 CFU/fish for this group of animals.

A second waterborne exposure trial was performed, in which only two (3.1%) of the directly infected fish (n=64) showed clinical signs of disease and were euthanized. These two animals cultured positive for *A. salmonicida*. However, all remaining animals, including those infected by direct inoculation, survived and were culture-negative. Quantitative PCR was not performed.

CHAPTER FOUR:

CONCLUSIONS

Feeding Trial by Direct Infection

In the first trial, the mLD_{50} dose was not reached; therefore, a beneficial effect was difficult to determine. There was no significant difference in mortality between the 0%, 0.5%, and 1% treatment groups, though there was a significant detrimental effect in the 2% group as compared to the lower allicin dose groups, indicating that there may be a negative effect at the highest dose group. There was no significant difference between the quantities of *A. salmonicida* bacteria in any of the treated groups, nor was there a difference in the proportion of each treatment group that tested positive by qPCR.

The second trial showed increased survivability (protection) of the 0.5% and 1% allicin groups as compared to the 0% and 2% allicin groups. There was no significant difference between the quantity of *A. salmonicida* bacteria in any of the treated groups, but there was a difference in the proportion of fish that tested positive per group, with the 0.5% and 1% groups having a lower proportion of positive animals when compared to the other groups, indicating that the fish fed the lowest allicin doses (0.5 and 1%) may have protection against disease or are able to clear the disease with higher incidence.

It is suspected that in vivo, the difference in survivability when challenged with *A. salmonicida* is based on immune up-regulation, rather than direct action on the bacteria. This idea is supported by the data showing no difference in quantity of bacteria per group, but lower percentages of animals being affected by disease in the low-dose (0.5% and 1%) allicin groups.

Feeding Trial by Direct Infection: Conclusions

1. **Rainbow trout fed allicin at 0.5% and 1% had improved survivability (protection) when challenged with the bacterial pathogen *A. salmonicida*.**
2. **Rainbow trout fed allicin at 2% had suspected detrimental effects denoted by reduced survival.**
3. **Despite improved survival with low-dose allicin dosing, the quantity of systemic bacteria present did not differ between treatment groups, indicating a suspected immune-up regulation affect rather than direct activity on the bacterium.**

Target Animal Safety Trial

Allicin supplementation did not negatively affect the average size of the animals, which is extremely important from a hatchery management position and in fact, other studies previously mentioned have shown that long-term garlic dosing can actually increase growth rate and feed efficiency (Fo, Han et al. 1990; Zeng, Ren et al. 1996; Jia, Hu et al. 1997; Hu 1999; Jia 1999).

Fish from both the control and high dose groups (2.5%) exhibited normal erythrocyte morphology with marked polychromasia in both groups, which is a normal finding in many species of fish, including those of the salmonid group (Yasutake and Wales 1983). White blood cell counts were not significantly different, though there was marked variability between individuals. There was a significant difference in the WBC differential between the control group and the high dose group (2.5%), with a significantly lower percentage of monocytes in the high dose group. This is suspected to be a result of monocytes being pulled out of circulation for redistribution into the renal tissue, as evidenced by the renal histopathology.

In the serum chemistry evaluations, the creatinine kinase was extremely high in all groups (data not shown), likely due to capture myopathy. In an attempt to reduce capture stress and reduce blood chemistry alterations, small groups of fish were netted and euthanized for immediate processing. The glutamate dehydrogenase (GDH) was significantly increased in all treatment groups from the control. In mammals and some non-mammalian species, GDH is a marker of hepatocellular damage, as it is released from the hepatocyte mitochondrial membranes during apoptosis and is a marker of irreversible damage. Aspartate aminotransferase (AST), a cytosolic enzyme, is released within hours of hepatic insult and is a more acute, sensitive indicator of damage in the liver, heart, skeletal muscle, and erythrocytes. In general, hepatic damage would be evidenced on a serum chemistry panel by an increase in AST as well as an increase in GDH (Boone, Meyer et al. 2005). As the results of this study demonstrated an increased GDH without AST increases and liver histology was normal, it is less likely that the increase was due to hepatic damage.

There are multiple alternate reasons explaining why the GDH may have been increased in the allicin treatment groups in the current study. Several studies determined that allicin acts as a hypolipidemic drug, increasing the activity of acetyl-coenzyme A, associated with increased beta-oxidation (Orellana, Kawada et al. 1992; Prager-Khoutorsky, Goncharov et al. 2007), leading to alterations in fat and glucose metabolism. As a result of increased activity driving the Krebs cycle, a higher level of GDH would be active, leading to an increase in the circulating levels of GDH (UCDavis 2010). Additionally, another study found that allicin induced p53-mediated autophagy, inhibited the viability of hepatic carcinoma lines, and induced degradation of mitochondria (Chu, Ho et al. 2012). Programmed cell death may have occurred as a result of allicin dosing, leading to increased levels of GDH released into the circulation.

Histology of gill, spleen, and liver were normal for all treatment groups. There was a notable, marked increase in the amount of pigment-containing macrophage centers of the kidney as the allicin dose increased. Pigment-containing macrophage centers are a normal feature in tissues of many species of fish and represent areas of surveillance; however, the amount of pigment increased notably as the allicin dose increase. This correlates to the decrease in circulating monocytes in the blood smear, as these cells were distributed to the renal tissue instead. This may indicate an inflammatory process occurring as a direct result of allicin supplementation. Inflammation is not necessarily a harmful process, but there comes a point where heavy inflammation starts to work against the body, causing adverse reactions. Together with the previous survival data indicating that the 2% dose had detrimental survival, it is suspected that there is indeed an adverse reaction to the allicin at levels 2% and higher.

Target Animal Safety: Conclusions

1. **Allicin fed at 0.5%, 1.5%, and 2.5% did not significantly alter the average length or weight of animals as compared to the control group.**
2. **Allicin concentration (0.5%, 1.5% and 2.5%) did not affect erythrocyte or leukocyte morphology or concentration; however, it did alter the WBC differential, with a decreased percentage of monocytes in the highest dose group. This is likely attributable to monocytes being removed from circulation for redistribution in the renal tissue.**
3. **In all allicin treatment groups, glutamate dehydrogenase was elevated compared to the controls. No hepatic damage was seen on histology. GDH may have been increased due to the increased effect allicin has on beta-oxidation, autophagy, and apoptosis, leading to increased cellular metabolism and hepatocyte turnover.**

4. **In the highest allicin group (2.5%), there was a significant trend towards increasing pigment-containing macrophage centers within the renal interstitium, supporting the idea that the highest dose had a potentially negative effect (inflammation).**

Waterborne Infection Trial

Waterborne infection trials were attempted to replicate a more natural route of infection. These trials were unsuccessful mainly because the LD_{50} dose of *A. salmonicida* was not optimized for this strain of fish. While the direct infection trials and target animal safety trials were performed with fish from the same hatchery (USGS Tunison), this final trial set was performed with fish obtained from a different hatchery (NYSDEC Bath). It is known that different populations of aquaculture fish have different genetic predispositions and resistance to varied pathogens; however, these genetic differences are not well recorded by hatchery management. After the initial waterborne challenge, none of the shedder fish cultured positive for *A. salmonicida*, indicating that the animals cleared the infection within 14 days of exposure to the bacteria at a dose of 10^3 CFU/fish.

A new LD_{50} was determined with a very small cohort of fish (10 fish per group, 5 doses) and it was determined that the LD_{50} for the Bath Hatchery fish was 10^5CFU/fish based on this limited study. A second waterborne challenge was attempted, with only several of the injected fish showing signs of disease and culturing positive for the infection. It is suspected that the LD_{50} was still too low as a result of the small sample size tested and this experiment was not optimized during this research phase.

Overall Conclusions

Feeding allicin to rainbow trout appears safe and effective against *A. salmonicida* at 0.5%.

This study adds to previous data indicating that garlic is a beneficial dietary supplement for use in rainbow trout, acting as an immunostimulant for the prevention of bacterial disease. A cost-benefit analysis should be performed prior to use in a hatchery setting, though garlic may also improve growth rate and feed efficiency in addition to its antimicrobial functions. It has been used for thousands of years to improve health and wellness to humans as well as other agricultural species and is an excellent environmentally-friendly natural product to consider for use in aquaculture.

BIBLIOGRAPHY

AFS (2012). Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens, Blue Book, American Fisheries Society.

Aly, S. M. and M. F. Mohamed (2010). "*Echinacea purpurea* and *Allium sativum* as immunostimulants in fish culture using Nile tilapia (*Oreochromis niloticus*)." Journal of Animal Physiology and Animal Nutrition **94**(5): 31-39.

Ankri, S., T. Miron, et al. (1997). "Allicin from garlic strongly inhibits cysteine proteinases and cytopathic effects of *Entamoeba histolytica*." Antimicrob Agents Chemother **41**(10): 2286-2288.

Ashley, P. J. (2007). "Fish welfare: Current issues in aquaculture." Applied Animal Behaviour Science **104**(3–4): 199-235.

Block, E. (1985). "The chemistry of garlic and onions." Sci Am **252**(3): 114-119.

Boone, L., D. Meyer, et al. (2005). "Selection and interpretation of clinical pathology indicators of hepatic injury in preclinical studies." Vet Clin Pathol **34**(3): 182-188.

Borek, C. (2006). "Garlic reduces dementia and heart-disease risk." J Nutr **136**(3 Suppl): 810S-812S.

Bullock, G. L., R. C. Cipriano, et al. (1983) "Furunculosis and Other Diseases Caused by *Aeromonas salmonicida*." US Fish & Wildlife Publications.

Cavallito, C. and J. H. Bailey (1944). "Allicin, the antibacterial principle of *Allium sativum*. Isolation, physical properties and antibacterial action." Journal of the American Chemical Society **66**: 1944-1952.

Chan, J. Y., A. C. Yuen, et al. (2013). "A review of the cardiovascular benefits and antioxidant properties of allicin." Phytother Res **27**(5): 637-646.

Chu, Y. L., C. T. Ho, et al. (2012). "Allicin Induces p53-Mediated Autophagy in Hep G2 Human Liver Cancer Cells." Journal of Agricultural and Food Chemistry **60**(34): 8363-8371.

Cipriano, R. C. (1983). Furunculosis: Pathogenicity, mechanisms of bacterial virulence, and the immunological response of fish to *Aeromonas salmonicida*. Bacterial and viral diseases of fish. Molecular Studies. J. H. Crose. Seattle, Washington, Washington Sea Grant Publication, University of Washington**:** 41-60.

Cipriano, R. C. and J. Bertolini (1988). "Selection for virulence in the fish pathogen *Aeromonas salmonicida*, using Coomassie Brilliant Blue agar." J Wildl Dis **24**(4): 672-678.

Colic, M. and M. Savic (2000). "Garlic extracts stimulate proliferation of rat lymphocytes in vitro by increasing IL-2 and IL-4 production." Immunopharmacol Immunotoxicol **22**(1): 163-181.

Cutler, R. R. and P. Wilson (2004). "Antibacterial activity of a new, stable, aqueous extract of allicin against methicillin-resistant *Staphylococcus aureus*." Br J Biomed Sci **61**(2): 71-74.

Ellis, A. E., T. S. Hastings, et al. (1981). "The role of *Aeromonas salmonicida* extracellular products in the pathology of furunculosis." Journal of Fish Diseases **4**(1): 41-51.

FAO/WHO/OIE (2006). Expert Consultation on Antimicrobial Use in Aquaculture and Antimicrobial Resistance. Seoul, Republic of South Korea.

FDA (2009). Guidance for Industry: Target Animal Safety for Veterinary Pharmaceutical Products. USDHHS. Rockville, MD, International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products.

FDA. (2013). "Aquaculture: Approved Drugs." Retrieved May 28, 2013, from http://www.fda.gov/AnimalVeterinary/DevelopmentApprovalProcess/Aquaculture/ucm132954.htm.

Feldberg, R. S., S. C. Chang, et al. (1988). "In vitro mechanism of inhibition of bacterial cell growth by allicin." Antimicrob Agents Chemother **32**(12): 1763-1768.

Fo, T. L., X. X. Han, et al. (1990). "Research and application of garlic residue premix." Feed Industry **11**: 12-13.

Francis-Floyd, R. (2012, November 2012). "Stress - Its Role in Fish Disease." Retrieved May 28, 2013, from http://edis.ifas.ufl.edu/fa005.

Gupta, N. and T. D. Porter (2001). "Garlic and garlic-derived compounds inhibit human squalene monooxygenase." J Nutr **131**(6): 1662-1667.

Gustafson, C. E., C. J. Thomas, et al. (1992). "Detection of *Aeromonas salmonicida* from fish by using polymerase chain reaction amplification of the virulence surface array protein gene." Applied and Environmental Microbiology **58**(12): 3816-3825.

Harris, J. C., S. L. Cottrell, et al. (2001). "Antimicrobial properties of *Allium sativum* (garlic)." Appl Microbiol Biotechnol **57**(3): 282-286.

Herman-Antosiewicz, A., A. A. Powolny, et al. (2007). "Molecular targets of cancer chemoprevention by garlic-derived organosulfides." Acta Pharmacol Sin **28**(9): 1355-1364.

Hu, S. J. (1999). "Effect of garlic as feed additive in Nile tilapia *Oreochromis niloticus* and carp *Cyprinus carpio* culture." Inland Fisheries **4**(15).

Huang, X. G., J. H. He, et al. (2001). "Primary research on the application effects of allicin on aquaculture of rice field eel *Monopterus albus*." Inland Fisheries **9**: 27.

Jia, W. B., B. Hu, et al. (1997). "Application research of iodinated allicin." China Feed **6**: 24-25.

Jia, W. B., P.T. Ren, and B. Hu (1999). "Research and application of allicin." Cereal & Feed Industry **5**: 31.

Jonkers, D., E. van den Broek, et al. (1999). "Antibacterial effect of garlic and omeprazole on *Helicobacter pylori*." J Antimicrob Chemother **43**(6): 837-839.

Ketola, H. G., C. E. Starliper, et al. (2012). Inhibition of *Aeromonas salmonicida* by Essential Oils and the Influence of Dietary Essential Oils on Resistance of Atlantic Salmon to Furunculosis. 37th Annual Eastern Fish Health Workshop, Lake Placid, NY.

Khanum, F., K. R. Anilakumar, et al. (2004). "Anticarcinogenic Properties of Garlic: A Review." Crit Rev Food Sci Nutr **44**(6): 479-488.

Klontz, G. W., W. T. Yasutake, et al. (1966). "Bacterial diseases of the Salmonidae in the Western United States: pathogenesis of furunculosis in rainbow trout." American Journal of Veterinary Research **27**(120): 1455-1460.

Lee, J.-Y. and Y. Gao (2012). "Review of the Application of Garlic, *Allium sativum*, in Aquaculture." Journal of the World Aquaculture Society **43**(4): 447-458.

Luna, L. G. (1968). Manual of histological staining of the Armed Forces Institute of Pathology, 3rd ed. New York, NY, McGraw-Hill.

Markwardt, N. M., Y. M. Gocha, et al. (1989). "A new application for Coomassie Brilliant Blue agar: detection of *Aeromonas salmonicida* in clinical samples." Diseases of Aquatic Organisms **6**: 231-233.

McCarthy, D. H. (1977). "Some ecological aspects of the bacterial fish pathogen *Aeromonas salmonicida*." Aquatic Microbiology **6**: 299-324.

McCarthy, D. H. and R. J. Roberts (1980). Furunculosis of fish - the present state of our knowledge. Advances in Aquatic Microbiology. M. R. D. a. H. W. Jannasch. London, Academic Press. **2:** 293-341.

NOAA. (2013). "Aquaculture in the United States." Retrieved May 28, 2013, from http://www.nmfs.noaa.gov/aquaculture/aquaculture_in_us.html.

Nya, E. J. and B. Austin (2009). "Use of garlic, *Allium sativum*, to control *Aeromonas hydrophila* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum)." J Fish Dis **32**(11): 963-970.

Nya, E. J. and B. Austin (2011). "Development of immunity in rainbow trout (*Oncorhynchus mykiss*, Walbaum) to *Aeromonas hydrophila* after the dietary application of garlic." Fish Shellfish Immunol **30**(3): 845-850.

Nya, E. J., Z. Dawood, et al. (2010). "The garlic component, allicin, prevents disease caused by *Aeromonas hydrophila* in rainbow trout, *Oncorhynchus mykiss* (Walbaum)." J Fish Dis **33**(4): 293-300.

Oliva-Teles, A. (2012). "Nutrition and health of aquaculture fish." J Fish Dis **35**(2): 83-108.

Orellana, A., M. E. Kawada, et al. (1992). "Induction of peroxisomal fatty acyl-coenzyme A oxidase and total carnitine acetyl-coenzyme A transferase in primary cultures of rat hepatocytes by garlic extracts." Toxicol Lett **60 (1)**: 11-17.

Prager-Khoutorsky, M., I. Goncharov, et al. (2007). "Allicin inhibits cell polarization, migration and division via its direct effect on microtubules." Cell Motility and the Cytoskeleton **64**(5): 321-337.

Rivlin, R. S. (2001). "Historical perspective on the use of garlic." J Nutr **131**(3s): 951S-954S.

Singh, B. B., S. P. Vinjamury, et al. (2007). "Ayurvedic and collateral herbal treatments for hyperlipidemia: a systematic review of randomized controlled trials and quasi-experimental designs." Altern Ther Health Med **13**(4): 22-28.

Talpur, A. D. and M. Ikhwanuddin (2012). "Dietary effects of garlic (*Allium sativum*) on haemato-immunological parameters, survival, growth, and disease resistance against *Vibrio harveyi* infection in Asian sea bass, *Lates calcarifer* (Bloch)." Aquaculture **364–365**(0): 6-12.

Trichet, V. V. (2010). "Nutrition and immunity: an update." Aquaculture Research **41**(3): 356-372.

UCDavis. (2010). "Kreb's Cycle." ChemWiki Retrieved July 27, 2013, from http://chemwiki.ucdavis.edu/Biological_Chemistry/Metabolism/Kreb's_Cycle.

Udey, L. R. and J. L. Fryer (1978). "Immunization of fish with bacterins of *Aeromonas salmonicida*." Marine Fisheries Review **40**: 12-17.

Weber, N. D., D. O. Andersen, et al. (1992). "In vitro virucidal effects of *Allium sativum* (garlic) extract and compounds." Planta Med **58**(5): 417-423.

Xiang, X. and C. Z. Liu (2002). "Effect of allicin on growth of *Colossoma barchpomum*." Fisheries Science and Technology Information **29**: 222-225.

Yang, F., X. W. Zuo, et al. (2010). "The effects of garlic extract on early growth and development of Manila clam *Ruditapes philippinarum*." Acta Ecologica Sinica **30**(989-994).

Yasutake, W. T. and J. H. Wales (1983). Microscopic Anatomy of Salmonids: An Atlas. Washington, D.C., United States Department of the Interior, Fish and Wildlife Service.

Zeng, H., Z. L. Ren, et al. (1996). "Application of allicin in tilapia feed." China Feed **21**: 29-30.

Zilberg, D., A. Tal, et al. (2010). "Dried leaves of *Rosmarinus officinalis* as a treatment for streptococcosis in tilapia." J Fish Dis **33**(4): 361-369.