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An Evaluation of Methodologies for Measuring Antibacterial Activity of the Epithelial Mucosa of Farmed Tilapia (*Oreochromis niloticus*) Against *Streptococcus agalactiae* and *Streptococcus iniae*

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AN EVALUATION OF METHODOLOGIES FOR MEASURING ANTIBACTERIAL ACTIVITY OF THE EPITHELIAL MUCOSA OF FARMED TILAPIA (*OREOCHROMIS NILOTICUS*) AGAINST *STREPTOCOCCUS AGALACTIAE* AND *STREPTOCOCCUS INIAE*

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ABSTRACT

Tilapiine cichlids are the third largest farmed fish worldwide and are among the easiest and most profitable fish to farm. The most common pathogens affecting farmed tilapia are *Streptococcus agalactiae* and *Streptococcus iniae*, which together account for losses of more than 150 million dollars annually. Fish are at the highest risk for developing a *Streptococcus* infection when they are in high density stock conditions. Research indicates that the secondary effects of high stock density such as low dissolved oxygen and high ammonia levels are of less significance to infection and mortality than the damage that over stocking causes to the mucosal immune system. However, assays used to determine the effectiveness of the tilapiine mucosal immune system have proven unreliable. The present work has been undertaken to evaluate mucus collection procedures and antibacterial assay effectiveness. One "spot-on-lawn" assay, four disk diffusion assays, and four micro titer plate assays were chosen based upon common levels of use in the literature. The assays were executed; however, each of the disk diffusion assays failed to accurately measure antibacterial activity when controlled for the antibacterial activity of additives. The microtiter plate assays successfully measured limited antibacterial activity at lower growth reduction levels of 10-30%. Additionally, these assays were administered on samples which were collected from tilapia in an actual aquaponics facility in contrast to the majority of experiments which are conducted on fish that have been purposely housed in clean and controlled conditions within research facilities. This deviation from standard methods introduced additional variables which influence assay outcomes; therefore, future research should address these discrepancies.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure Page

LIST OF TABLES

Table Page

CHAPTER ONE

REVIEW OF LITERATURE

Introduction

Tilapia is the common name for approximately one hundred species of cichlids that can be found among three separate genera. The wide range of the term results from the etymology of the word "tilapia" which evolved from a Tswana word that simply means "fish." In modern day food production, the term "tilapia" primarily relates to those fish of the genus *Oreochromis*, which traces its genetic roots back 4,000 years to northern African waterways where ancient Egyptians began actively farming cichlids. Recent decades have seen the growth of the tilapia industry into a worldwide phenomenon with experts ranking tilapia as the second most important farmed fish worldwide, and it has been hailed the "most important aquaculture species of the 21st century" (Shelton 2002). As our world population continues to rise toward critical levels, agricultural research must work to maximize food production with minimal space and less than ideal resources.

In this potential future where the human race exceeds the planet's carrying capacity, tilapia species may become instrumental in feeding a hungry world, as tilapia can be raised in small spaces with nominal energy and water input, yet they quickly become a high quality protein source loaded with essential fatty acids. In many respects, tilapia represents the perfect species for the constraints of future production. *Oreochromis* is tolerant of salinity levels from freshwater to brackish (Kamal 2005), as well as, being tolerant of extended periods of low dissolved oxygen levels due to their ability to down

regulate cardiac function (Lague 2012). Tilapia can thrive with stocking rates as high as 50-60 large fish per single 4 m^3 tank (Yi 1996) while being prolific and having a growth rate of 2-3% of body weight per day (USDA 1991). Additionally, tilapia offer these benefits while consistently being low trophic level feeders (Waite 2014).

Although tilapia farming is on the rise world-wide, the greatest current threat to commercial tilapia production is *Streptococcus* infection. Both *Streptococcus agalactiae* and *Streptococcus iniae* are opportunistic diseases that arise when tilapia are stocked at high densities, such as is common in commercial facilities. These infections are often fatal for tilapia within three to five days of exposure. To prevent major profit loss, aquaculturists often resort to extensive use of antibiotics, which poses the potential risk of providing an environment where antimicrobial resistance genes could pool. The World Health Organization has been working since 2006 to quantify this risk; however, based upon emerging scientific consensus, WHO guidelines currently recommend minimizing the use of antibiotics that are considered "critically important to human medicine" (WHO 2011). Many of these antibiotics, including penicillins, sulphonimides, and tetracyclines, have seen widespread used in worldwide aquaculture (Sapakota 2008). In an effort to minimize the use of antibiotics in aquaculture, research is underway by investigators who seek to find novel approaches that enhance the tilapia's immune system, specifically the quality, quantity, and composition of the fish's external mucosal immune system (Gomez 2013).

The Teleost Immune System: an Overview

Teleost (finned) fish were the first group to evolve at the beginning of the adaptive radiation of vertebrates. Therefore, jawed fishes emerged at the crossroads between animals with simple innate immunity and animals that have both innate and highly developed adaptive immune systems. Teleost fish have both innate and adaptive systems; however, their adaptive immune system is significantly less effective than the mammalian system while their innate pathways offer more robust responses than those of their mammalian counterparts. More specifically, antibody affinity is lower in fish than mammals, memory response is weaker, and affinity maturation is absent due to a lack of lymph nodes and germinal centers (Tort 2003). However, recent research has pointed toward the presence of melano-macrophage centers in the stroma of the spleen which function as proto-germinal centers; however, only preliminary data currently exists (Magor 2015). This inhibition of adaptive immunity also hinders the ability of immunizations to prevent disease in fish.

In general terms, the teleost immune system protects the fish with non-specific barriers like the scales and mucosa which act as both a physical barrier sequestering invading microbes until sloughing and a chemical barrier which inactivates potential infectious agents through the actions of antimicrobial peptides, lysozymes, and interferons. The telost immune system also protects the fish in specific ways using antibody production, though the memory response is slower, weaker, and of shorter duration than that of mammals (Iwama 1996).

Tilapiine Immune Anatomical Structures

The tilapia's lymphoid organs include the kidney, thymus, and spleen. In tilapia, as with other jawed fishes, the kidney runs the length of the animal's medial axis, and the head of the kidney is responsible for hemopoietic functions, in addition to being the site of antigen processing and phagocytosis (Zapata 1996). Early B-cell development occurs in the anterior portion of the head kidney which allows naïve B cells to be transferred to the spleen and posterior kidney through the bloodstream. The tilapiine thymus produces functional T lymphocytes which drive the adaptive immune response (Bowden 2005). The spleen is primarily involved in macrophage phagocytosis of antigens and detaining antigens for adequate periods of time to allow for the work of immunological memory (Uribe 2011). Tilapia leukocytes can be found in most organs and systems of its body; however, they are primarily found in the mucosal associated lymphoid tissue which can be found in the gut, skin, and gills (Beck 2015). (See section 1.5 for more information on the mucosal immune system).

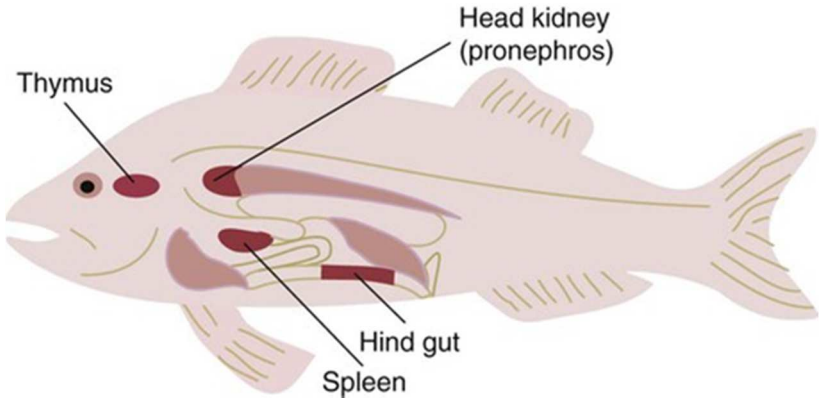


Figure 1.1: Anatomical Structures of the Teleost Immune System (Evolution 2016).

Tilapiine Immune Cellular Components

Tilapia exhibit versions of the immune cells one expects to find in vertebrates including: B and T lymphocytes, natural killer cells, monocytes, macrophages, neutrophils, eosinophils, mast cells, and thrombocytes (Whyte 2007). B and T cells both undergo the somatic VDJC rearrangement necessary to form antigen specific receptors, leading to an extensive repertoire of unique cells. In all teleost fish, there are four known immunoglobulins produced: IgM, IgZ, IgT, and IgD; however, isotype switching is not possible in tilapia (Beck 2015).

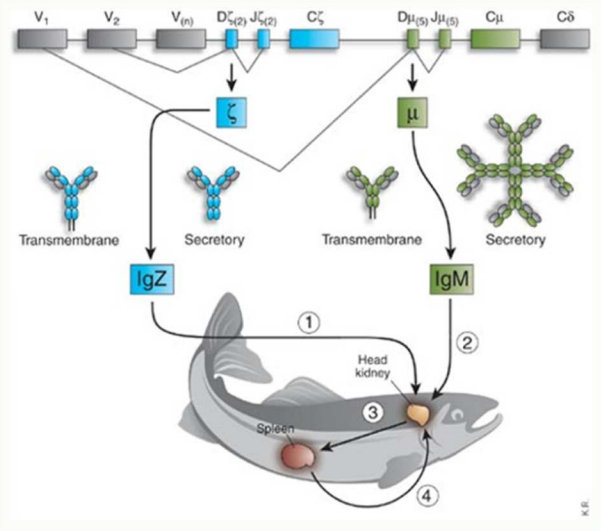


Figure 1.2: Diagram of Immunoglobulin Production in Teleosts (Flajnik 2005).

IgZ is only produced in the head kidney (1). IgM is produced in the head kidney (2), but IgM producing cells may then migrate to the spleen (3). Upon stimulation by antigen, IgM producing cells may return to the head kidney (4).

As with mammals, B cells function in antigen presenting and antibody production; however, teleost B cells also have additional phagocytic abilities (Salinas 2011). Genetic studies of T cells in teleosts have shown that all of the genetic components necessary for mammalian-level T cell function are present; however, much of this research is often still in question. For example, CD4 is expressed on T cells in teleosts, but a lack of specific blocking/labeling-antibodies has led to a lack of information on the exact nature of this receptor's function (Beck 2015). Two natural killer cell homologs can be found in tilapia: nonspecific cytotoxic cells (NCCs) and NK-like cells. These cells target non-self recognized invaders, particularly protozoans (Evans 1990). Macrophages were long thought to work similarly to those in mammals with certain macrophages being activated by Th1 while others by Th2; however, more recent work indicates that teleost macrophages (and monocytes) can also be activated alternatively. Teleost macrophages are divided into four phenotypes: innately activated macrophages which are activated by antigen, classically activated macrophages which are activated by antigen plus IFNγ, alternatively activated macrophages which are activated in the presence of interleukin 4 and interleukin 13, and macrophages which are activated in the presence of interleukin 10. Those alternatively activated in the presence of IL 4 and IL 13 function in wound healing while those alternatively activated by IL 10 service a regulatory function by reducing inflammatory responses (Forlenza 2011). Current knowledge indicates that neutrophils, eosinophils, mast cells, and thrombocytes have identical functions to those known in vertebrates (Beck 2015).

Tilapiine Mucosal Immune System

The mucus producing tissues of the gills, intestines, and skin comprise the mucosal immune system. The specific structures and functions of each of these regions vary slightly; however, they share the same basic structure. This structure includes the epithelial surface with mucus secreting goblet cells, supportive stromal tissues, vascular supply, musculature, and immunologically reactive cells (Beck 2015). The tilapiine mucosa is structurally similar to its mammalian counterparts. However, the tilapia's aquatic environment allows for constant contact between the animal and the surrounding microflora. This increased contact results in a mucosal immune system that is much more dependent on the conditions of the environment to be functional; therefore, it is sensitive the effects of pH, temperature, dissolved oxygen, as well as, other factors (Beck 2015).

The mucosa of the integument makes up the largest portion of the mucosa-associated immune system in tilapia. The integument contains a multitude of mucus producing goblet cells which are interspersed among simple squamous cells which have underlying dermal, sub dermal, and muscle layers. Goblet cells produce highly branched, viscous glycoproteins called mucins. These goblet cells are continually being sloughed off along with the squamous cells, but new cells are in continuous production in the lower tissues of the integument (Beck 2015). The epidermal mucus layer produced by these goblet cells contains lysozymes, antimicrobial peptides, proteases, peroxidases, and lectins (Salinas 2011). Additionally, resident dendritic cells are present to process antigen. B cells are also present, along with antibodies in the form of IgM and IgT. These

immunoglobulins are assisted across mucosal barriers via the polymeric immunoglobulin receptor (Gomez 2013). Mast cells and eosinophils have been found in the dermal layer but not in the mucosa itself. T cells are also present, but the exact nature of T cell action in the fish mucosa and integument is still in question (Salinas 2015). The external mucosa is also home to commensal bacteria which have unique adhesion properties that allow them to claim regions of the tilapiine mucosa which they defend against all invaders, pathogenic and otherwise (Grześkowia 2011).

The gills are a mucosal tissue with the primary role of gas exchange; however, the surface mucus layer also functions in immunity. The surface of the gill features both primary lamellae which are responsible for increasing surface area and allowing for additional exchange. Secondary lamellae then branch from the primary, further extending surface area. Among these secondary lamellae are goblet cells, such as those found in the integument previously discussed. While these structures add to the oxygenating capabilities of the gills, they could potentially hinder the immune response because they are so dense that the ability of immunoglobulin and other cell types to have the space necessary for movement and interactions with pathogens. The gill mucosa overcomes this barrier with specialized cuffs which surround the vasculature of each individual filament. These cuffs are able to secrete localized IgM in response to the presence of antigen, thus removing the need for the antibodies to circulate over larger distances on the gill (Davidson 1997).

The intestinal mucosa of the tilapia is similar in structure and function to the mammalian gut. There is an initial aqueous layer, followed by a thick mucosal layer, which covers a third layer of columnar epithelial cells: the functional enterocytes responsible for nutrient absorption. As with the integumentary and gill systems, goblet cells are interspersed between enterocytes, though the frequency of goblet cells decreases near the posterior end of the intestines (Beck 2015). Though the mucosa of the tilapiine gut functions largely in the same manner as that of the gills and skin, a primary difference lies in a significant reduction in the number of resident dendritic cells. This lack of dendritic cells is likely due to the increased need for commensal bacteria in the gut that are necessary for proper digestion (Gomez 2013).

Relevant Tilapiine Pathogens

Streptococcus agalactiae/ Streptococcus iniae

S. agalactiae represents the only Group B streptococcus according to the Lancefield classification system. *S. agalactiae* forms chains of Gram positive cocci. It is a facultative anaerobic bacterium that exhibits beta hemolysis and produces alkaline phosphatase; it also produces acid in the presence of ribose and trehalose sugars (Whiley 2009; Holt 1994). *S. agalactiae* is a largely harmless commensal gut and reproductive tract bacteria in humans; however, it can be an opportunistic infection in neonates and adults with compromised immune systems (Edwards 2011). *S. agalactiae* is also one of the primary causes of mastitis in dairy cattle (Stableforth 1950).

In tilapia, *S. agalactiae* infection is primarily observed in farmed fish. The initial symptom of the infection is an isolated lesion found near the lip or tail line of the fish. These lesions are small enough to be easily missed by casual inspection of an aquaculture crop. Untreated infection leads to septicemia, hemorrhaging, swirling behavior, bent bodies, anorexia, lethargy, and death, often within seven days of the emergence of visible symptoms. Cases have been confirmed of initial asymptomatic infection that also proved fatal (Pretto-Giordano 2010).

Much like *S. agalactiae*, *S. iniae* is a Gram-positive coccus and facultative anaerobe which exhibits beta hemolysis; however, it cannot be grouped according to the Lancefield system because it does not express any of the antigens necessary for categorization. Genomic analysis indicates that it is closely related to *S. agalactiae*, and the pathology of the two streptococci is nearly identical, requiring bacterial isolation to identify (Russo 2006). *S. iniae* is a human pathogen, causing cellulitis in a small number of confirmed cases worldwide, two of which involved injuries at a fish processing facility (Lau 2003). Both species of *Streptococcus* thrive on blood agar plates and in tryptic soy broth when incubated at 37°C.

Figure 1.3: Tail line ulceration on tilapia infected with *Streptococcus agalactiae* (Vetbook 2015).

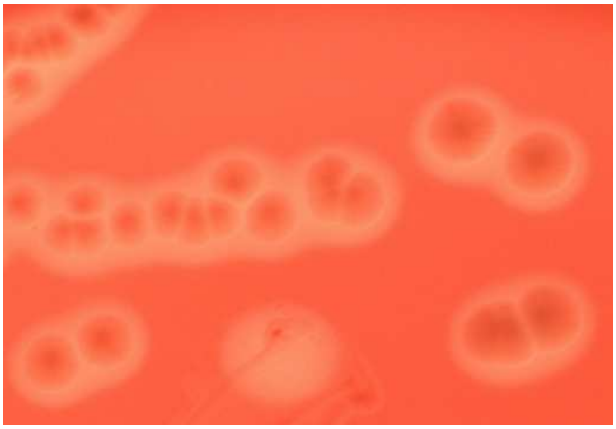


Figure 1.4: *Streptococcus agalactiae* colonies on sheep's blood agar.

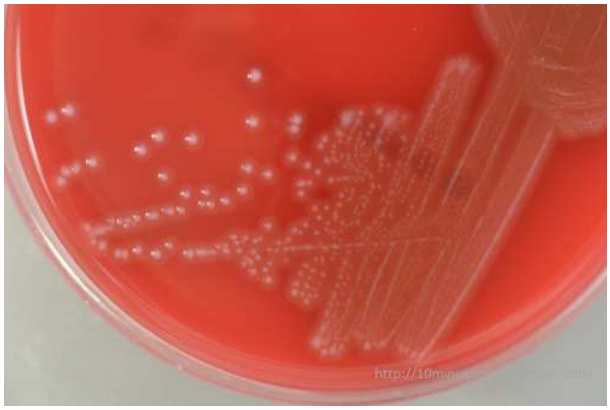


Figure 1.5 *Streptococcus iniae* colonies on sheep's blood agar.

Aeromonas salmonicida

A. salmonicida was included in this study to comparatively observe the antibacterial activity of tilapia mucus against a Gram-negative organism. Although tilapia are susceptible to this disease, *A. salmonicida* does not pose the threat to tilapia aquaculture production that *S. iniae* and *S. agalactiae* do (Austin 2016). *A. salmonicida* is a rod shaped non-motile bacterium that is a facultative anaerobe; it grows well on blood agar plates and in tryptic soy broth at ambient temperatures of approximately 25°C, though the colonies diffuse a brown pigment into blood agar. *A. salmonicida* is not a known human pathogen (Shaw 1992).

A. salmonicida is found primarily in aquaculture sites with poor water quality where it causes a disease known as funrunculosis or "tail rot." Additional external signs of infection include swelling around the vents, ulcers, and liquefied feces. Internally, fish with *A. salmonicida* suffer from hemorrhaging which can be acute or chronic. Once the fish exhibits extreme lethargy or swirling behavior, death is imminent (Noga 2010).

Figure 1.6: *Aeromonas salmonicida* colonies on sheep's blood agar.

Yersinia ruckeri

Y. ruckeri was also included in this study to provide comparative information about additional fish diseases that tilapia reportedly have more resistance to than *S. agalactiae* and *S. iniae*. While *Y. ruckeri* causes significant loses to other species in the aquaculture industry, tilapia are not highly susceptible. *Y. ruckeri* is a Gram-negative rod-shaped enterobacterium that is a facultative anaerobe (Tobback 2007).

Y. ruckeri causes enteric red mouth disease in many species of fish, as the redness is the result of hemorrhaging of the gums and the inside of the mouth. Other symptoms include inflammation of the intestines which leads to anorexia, darkening of the skin, enlargement of the spleen, and protrusion of the eye. Untreated, *Y. ruckeri* results in massive internal hemorrhaging and death (Noga 2010).

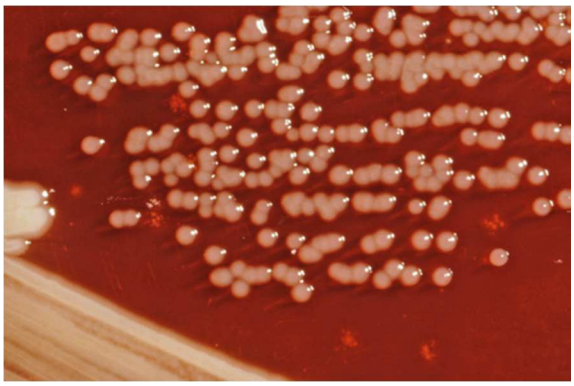


Figure 1.7: *Yersinia ruckeri* colonies on sheep's blood agar.

Importance of Mucosa in Preventing *Streptococcus* Infection

An evaluation of multiple studies indicates that the mucosal immune system plays an important role in preventing *Streptococcus* infection. In 2000, Shoemaker, et al. produced evidence that stocking densities influence infection rate more than the overall concentration of bacteria. This result is potentially a byproduct of the conditions that overstocking creates i.e. low dissolved oxygen and/or high ammonia which affect the overall health of the tilapia, leading to immunocompromised stock. However, this result is also potentially the result of physical damage that overstocking causes to the fish external mucosa, or a combination of multiple factors. In an effort to determine the underlying issues with overstocking, a study was designed in which tilapia were challenged with *Streptococcus agalactiae* via injection while being held in tanks with both normal dissolved oxygen and low dissolved oxygen. In essence, this study mimicked the conditions of high density stocking without the physical damage to the mucosa. The results of this particular study indicate that low oxygen does affect mortality rates but not

infection rates (Evans 2003). In contrast, a separate research team conducted a similar experiment by mimicking the conditions of high stocking density (low dissolved oxygen and high ammonia concentration). In this study, the fish were challenged with *Streptococcus agalactiae* via immersion. Results showed no difference in infection or mortality rates between fish housed in poor conditions (dissolved oxygen <1.0 mg/L and ammonia >10 mg/L) and those housed in normal conditions (dissolved oxygen >5 mg/L and ammonia <2 mg/L). Therefore, fish that were challenged in such a way that the mucosa was bypassed (injection) were further affected by the conditions of the tank, and fish that were challenged in such a way that the mucosa was not bypassed (immersion), did not have higher infection rates in poor conditions (Bowser 1998). The combination of this research belies the importance of the epidermal mucosa in preventing *Streptococcus* infection, as well as, the importance that stocking density plays in infection rates, though further research is necessary. Therefore, the purpose of the present study is to determine the ideal methodology for quantifying the antibiotic activity of tilapia external mucus in tilapia being housed in the practical conditions of aquaculture facilities so that this activity can be evaluated in terms of resistance to *Streptococcus* disease.

CHAPTER TWO

METHODS AND MATERIALS

Institutional Approval

The following research methods gained approval from the Clemson University Office of Research Compliance's Institutional Biosafety Committee. This approval was granted for the period of June 7, 2016 until June 6, 2017 under the protocol number #IBC2016-21.

Collection Methods

Mucus was collected from tilapia at two locations: the Clemson University Aquaponics Facility and a professional aquaponics farm in Anderson, South Carolina. Three different mucus collection methods were evaluated. For each method, the tilapia specimen was rinsed with sterile distilled water and placed on a large tempered glass surface sterilized with 70% ethanol. Glass microscope slides which had been autoclaved (15 min gravity cycle), a spatula which had been autoclaved (20 min gravity cycle), and a Corning Incorporated 3011 sterile cell scraper were each used to scrape mucus from the dorsolateral surfaces of the fish, avoiding the urogenital area. Using each of these methods, mucus was scraped from fish on to the large glass surface. Sterile microscope slides were then used to move the mucus from the glass surface into the 50 mL conical tube.

Quantification of Protein

For the purposes of this study, the protein was quantified via electrophoresis and spectrophotometry. Prior to the electrophoresis protein quantification, a chloroform methanol precipitation was undertaken to concentrate the protein following the method set forth by Wessel and Flugge in their textbook *Analysis of Biochemistry* (Wessel 1984). Four hundred μL of methanol was added to 100 μL of crude mucus sample agitated on a Vortex Genie 2 for 5 min. An additional 100 μL of chloroform was added to the solution, and it was again agitated for 5 min. Three hundred μL of distilled water were added, followed by an additional 5 min of agitation. The solution was then centrifuged at 21,913 x *g* for 1 min. The top aqueous layer was removed, 400 μL of methanol were added, and the solution was agitated for 5 min and centrifuged at 21,913 x *g* for 2 min. The remaining methanol was removed via micropipette as not to disturb the pellet. The sample was then dried using a speedvac. Finally, 20 μL of loading buffer were added, and the sample was prepared for electrophoresis using a BioRad Mini-protein TGX Precast trisglycine gel. Upon completion, the gel was compared to the blue standard from BioRad Precision Protein Plus.

In order to quantify the protein in the mucus samples, a Eppendorf Biospectrophotometer 2 was used to compare the sample absorbance at 280 nm to a standard curve. Crude samples of 5 mL were diluted with an additional 5 mL of phosphate buffered saline prior to quantification. The samples were then centrifuged at 21,913 x *g* for 15 min using a Pall Life Sciences Nanosep Centrifugal Device for

concentrating and desalting. Thereafter, the filtrate and the sample were measured via A280 for protein analysis.

Standardizing Bacterial Cultures

Cultures of *Streptococcus agalactiae* (ATCC 13813)*, Streptococcus iniae* (ATCC 29177)*, Aeromonas salmonicida* (ATCC 33658), and *Yersinia ruckeri* (ATCC 29473) were started from Microbiologics Kwik Stik preparations and grown in tryptic soy broth (TSB) in 50 mL sterile, conical tubes. The *Streptococcus* species were incubated at 37°C for 24 hours while the *A. salmonicida* and *Y. ruckeri* cultures were grown at ambient room temperature. Cultures were plated to ensure the presence of a monoculture which was consistent with known growth patterns including color, shape, and hemolysis for each species of bacteria. Under sterile conditions, the Thermo Scientific Genesys 20 spectrophotometer was set to absorbance 600 nm, and a cuvette of sterile TSB was read as a blank. Ratios of TSB to culture were used until an absorbance of 0.5 to 0.6 was achieved for initial experiments and the concentration was reduced to 0.2 for later experiments (see discussion). These ratios were then used to create 10 mL of culture in a sterile conical tube.

Contamination Prevention

In order to evaluate the amount of bacterial contamination of the mucus, samples were streaked four ways onto plates with a variety of media including the following:

- Sheep Blood Agar

- Tryptic Soy Agar
- MacConkey Agar
- Eosin Methylene Blue Agar
- Mannitol Salt Agar
- Phenylethyl Alcohol Agar
- CHROMagar™ Orientation Agar

The plates were then evaluated for colony growth by a visual inspection of size, hemolysis, color, and shape. Once it was determined that contamination had occurred, methods were evaluated to eliminate cellular growth without the degradation of proteins. One portion of sample was freeze dried in the Lyo-Centre lyophilizer, resuspended, and plated on a sterile plate with sheep blood agar. Another portion of sample in PBS was thinly spread across a 100 mm x 15 mm sterile plate under a Thermo Scientific 1300 Series A2 hood while under direct ultraviolet light for 20 min. The sample was then collected into a sterile conical tube and plated on sterile sheep blood agar to assess bacterial growth. Plates were examined at 12 and 24 hr.

Antibacterial Assays

Spot-on-Lawn Assay

Twenty-four hour cultures of *Streptococcus agalactiae*, *Streptococcus iniae, Aeromonas salmonicida*, and *Yersinia ruckeri* were standardized and streaked onto sheep blood agar plates. Five mL of mucus was collected from two fish into sterile tubes one of which contained 5 mL of sterile distilled water, while the other contained 5 mL of a 3%

acetic acid solution. This mixture was taken immediately into the lab where it was agitated for 5 min. Thirty μL of each sample was spotted onto the blood agar plate in each of four quadrants. The plates were then incubated at 20°C, and the inhibition zones were measured at 24 hrs. Sterile distilled water and bleach were also spotted as positive and negative controls, respectively.

Disk Diffusion Assay with Lyophilization

Five mL samples of mucus were collected into sterile conical tubes which contained 5 mL of sterile distilled water. The mixture was agitated for 5 min and stored overnight at -80°C. The sample was then lyophilized for 24 hrs. The dried sample was then divided into two parts by weight. One part was dissolved in 3% acetic acid solution and the remaining portion was dissolved in sterile, triple distilled water. Acetic acid was included to enhance the solubility of the proteins (Kumari et al. 2011, Subramanian et al. 2008, Nigam et al. 2015, Wei et al. 2010). Each aliquot was agitated for 5 min and centrifuged at 10,000 x *g* at 4°C for 15 min. Thirty µL of each sample was impregnated onto sterile 6 mm disks and placed onto the surface of the impregnated agar with sterile forceps. Thirty µL of distilled water, bleach, and 3% acetic acid were added to sterile disks. Bleach served as a positive control while distilled water is a negative control, and acetic acid is a component control. The *Streptococcus* plates were then incubated at 37°C, while the *Aeromonas salmonicida* and *Yersinia ruckeri* plates were incubated at 20°C. Zone of inhibition measurements were taken at 24 hrs.

Disk Diffusion Assay with UV Sterilization of Liquid Sample

Five mL samples of mucus were collected into sterile conical tubes which contained either 5 mL of sterile distilled water or 5 mL of 3% acetic acid. The mixture was taken straight to the lab where it was agitated for 5 min. While in sterile conditions, the samples were poured into sterile petri dishes and placed within 15 cm of direct UV light for 20 min. Thirty µL of sample were then pipetted onto 6 mm sterile disks and

placed upon the surface of impregnated agar plates. Thirty μL of distilled water, bleach, and 3% acetic acid were added to sterile disks. Bleach serves as a positive control while distilled water is a negative control, and acetic acid is a component control. The *Streptococcus* plates were then incubated at 37°C, while the *Aeromonas salmonicida* and *Yersinia ruckeri* plates were incubated at 20°C. Zone of inhibition measurements were taken at 24 hrs.

Disk Diffusion Assay with UV sterilization of Crude Mucus Samples

Under sterile conditions, 6 mm sterile paper disks were used to wipe mucus directly from the surface of the fish. These disks were then placed onto sterile petri dishes and placed 15 cm from direct UV light for 10 min. These were then turned with sterile forceps and left for an additional 10 min. Disks were rewet with 50 μL of phosphate buffered saline. These disks were then placed upon the surface of blood agar plates which had been impregnated with standardized bacteria. The *Streptococcus* plates were then incubated at 37°C, while the *Aeromonas salmonicida* and *Yersinia ruckeri* plates were incubated at 20°C. Zone of inhibition measurements were recorded at 24 hrs.

Disk Diffusion Assay with Chloroform Methanol Precipitation

Four hundred μL of methanol was added to 100 μL of crude mucus sample and vortexed for 5 min. An additional 100 μL of chloroform was added to the solution, and it was again vortexed for 5 min. Three hundred μL of distilled water were added, followed by an additional 5 min of agitation. The solution was then centrifuged at 21,913 x *g* for 1

min. The top aqueous layer was removed, 400 µL of methanol were added, and the solution was vortexed for 5 min and centrifuged at 21,913 x *g* for 2 min. The remaining methanol was removed via micropipette as not to disturb the pellet. The sample was then dried using a speedvac (Wessel 1984). This precipitation was undertaken to concentrate the protein, as well as, eliminate any bacterial contamination. The dried pellet was then reconstituted with phosphate buffered saline by repeated pipetting, shaking, and vortexing at 10 min intervals under sterile conditions. The resuspension was incomplete, but spectrophotometer A28 readings showed significant protein had gone into solution. The solution was then drawn from the tubes with a micropipetter, accumulated into a larger sterile tube, and agitated for 5 min. Thirty µL of the solution was pipetted onto 6 mm sterile paper disks which had been placed upon the surface of inoculated sheep blood agar plates. The *Streptococcus* plates were then incubated at 37°C, while the *Aeromonas salmonicida* and *Yersinia ruckeri* plates were incubated at 20°C. Zone of inhibition measurements were recorded at 24 hrs.

Microtiter Broth Dilution Assay with Minimal Sample Processing

Five mL of sample was collected, using the cell scraper method described in detail in Chapter 2.1, into a sterile conical tube containing an additional 5 mL of phosphate buffered saline. The sample was taken immediately to the lab and centrifuged for 10 min at 21,913 x *g*/4°C to remove any epidermal skin cells. The supernatant was poured into a sterile petri dish and placed within 15 cm of UV light under sterile conditions for 20 min.

A 96-well plate was then prepared with a 1:2 serial dilution of the sample in Rows A-D. One hundred fifty µL of 24 hr liquid bacterial cultures (details on culture standardization can be found in Section 2.3) were then added to each well. Rows E-F were growth control and contained only phosphate buffered saline and standardized liquid cultures. The plates were then immediately read on a plate spectrophotometer at 595 nm following medium shaking. The *Streptococcus* plates were incubated at 37°C while the *Aeromonas salmonicida* and *Yersinia ruckeri* plates were incubated at room temperature (approximately 20°C). Spectrophotometer readings were taken after 12 and 24 hrs of incubation.

Microtiter Broth Dilution Assay with Sodium Acetate

Five mL of sample was collected, using the cell scraper method described in detail in Chapter 2.1, into a sterile conical tube containing 200 µL of sodium acetate (NaAc). The sample was taken immediately to the lab and centrifuged at 4°C for 15 min at 21,913 x *g.* The supernatant was the transferred to a larger tube. The pellet was resuspended with 300 µL of NaAc, vortexed, and centrifuged for an additional 15 min at 21,913 x *g* and 4°C. The second supernatant was merged with the initial and the sample was poured into a sterile petri dish where it was held under UV light for 20 min in a sterile field.

A 96-well plate was then prepared with a 1:2 serial dilution of the sample in rows A-D. One hundred fifty µL of 24 hr liquid bacterial cultures were then added to each well. Rows E-F were growth control and contained only phosphate buffered saline and

standardized liquid cultures. The plates were then immediately read on a plate spectrophotometer at 595 nm with medium shaking. The *Streptococcus* plates were incubated at 37°C while the *Aeromonas salmonicida* and *Yersinia ruckeri* plates were incubated at room temperature (approximately 20°C). Spectrophotometer readings were recorded after 12 and 24 hrs of incubation.

Microtiter Broth Dilution Assay to Evaluate Buffer Effectiveness

A plate assay was run without sample using either NaAc or PBS in 1:2 serial dilution along with 150 µL of 24 hr standardized bacterial cultures of *Streptococcus agalactiae, Streptococcus iniae, Aeromonas salomicida*, and *Yersinia ruckeri.* The plates were then immediately read on a Thermo Scientific Multiscan FC plate spectrophotometer at 595 nm following medium shaking. The *Streptococcus* plates were incubated at 37°C while the *Aeromonas salmonicida* and *Yersinia ruckeri* plates were incubated at room temperature (approximately 20°C). Spectrophotometer readings were taken after 12 and 24 hr of incubation.

Microtiter Broth Dilution Assay with PBS and Nanosep Centrifugal Filter

Five mL of mucus sample was collected using the cell scraper method and added to a sterile, conical tube containing 5 mL of PBS. The sample was then centrifuged twice at 21,913 x *g*/4°C, and the supernatants combined. Pall Life Sciences nanosep centrifugal device was then used to concentrate and desalt the sample. The nanoseps were prepared per instructions by running sterile PBS through the filters with two 10-min centrifugations at 21,913 x *g*/4°C. The sample was then separated into 10 nanosep tubes

with a micropipette. The tubes were centrifuged for 10 min at 21,913 x *g* /4°C, examined, and centrifuged for an additional 10 min. The filtrates from each tube were combined. One hundred µL of PBS was added to the top portion of each filter (per instructions), resuspended, and vortexed. The retentate from each nanosep tube was then combined into one sterile tube.

A 96-well plate was then prepared with a 1:2 serial dilution of the sample in Rows A-D. One hundred fifty µL of 24-hr liquid bacterial cultures were then added to each well. Rows E-F were growth control and contained only phosphate buffered saline and standardized liquid cultures. The plates were then immediately read on a plate spectrophotometer at 595 nm following medium shaking. The *Streptococcus* plates were incubated at 37°C while the *Aeromonas salmonicida* and *Yersinia ruckeri* plates were incubated at room temperature (approximately 20°C). Spectrophotometer readings were taken after 12 and 24 hrs of incubation.

CHAPTER THREE

RESULTS AND DISCUSSION

Sample Collection

Upon visual inspection and inspection under a light microscope at 4x magnification (Figure 3.1), the samples taken via scraping with a microscope slide contained a significant amount of skin cells, smaller scales, and other debris which required additional centrifugation. The samples taken via cell scraper and spatula appeared significantly cleaner. When attempting to collect the mucus, with all three methods, it was difficult to get the mucus from the fish into the specimen tube. The most efficient method found was to use a cell scraper in conjunction with a sterile microscope slide. By scraping the mucus onto the slide at the base of the tail, the mucus could then be transferred to the tube by scraping it off the slide. The cell scraper/microscope slide method was used for the remainder of this study for convenience as it was pre-sterilized while the spatula required a 20 min autoclave gravity cycle.

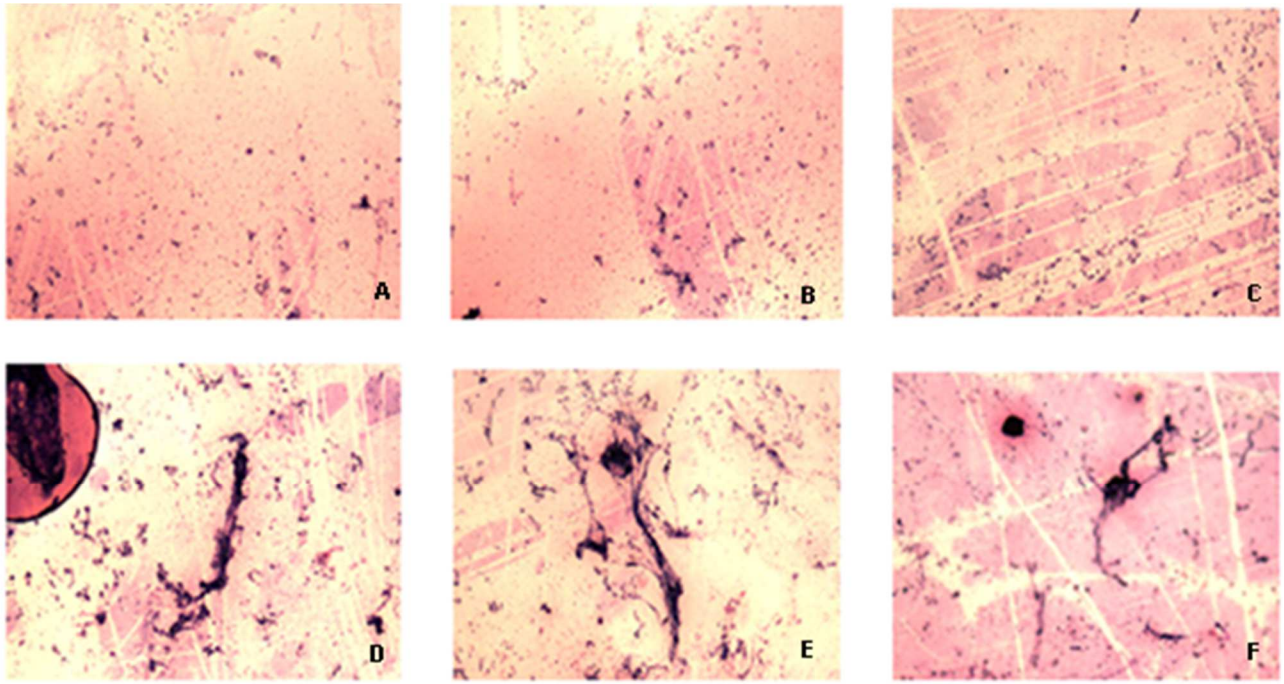


Figure 3.1: Photographs of sample mucus smears stained with Wright's stain. Figures A, B and C are photos of smears of mucus taken via cell scraper. Figures D, E, and F are photos of smears of mucus taken via scraping with a sterile glass microscope slide. All photos were taken of the same size field of vision under 4x magnification.

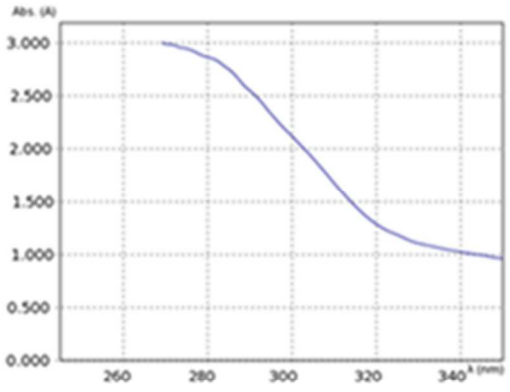
Protein Quantification Results

Analysis of Protein via BioSpectrophotometer

Figure 3.2 (below) represents the protein quantification via spectrophotometer at absorbance 280 for the crude mucus samples, with dilution factors taken into account. The three trials of quantification for crude mucus detected the large quantities of protein one would expect from unprocessed mucus at 28.598, 27.775 and 28.021 mg/mL. This result indicates that the samples, taken from three different tanks of fish at two locations, are composed of similar amounts of total protein, though the composition of those proteins was not determined.

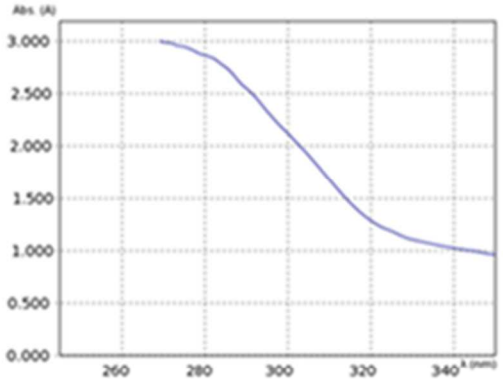
Figure 3.3 represents the protein quantification for the samples in the rententate following the use of a centrifugation filter which concentrates proteins while Figure 3.4 represent the protein quantification for the samples in the filtrate following the same protein concentration centrifugation. The purpose of this measurement was to determine if the concentrating filter was successful in retaining protein, as the literature reports that the antibacterial proteins in fish mucus are small peptides (Ebram 1999). Measurements taken of the retentate resulted in readings of 8.846, 9.032, and 8.537 mg/mL while those taken of the filtrate resulted in readings of 1.172, 0.613, and 1.684 mg/mL. This result indicates that the concentrating centrifugation filter had similar effects on each of the three samples and that more protein remained in the retentate than the filtrate. However, because protein was found in the filtrate, both retentate and filtrate were used in antibacterial assays to assess activity.

Figure 3.2: Curves representing density of crude protein sample per absorbance 280 for three trials.



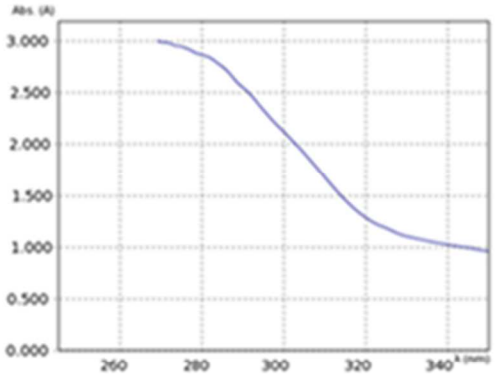
Trial 1

28.598 mg/mL



Trial 2

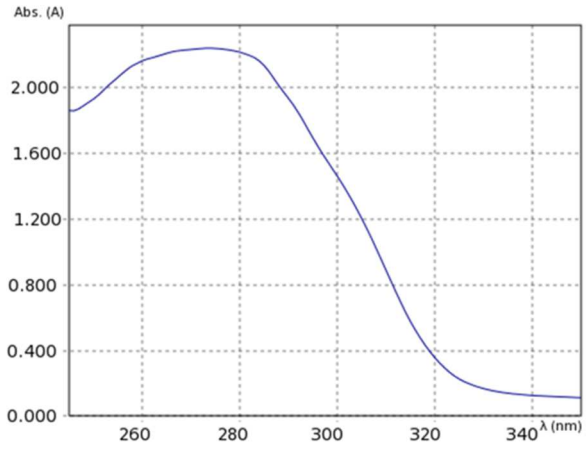
27.775 mg/mL



Trial 3

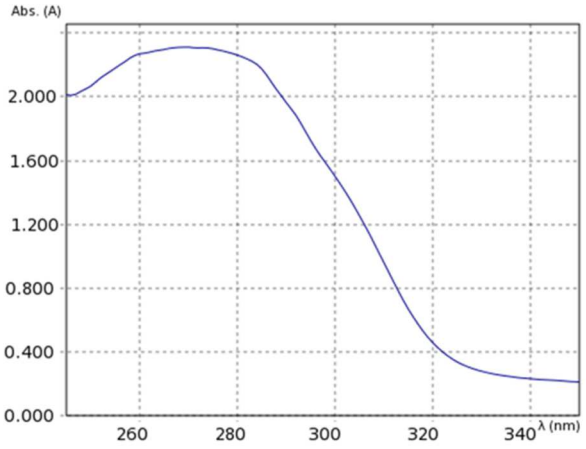
28.021 mg/mL

Figure 3.3: Curves representing density of retentate protein sample per absorbance 280 for three trials.



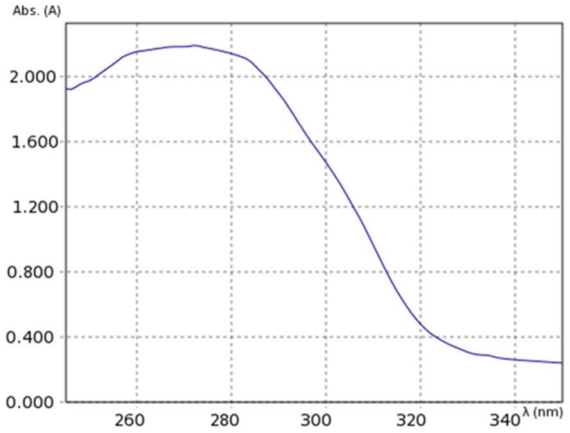
Trial 1

8.846 mg/mL



Trial 2

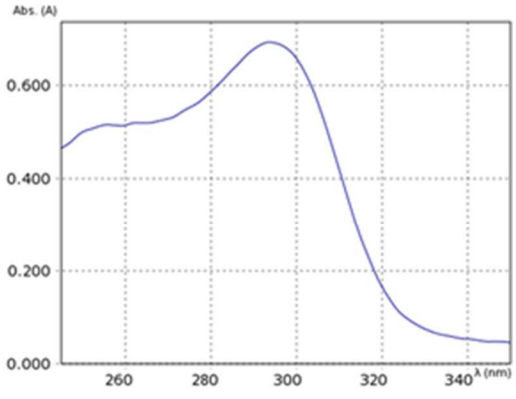
9.032 mg/mL



Trial 3

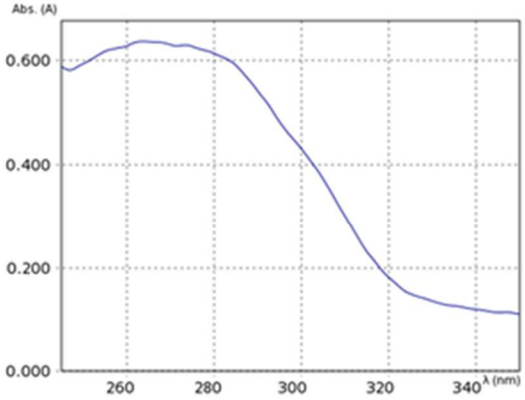
8.537 mg/mL

Figure 3.4: Curves representing density of filtrate protein sample per absorbance 280 for three trials.



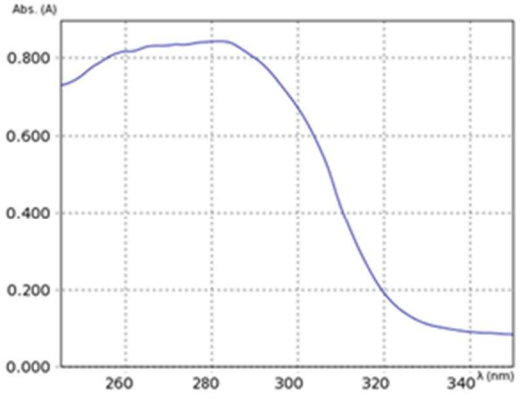
Trial 1

1.172 mg/mL



Trial 2

0.613 mg/mL



Trial 3

1.684 mg/mL

Protein Analysis via Electrophoresis

Electrophoresis (Figure 3.5) was used as verification that the proteins contained within the mucus were of the same approximate molecular weights as those that have been indicated in the literature. The second column of the gel is the Biorad Precision Protein Plus blue standard (seen also in the left) and experimental sample is seen in the first and third columns. This comparison indicates that the molecular weights of the proteins found in the samples for this experiment (approximately 250, 100, 75, 35, and 25 kDa for the most visible results) are consistent with those found in other experiments such as the 233, 104, 88, 78, 51, 46, 38, 31, and 25 previously reported present in tilapia mucus (Wibowbo 2015).

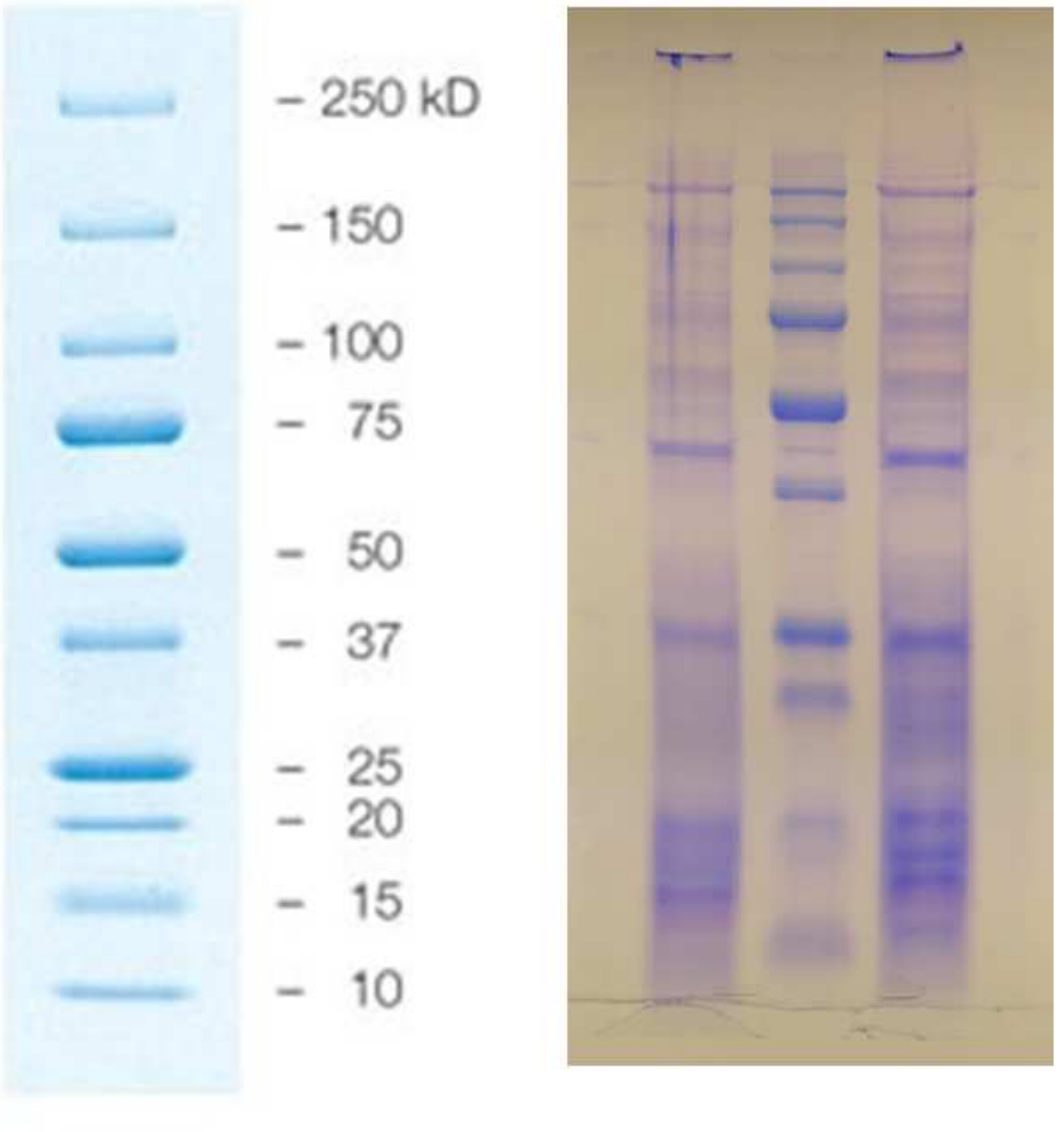


Figure 3.5: Ladder for the blue standard used from Biorad Precision Protein Plus (left). Electrophoresis results with sample in Columns 1 and 3 and blue standard in Column 2 (right).

Contamination Prevention Results

Contamination was suspected of the disk diffusion assay based upon the presence of colonies which have morphologies that were significantly different than the target species. Therefore, samples were plated on seven agar types to verify this conclusion and to point toward a possible source of contamination. Based upon the color changes indicated in the orientation agar and similarities of potential morphologies selected for by each agar, the genera of the contaminants is listed below in Table 3.1. This result indicates contamination from either the urogenital area of the tilapia during scraping or the presence of bacteria found in the water which was heavily contaminated with fecal matter in one sampling location.

Table 3.1: Suspected contamination species based upon agar selection

Agar Type	**Selects For**	**Suspected Contamination**
Sheep Blood Agar	General bacterial growth, detects hemolytic activity	Unidentified colonies of 3 different morphologies
Tryptic Soy Agar	General bacterial growth	Unidentified colonies
MacConkey Agar	Gram negative bacilli and enteric bacilli, differentiates lactose fermentation	Unidentified colonies of 2 different morphologies
Eosin Methylene Blue Agar	Gram negative bacilli, particularly E. coli	*E.coli,* unidentified colonies
Mannitol Salt Agar	Bacteria capable of fermenting mannitol and/or surviving in a high salt environment	No colony growth
Phenylethyl Alcohol Agar	Staphylococcus species	*Staphylococcus* spp.
CHROMagar™ Orientation Agar	Urinary and enteric pathogens, color change per species	*Enterococcus*, *E. coli*, unidentified colonies

Once contamination was confirmed, methods were evaluated to reduce or eliminate the effects of the additional species of bacteria without degrading proteins. Two methods were evaluation, treatment of the diluted sample with ultraviolet radiation and chloroform methanol precipitation. Ultraviolet treatment was proposed based upon its widespread use in sewage treatment facilities where the presence of suspended solids does not diminish UV's effectiveness when the dosage is sufficiently high for the volume for liquid being sterilized (Wolfe 1990). Chloroform methanol precipitation was used both to decontaminate the sample and concentrate the protein. Chloroform works as an antibacterial agent by disrupting the lipids in the cell membrane (Reigada 2013). Following each treatment, samples were streaked onto sheep blood agar plates and left to incubate for 24 hrs with visual inspection at 12 and 24 hrs. Though both methods were largely successful at eliminating or reducing bacterial contamination (see Tables 3.2 and 3.3 below), the chloroform methanol precipitation requires a speedvac drying step which prevented the proteins from completely resuspending in solution; therefore, further experimentation involved decontamination via UV light.

Table 3.2: Twelve-hour plate growth following UV and Chloroform treatment of sample

Treatment	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
UV	-	-	-	-	-
Chloroform	-	-	-	-	-

+indicates growth

–indicates no visible colony growth

Table 3.3: Twenty-four-hour plate growth following UV and Chloroform treatment of sample

Treatment	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
UV	-	-	-	-	-
Chloroform	-	+	-	-	-

+indicates growth

–indicates no visible colony growth

Spot on Lawn Assay Results

Disk antibacterial assays are considered qualitative but not quantitative (Antibiotic 2017); therefore, the results of these assays will be presented as either exhibiting antibacterial activity or not. The initial trials of spot-on-lawn assays were done prior to decontamination experimentation during which every spot exhibited significant growth of bacterial colonies that did not match the morphology of the target organism; therefore, the results have not been included in the following discussion.

When antibacterial activity was detected (Table 3.4), it appeared to be the result of two possible factors: contamination with species which produced substances that prevented the growth of other colonies around the disk or the presence of 3% acetic acid. The contamination issue was dealt with by modifying the protocol to include antibiotic measures that should not degrade the necessary proteins. Acetic acid was initially included in the protocol because of its frequent use in other published works (Hellio 2002, Subashimi 2013, Kumari 2011, Nigam 2015, Wei 2010). The inclusion of acetic acid in the protocol is linked to its ability to enhance the solubility of the proteins; however, in this assay, the 3% acetic acid control produced zones of inhibition without the inclusion of sample. Therefore, the inclusion or concentration of acetic acid in antibacterial assays merits further research.

Table 3.4: Results of spot-on lawn assay trials 1-12/controls

Trial	*S. agalactiae*	*S. iniae*	*A. salmonicida*	*Y. ruckeri*
1 (aqueous)	–	–	–	–*
2 (aqueous)	–	°	–	–*
3 (aqueous)	–	–	–	–
4 (aqueous)	–	–	–	–
5 (aqueous)	–	–	–	–
6 (aqueous)	–	–	–	–
7 (acidic)	+*	+	+*	+
8 (acidic)	+	+	+	+
9 (acidic)	+	°	+	+*
10 (acidic)	–	+	+	+*
11 (acidic)	°	+	–	+
12 (acidic)	–	–	+	+
Bleach	+	+	+	+
D.I.Water	–	–	–	–

*indicates contamination

$^{+}$indicates antibacterial activity

$^{-}$indicates a lack of antibacterial activity

°indicates insufficient bacterial growth

Disk Diffusion Assay with Lyophilization Results

As seen in the spot-on-lawn assays, in the disk diffusion assay with lyophilizaion (Tables 3.5, 3.6, 3.7, and 3.8 below) antibacterial activity was only detected when in the presence of bleach or acetic acid. Lyophilization was also tested within this protocol because it is a commonly used practice in the processing of mucus for antibacterial testing; however, it has been theorized that the water within the mucus may have been "bound irreversibly to the glycoprotein" (Mucus Symposium 2009). If so, the structure of the mucus would be damaged significantly, which may affect its ability to act as an antibacterial substance. Therefore, future trials were undertaken without lyophilization to potentially determine its effect on the antibacterial properties of the mucus (see below).

Disk diffusion assays cannot distinguish between bacteriostatic and bactericidal activities; however, neither of these conditions were detectable in this assay. There are a minimum of three possible explanations for this result: diffusion rate, solubility, or a lack of antibacterial activity in tilapia mucus. Further research should be undertaken to alter each of these variables to determine if another result is possible.

Table 3.5: Results of disk diffusion assay with Lyophilization trials 1-12/controls for *Streptococcus agalactiae*

Trial 1 (aqueous)	–	Trial 1 (acetic)	–
Trial 2 (aqueous)	–	Trial 2 (acetic)	+
Trial 3 (aqueous)	–	Trial 3 (acetic)	+
Trial 4 (aqueous)	–	Trial 4 (acetic)	+
Trial 5 (aqueous)	–	Trial 5 (acetic)	–
Trial 6 (aqueous)	–	Trial 6 (acetic)	+
Trial 7 (aqueous)	–	Trial 7 (acetic)	–
Trial 8 (aqueous)	–	Trial 8 (acetic)	+
Trial 9 (aqueous)	–	Trial 9 (acetic)	+
Trial 10 (aqueous)	–	Trial 10 (acetic)	+
Trial 11 (aqueous)	–	Trial 11 (acetic)	+
Trial 12 (aqueous)	–*	Trial 12 (acetic)	+
Bleach	+	3% Acetic	+
D.I.Water	–	D.I. Water	–

*indicates contamination
$^{+}$indicates antibacterial activity
$^{-}$indicates a lack of antibacterial activity
°indicates insufficient bacterial growth

Table 3.6: Results of disk diffusion assay with Lyophilization trials 1-12/controls for *Streptococcus iniae*

Trial 1 (aqueous)	–	Trial 1 (acetic)	+
Trial 2 (aqueous)	–	Trial 2 (acetic)	+
Trial 3 (aqueous)	–	Trial 3 (acetic)	+
Trial 4 (aqueous)	–	Trial 4 (acetic)	+
Trial 5 (aqueous)	–	Trial 5 (acetic)	+
Trial 6 (aqueous)	–	Trial 6 (acetic)	+
Trial 7 (aqueous)	–	Trial 7 (acetic)	+
Trial 8 (aqueous)	–	Trial 8 (acetic)	+
Trial 9 (aqueous)	–	Trial 9 (acetic)	–
Trial 10 (aqueous)	–	Trial 10 (acetic)	+
Trial 11(aqueous)	–	Trial 11 (acetic)	+
Trial 12 (aqueous)	–	Trial 12 (acetic)	–
Bleach	+	3% Acetic	+
D.I.Water	–	D.I. Water	–

*indicates contamination

$^{+}$indicates antibacterial activity

$^{-}$indicates a lack of antibacterial activity

°indicates insufficient bacterial growth

Table 3.7: Results of disk diffusion assay with Lyophilization trials 1-12/controls for *Aeromonas salmonicida*

Trial 1 (aqueous)	–	Trial 1 (acetic)	+
Trial 2 (aqueous)	–*	Trial 2 (acetic)	+
Trial 3 (aqueous)	–	Trial 3 (acetic)	–
Trial 4 (aqueous)	–	Trial 4 (acetic)	+
Trial 5 (aqueous)	–*	Trial 5 (acetic)	+*
Trial 6 (aqueous)	–	Trial 6 (acetic)	+
Trial 7 (aqueous)	–	Trial 7 (acetic)	+
Trial 8 (aqueous)	–	Trial 8 (acetic)	–
Trial 9 (aqueous)	–	Trial 9 (acetic)	–
Trial 10 (aqueous)	–	Trial 10 (acetic)	+
Trial 11(aqueous)	–	Trial 11 (acetic)	+
Trial 12 (aqueous)	–	Trial 12 (acetic)	+*
Bleach	+	3% Acetic	+
D.I.Water	–	D.I. Water	–

*indicates contamination

$^{+}$indicates antibacterial activity

$^{-}$indicates a lack of antibacterial activity

°indicates insufficient bacterial growth

Table 3.8: Results of disk diffusion assay with Lyophilization trials 1-12/controls for *Yersinia ruckeri*

Trial 1 (aqueous)	–	Trial 1 (acetic)	–
Trial 2 (aqueous)	–	Trial 2 (acetic)	–
Trial 3 (aqueous)	–	Trial 3 (acetic)	+
Trial 4 (aqueous)	–	Trial 4 (acetic)	+
Trial 5 (aqueous)	–	Trial 5 (acetic)	+
Trial 6 (aqueous)	–	Trial 6 (acetic)	+
Trial 7 (aqueous)	–	Trial 7 (acetic)	+
Trial 8 (aqueous)	–	Trial 8 (acetic)	+
Trial 9 (aqueous)	–	Trial 9 (acetic)	+
Trial 10 (aqueous)	–	Trial 10 (acetic)	+
Trial 11(aqueous)	–	Trial 11 (acetic)	+
Trial 12 (aqueous)	–	Trial 12 (acetic)	+
Bleach	+	3% Acetic	+
D.I.Water	–	D.I. Water	–

*indicates contamination

$^{+}$indicates antibacterial activity

$^{-}$indicates a lack of antibacterial activity

°indicates insufficient bacterial growth

Disk Diffusion Assay with UV Sterilization of Liquid Sample Results

In further attempts to evaluate the disk diffusion assay, a second trial was undertaken which removed the lyophilization step (see Tables 3.9, 3.10, 3.11, and 3.12) As with the spot-on-lawn assay and the lyophilization disk assay, antibacterial activity was only detected in those samples which had been added to the 3% acetic acid solution, yet the control for acetic acid without sample was again positive. Therefore, it could not be determined if the lyophilizing had an effect on the antibacterial properties of the mucus samples.

Table 3.9: Results of disk diffusion assay with UV sterilization for *Streptococcus agalactiae*

Trial 1 (aqueous)	–	Trial 1 (acetic)	–
Trial 2 (aqueous)	–	Trial 2 (acetic)	+
Trial 3 (aqueous)	–	Trial 3 (acetic)	–
Trial 4 (aqueous)	–	Trial 4 (acetic)	+
Trial 5 (aqueous)	–	Trial 5 (acetic)	+
Trial 6 (aqueous)	–	Trial 6 (acetic)	+
Trial 7 (aqueous)	–	Trial 7 (acetic)	+
Trial 8 (aqueous)	–	Trial 8 (acetic)	–
Trial 9 (aqueous)	–	Trial 9 (acetic)	–
Trial 10 (aqueous)	–	Trial 10 (acetic)	+
Trial 11(aqueous)	–	Trial 11 (acetic)	+
Trial 12 (aqueous)	–	Trial 12 (acetic)	+
Bleach	–	3% Acetic	+
D.I.Water	–	D.I. Water	–

*indicates contamination
$^{+}$indicates antibacterial activity
$^{-}$indicates a lack of antibacterial activity
°indicates insufficient bacterial growth

Table 3.10: Results of disk diffusion assay with UV sterilization for *Streptococcus iniae*

Trial 1 (aqueous)	–	Trial 1 (acetic)	+
Trial 2 (aqueous)	–	Trial 2 (acetic)	+
Trial 3 (aqueous)	–	Trial 3 (acetic)	–
Trial 4 (aqueous)	–	Trial 4 (acetic)	–
Trial 5 (aqueous)	–	Trial 5 (acetic)	+
Trial 6 (aqueous)	–	Trial 6 (acetic)	+
Trial 7 (aqueous)	–	Trial 7 (acetic)	+
Trial 8 (aqueous)	+*	Trial 8 (acetic)	–
Trial 9 (aqueous)	–	Trial 9 (acetic)	+
Trial 10 (aqueous)	–	Trial 10 (acetic)	+
Trial 11(aqueous)	–	Trial 11 (acetic)	+
Trial 12 (aqueous)	–	Trial 12 (acetic)	–
Bleach	+	3% Acetic	+
D.I.Water	–	D.I. Water	–

*indicates contamination

$^{+}$indicates antibacterial activity

$^{-}$indicates a lack of antibacterial activity

°indicates insufficient bacterial growth

Table 3.11: Results of disk diffusion assay with UV sterilization for *Aeromonas salmonicida*

Trial 1 (aqueous)	–	Trial 1 (acetic)	+
Trial 2 (aqueous)	–	Trial 2 (acetic)	+
Trial 3 (aqueous)	–	Trial 3 (acetic)	+*
Trial 4 (aqueous)	–	Trial 4 (acetic)	+
Trial 5 (aqueous)	–	Trial 5 (acetic)	+
Trial 6 (aqueous)	–	Trial 6 (acetic)	–
Trial 7 (aqueous)	–*	Trial 7 (acetic)	+
Trial 8 (aqueous)	–	Trial 8 (acetic)	–
Trial 9 (aqueous)	–	Trial 9 (acetic)	+
Trial 10 (aqueous)	–	Trial 10 (acetic)	–
Trial 11(aqueous)	–*	Trial 11 (acetic)	–
Trial 12 (aqueous)	–	Trial 12 (acetic)	+
Bleach	+	3% Acetic	+
D.I.Water	–	D.I. Water	–

*indicates contamination

$^{+}$indicates antibacterial activity

$^{-}$indicates a lack of antibacterial activity

°indicates insufficient bacterial growth

Table 3.12: Results of disk diffusion assay with UV sterilization for *Yersinia ruckeri*

Trial 1 (aqueous)	–*	Trial 1 (acetic)	+*
Trial 2 (aqueous)	–	Trial 2 (acetic)	+
Trial 3 (aqueous)	–	Trial 3 (acetic)	–
Trial 4 (aqueous)	–*	Trial 4 (acetic)	+*
Trial 5 (aqueous)	–*	Trial 5 (acetic)	+*
Trial 6 (aqueous)	–	Trial 6 (acetic)	+
Trial 7 (aqueous)	–	Trial 7 (acetic)	+
Trial 8 (aqueous)	–	Trial 8 (acetic)	–
Trial 9 (aqueous)	–	Trial 9 (acetic)	–
Trial 10 (aqueous)	–	Trial 10 (acetic)	+
Trial 11(aqueous)	–	Trial 11 (acetic)	+
Trial 12 (aqueous)	–	Trial 12 (acetic)	–
Bleach	+	3% Acetic	+
D.I.Water	–	D.I. Water	–

*indicates contamination

$^{+}$indicates antibacterial activity

$^{-}$indicates a lack of antibacterial activity

°indicates insufficient bacterial growth

Disk Diffusion Assay with UV sterilization of Crude Mucus Results

Given the results of the previous assays, questions arose about a possible lack of uniformity of the density of the proteins within the mucus. In other words, are the mucus proteins evenly spread throughout the solution, and if not, could this account for the lack of antibacterial activity? Therefore, an additional assay was devised which evenly spread mucus across the disk directly from the surface (results in Table 3.13 below). The disks were held with sterile forceps and used to wipe the mucus directly from the surface of the fish. The disks were then held under UV and resuspended with 50 μL of sterile distilled water. As with previously discussed disk diffusion assays, no antibacterial activity was measured except in cases of contamination. These zones of inhibition were likely caused by the invading bacteria. It is unclear if the lack of antibacterial activity is the result of a lack of solubility of the sample which may have adhered too well to the disk. This result led to further experimentation to test chloroform as an antimicrobial agent and to ensure that the UV light was not degrading any of the necessary peptides and proteins found in the sample.

Table 3.13: Results of disk diffusion assay with UV sterilization of crude mucus for all diseases

	S. agalactiae	*S. iniae*	*A. salmonicia*	*Y. ruckeri*
Trial 1	–	–	–	+*
Trial 2	–	–	–	–
Trial 3	–	–	–	–
Trial 4	–	–	–	–
Trial 5	–	–	–	–
Trial 6	–	–	–	–
Trial 7	–	–	–	–
Trial 8	+*	–	–	–
Trial 9	–	–	–	–
Trial 10	–	–	–	–
Trial 11	–	–	–	–
Trial 12	–	–*	–	–
Bleach	+	+	+	+
D.I.Water	–	–	–	–
PBS	–	–	–	–

*indicates contamination
$^{+}$indicates antibacterial activity
$^{-}$indicates a lack of antibacterial activity
°indicates insufficient bacterial growth

Disk Diffusion Assay with Chloroform Methanol Precipitation Results

A chloroform methanol precipitation assay was carried out to evaluate chloroform as a possible method of removing contaminating bacteria. The chloroform would then be evaporated during the speedvac process and would not affect the antibacterial activity of the assay itself. The additional benefit of this precipitation is protein concentration which should amplify the effects of the antimicrobial proteins in the same. Unfortunately, the protein pellet that remained after speedvac drying did not go back into solution well even upon repeated agitation via vortex and micropipetting. The results of the assay can be seen in Table 3.14 below; however, it is unlikely that enough of the protein entered into solution for any antibacterial results to occur.

Table 3.14: Results of disk diffusion assay with chloroform methanol for all diseases

	S. agalactiae	*S. iniae*	*A. salmonicida*	*Y. ruckeri*
Trial 1	–	+*	–	–
Trial 2	–	–	–	–
Trial 3	–	–	–	–
Trial 4	–	–	–	–
Trial 5	–	–	–	–
Trial 6	–	–	–	–
Trial 7	–	–	–	–
Trial 8	–	–	–	–
Trial 9	–	–	–	–
Trial 10	–	–	–	–
Trial 11	–	–	–	–
Trial 12	–	–	–	–
Bleach	+	+	+	+
D.I.Water	–	–	–	–
PBS	–	–	–	–

*indicates contamination
+indicates antibacterial activity
–indicates a lack of antibacterial activity
°indicates insufficient bacterial growth

Microtiter Plate Assay with Minimal Sample Processing Results

The results of the spot-on-lawn and disk diffusion assays indicated a lack of sensitivity in measuring the antibacterial activity in fish mucus, which the literature overwhelmingly supports. Therefore, experiments using a microtiter method of reading growth via spectrophotometer were undertaken as a more sensitive method of determining antimicrobial activity. In this initial run, the sample was simply centrifuged to remove epidermal cells, placed under UV, and diluted in a small amount of phosphate buffered saline prior to the plate assay.

For the purposes of this assay, antibacterial activity was calculated by taking a percentage of the growth control mean and subtracting it from the change in turbidity over time. The resulting cells that were less than zero were considered to indicate antibacterial activity, assessed as a total of antibacterial units based upon the dilution factor of each well. An antibacterial unit is defined as the reciprocal of the dilution factor for each well that inhibited growth by 50% (Faye *et al*. 2002, Singh *et al*. 2012, Wang 2010). For the purposes of this study, growth inhibition percentages lower than 50% were also calculated. The measurements are then averaged from three trials and displayed in Tables 3.15 and 3.16 below. Percentages are calculated based on percentage of growth control mean and represent a reduction in growth by that factor. This assay was successful in detecting significant antibacterial activity of the sample for all diseases: *S. agalactiae, S.iniae, A. salmonicida*, and *Y. ruckeri* (Table 3.15 and 3.16 below). The raw data for this assay can be found in Appendix A.

Table 3.15 Antibacterial activity for minimal processing assay at 12 hours for all diseases calculated in antibacterial units.

Antibacterial Activity at 12 hours (in Antibacterial Units/ml)

	S. agalactiae	***S. iniae***	***A. salmonicida***	***Y. ruckeri***
10% growth reduction	44.33	2731.67	2.33	1279.67
20% growth reduction	40.00	2696.67	1.67	1311.67
30% growth reduction	29.00	0.00	1.00	586.00
40% growth reduction	21.00	0.00	1.00	148.67
50% growth reduction	22.33	0.00	0.33	137.33

% growth reduction indicates that the bacterial culture challenged with the mucus sample grew x% less than the average of the control wells.
An antibacterial unit is defined as the reciprocal of the dilution factor for each well that inhibited growth by x%

Table 3.16 Antibacterial activity for minimal processing assay at 24 hours for all diseases calculated in antibacterial units.

Antibacterial Activity at 24 hours (in Antibacterial Units/ml)

	S. agalactiae	***S. iniae***	***A. salmonicida***	***Y. ruckeri***
10% growth reduction	0.00	2732.33	3,625.67	83.33
20% growth reduction	0.00	2728.33	166.33	82.00
30% growth reduction	0.00	0.00	412.00	59.00
40% growth reduction	0.00	0.00	86.67	41.67
50% growth reduction	0.00	0.00	20.67	37.33

% growth reduction indicates that the bacterial culture challenged with the mucus sample grew x% less than the average of the control wells.
An antibacterial unit is defined as the reciprocal of the dilution factor for each well that inhibited growth by x%

Microtiter Broth Dilution Assay with Sodium Acetate Results

An additional microtiter broth dilution assay was carried out to evaluate the effectiveness of various solvents used in assays which measure antibacterial activities of epidermal mucus. An assay recommended by the Department of Marine Biotechnology at the University of Tromso, Norway advises the use of sodium acetate (pH 6.0) as a buffer in a microtiter dilution assay that measures antibacterial activity of tilapia mucus (Mozumder 2005). However, the growth control wells for each disease failed to grow with this buffer. The growth control mean for *S. agalactiae* was -0.05867 at 12 hrs and -0.02458 at 24 hrs. The growth control mean for *S. iniae* was -0.003667 at 12 hrs and -0.01508 at 24 hrs. The growth control mean for *A. salmonicida* was -0.1295, and the growth control mean for *Y. ruckeri* was -0.01625. This indicates a complete lack of culture growth for the entire assay; therefore, antibacterial activity could not be calculated. The raw data for three trials of this assay can be found in Appendix A.

Microtiter Broth Dilution Assay to Evaluate Buffer Effectiveness Results

Given the results of the microtiter assay using sodium acetate as a buffer, an experiment was conducted which sought to compare the use of sodium acetate buffer to phosphate buffered saline. The difference between the 12-hr reading and the 0-hr reading for three trials of each buffer with each of the four diseases indicates negative growth for all sodium acetate wells and positive growth for PBS wells (Table 3.17). The growth means for the sodium acetate test were negative for all diseases, and the growth means for all phosphate buffered saline test were positive. This result clearly encourages the use of PBS as a buffer in microtiter broth dilution assays.

Table 3.17: Growth control means for all diseases grown in sodium acetate or phosphate buffered saline.

Treatment	**Growth Control Mean**
S. agalactiae + NaAc	-0.00425
S. iniae. + NaAc	-0.00167
A. salmon. + NaAc	-0.38017
Y. ruckeri + NaAc	-0.12067
S. agalactiae + PBS	0.00667
S. iniae + PBS	0.05
A. salmonicida + PBS	0.0167
Y. ruckeri + PBS	0.0417

Microtiter Broth Dilution Assay with PBS and Nanosep Centrifugal Filter Results

A final assay was devised which sought to concentrate the protein in the sample to see what if any effect this might have on the antibacterial activity measured by the plate spectrophotometer. Once the sample was concentrated and desalted with the centrifugal filter, absorbance 280 biospectrophotometer readings indicated that protein was present both in the rententate and the filtrate. Therefore, the antibacterial assay was run on both portions of the sample. It was thought that perhaps the smallest of the antimicrobial peptides found in the mucus samples could have been spun into the filtrate..

The assays carried out with the rententate from this centrifugation filter measured no antibacterial activity against any of the four tested diseases (Tables 3.18 and 3.19). The results of the assays run on the filtrate (Tables 3.20 and 3.21) illustrate no antibacterial activity was present for *S. agalactiae*, *A. salmonicida*, or *Y. ruckeri* at 12 hrs. There was; however, significant antibacterial activity measured for *A.salmonicida* at the 10% and 20% growth reduction levels with 2,004.33 antibacterial units and 5.33 antibacterial units respectively.

This testing method was used on identical samples to the minimal processing assay previously discussed. Therefore, the addition of the concentrating filter to the process resulted in a reduction in measureable antibacterial activity. This reduction could be due to the protein's exposure to ambient temperatures during the additional time needed for processing; however, the samples were maintained on ice and centrifuged at 4°C. The reduction in activity could also be due to the loss of cross linkages in the mucus

during the filtering or the loss of interactions between the larger proteins with the smaller peptides. Further research should be undertaken to determine the underlying causes.

Table 3.18: Antibacterial activity for centrifugal filter assay retentate sample at 12 hours for all diseases calculated in antibacterial units.

Antibacterial Activity at 12 hours (in Antibacterial Units/ml)

	S. agalactiae	***S. iniae***	***A. salmonicida***	***Y. ruckeri***
10% growth reduction	0.00	0.00	0.00	0.00
20% growth reduction	0.00	0.00	0.00	0.00
30% growth reduction	0.00	0.00	0.00	0.00
40% growth reduction	0.00	0.00	0.00	0.00
50% growth reduction	0.00	0.00	0.00	0.00

% growth reduction indicates that the bacterial culture challenged with the mucus sample grew x% less than the average of the control wells.

An antibacterial unit is defined as the reciprocal of the dilution factor for each well that inhibited growth by x%

Table 3.19: Antibacterial activity for centrifugal filter assay retentate sample at 24 hours for all diseases calculated in antibacterial units.

Antibacterial Activity at 24 hours (in Antibacterial Units/ml)

	S. agalactiae	***S. iniae***	***A. salmonicida***	***Y. ruckeri***
10% growth reduction	1.00	0.00	0.00	0.67
20% growth reduction	0.33	0.00	0.00	0.00
30% growth reduction	0.00	0.00	0.00	0.00
40% growth reduction	0.00	0.00	0.00	0.00
50% growth reduction	0.00	0.00	0.00	0.00

% growth reduction indicates that the bacterial culture challenged with the mucus sample grew x% less than the average of the control wells.

An antibacterial unit is defined as the reciprocal of the dilution factor for each well that inhibited growth by x%

Table 3.20: Antibacterial activity for centrifugal filter assay filtrate sample at 12 hours for all diseases calculated in antibacterial units.

Antibacterial Activity at 12 Hours (in Antibacterial Units/ml)

Sample	***S. agalactiae***	***S. iniae***	***A. salmonicida***	***Y. ruckeri***
10% growth reduction	0.00	0.00	0.00	0.00
20% growth reduction	0.00	0.00	0.00	0.00
30% growth reduction	0.00	0.00	0.00	0.00
40% growth reduction	0.00	0.00	0.00	0.00
50% growth reduction	0.00	0.00	0.00	0.00

% growth reduction indicates that the bacterial culture challenged with the mucus sample grew x% less than the average of the control wells.

An antibacterial unit is defined as the reciprocal of the dilution factor for each well that inhibited growth by x%

Table 3.21: Antibacterial activity for centrifugal filter assay filtrate sample at 24 hours for all diseases calculated in antibacterial units.

Antibacterial Activity at 24 Hours (in Antibacterial Units/ml)

Sample	***S. agalactiae***	***S. iniae***	***A. salmonicida***	***Y. ruckeri***
10% growth reduction	1.33	0.00	2004.33	5.00
20% growth reduction	0.33	0.00	5.33	0.00
30% growth reduction	0.00	0.67	0.00	0.00
40% growth reduction	0.00	0.00	0.00	0.00
50% growth reduction	0.00	0.00	0.00	0.00

% growth reduction indicates that the bacterial culture challenged with the mucus sample grew x% less than the average of the control wells.

An antibacterial unit is defined as the reciprocal of the dilution factor for each well that inhibited growth by x%

CONCLUSIONS

The disk diffusion and spot-on-lawn assays were not successful in measuring antibacterial activity unless the sample was diluted into 3% acetic acid. When acetic acid was used as a control, it alone resulted in zones of inhibition. Therefore, future studies should discontinue use of 3% acetic acid as a solvent. Additional studies should be conducted to confirm this finding and to further determine the usefulness of diffusion assays on mucus antibacterial testing.

The results of the microtiter broth dilution assay also indicate a need to carefully examine the role that solvents play in the determination of antibacterial activity. Just as in the disk diffusion assay, the addition of sodium acetate (NaAc) to the assay resulted in antibacterial activity that was likely the result of the solvent due to the fact that the growth control wells, which contained only NaAc and culture, failed to grow. This finding was strengthened by an additional assay which compared culture growth with phosphate buffered saline and NaAc. Further research should be done to confirm this result, and future experimental models should adapt accordingly.

In addition, statistical analysis indicates that mucus which has been concentrated by a centrifugation filter exhibits less antibacterial activity than that which has not been processed beyond simple centrifugation and removal of contaminating bacteria. Using a t-test for means comparison, the data collected indicate that against *A. salmonicida* (Table 4.1*)*, this most simple method revealed more antibacterial activity for 10%, 20%, 30% and 40% for the duration of the assay. Against *S. agalactiae* (Table 4.2), it detected more antibacterial activity for 20-30% at the 12-hour mark but did not provide measure of

additional activity at 24 hrs. Against *S. iniae* (Table 4.2), the simple spin methodology provided more measured antibacterial activity for 10-20% for both 12 and 24 hrs. Finally, when the simple spin sample was measured against *Y. ruckeri*, the assay detected more antibacterial activity at both time points and for all growth reduction percentages.

As the simple processed microtiter dilution assay was the only method tested which measured any significant antibacterial activity, the biological activity found bears further discussion, particularly for the 10% growth reduction level (Table 3.15). Ten percent of *Y. ruckeri's* growth was inhibited at dilution rates up to 1:1024 (1279.67 antibacterial units) for the first 12 hrs, which is consistent with the tilapiine ability to resist this disease, as it is primarily a pathogen that affects species of salmon (Eissa 2008). *S. agalactiae* saw a 10% reduction in growth up to 1:32 dilution during the first 12-hour period (44.33 antibacterial units) but no change over the second 12 hrs. During the first 12-hour period, the mucus sample only reduced *A. salmonicida's* growth by 10% up to a dilution factor of 1:2; however, by the 24-hour mark, that 10% reduction was measured at a dilution factor of 1:2048 (3,625.67 antibacterial units), which indicates that the antibacterial activity of the mucus is successful against *A. salmonicida* but requires a longer period of time. Most surprising was the extensive antibacterial activity that the mucus samples exhibited against *S. iniae* with 10% growth reduction up to the 1:2048 dilution factor at both 12 hrs (2731 antibacterial units) and 24 hrs (2732 antibacterial units).

When measuring for 20% reduction in growth, the sample was even more limited at fighting *S. agalactiae* at only 40 antibacterial units at the 12-hour measurement and no additional reduction of growth was measured at 24 hrs. *A. salmonicida* had non-significant reduction of growth for 20% (1.67 antibacterial units) at 12 hrs. Again, the mucus was more successful at reducing *A. salmonicida* at the 24-hour mark (167 antibacterial units), further indicating that additional time is needed for the mucus to successfully fight this bacterium. In contrast, the data indicate that the mucus reduces the growth of *Y. ruckeri* by 20% quickly and completely (1,311.67 antibacterial units) within the first 12 hrs then does little to further reduce the growth during the second 12-hour period. Finally, as with the 10% reduction rate, *S. iniae* was significantly reduced for 20% during both the 12-hour (2,696.67 antibacterial units) and 24-hour (2,728.33 antibacterial units) time frames.

The data indicate that the tilapia mucus is much less likely to reduce disease growth to higher levels of 30-50%. *Y. ruckeri* was the most reduced by the mucus samples at these levels in the first 12 hrs with 30% reduction by 586 antibacterial units, 40% reduction by 148.67 antibacterial units, and 50% reduction by 137.33 antibacterial units. During the second 12-hour period, the mucus sample was less able to reduce *Y. ruckeri's* growth to 30% (59.00 antibacterial units), 40% (41.67 antibacterial units), and 50% (37.33 antibacterial units). The mucus failed to reduce *S. iniae* at rates of 30-50% at all, and it only reduced *S. agalactiae* in the first 12 hrs to 30% (29 antibacterial units), 40% (21 antibacterial units), and 50% (22.33 antibacterial units). The sample's ability to kill *A. salmonicida* to higher levels (30-50%) continues the trend of limited potency by

the 12-hour mark with only 1 antibacterial unit for 30% and 40%, and 0.33 antibacterial unit for 50% reduction. However, by 24 hrs, those numbers had increased exponentially to 412 antibacterial units for 30% reduction, 86.67 antibacterial units for 40%, and 20.67 antibacterial units for 50% reduction. Overall, the mucus with limited processing was able to successfully defend against all four bacterial cultures, though *S. agalactiae* to a lesser degree. This result is consistent with reports of the virulence of *S. agalactiae*.

When the dilution factor is isolated as a variable with the standard of inhibition simply being whether it grew more or less than the Control, it becomes apparent that at certain dilution factors, the addition of the mucus sample may have aided in the growth of the bacteria culture. This is particularly true for the *Streptococcus* species (Figures 4.1 and 4.2). At the 12-hour reading, *S. agalactiae* was significantly different from control at the 1:64, 1:128, 1:256, 1:512 dilution factors, and each of these data points showed an increase in colony growth with the addition of the mucus. It is possible at this low level of antibacterial activity that the bacteria are able to feed off of the sugar portion of the glycoprotein. Similar behavior has been observed in bacteria which feed off of the mucus within the intestines of mice (Berry 2013). However, at the 24-hour reading for *S. agalactiae*, 1:4, 1:8, and 1:64 also showed the bacteria appear to benefit from the addition of the mucus. In contrast, *S. iniae*'s 12-hour reading (Figure 4.2) included significant differences from control at dilution factors 1:1, 1:8, 1:16, 1:32, 1:64, 1:128, 1:512, and 1:2048. Each of these data points was less than the control mean; therefore, statistically significant growth reduction occurred during this time period. The 24-hour reading, however, indicates a significant difference at 1:1, 1:2, 1:4, 1:64, and each of these is

above the growth mean, indicating that the culture grew better in the mucus wells than in the control wells. More research needs to be done to determine the underlying causes of this result.

When looking at the dilution factor treatments for *A. salmonicida* and *Y. ruckeri* (Figures 4.3 and 4.4), the most apparent finding is that *A. salmonicida* does significant growing during the first 12-hour period and is inhibited during the second 12-hour period. *Y. ruckeri* does the opposite, rebounding in the second phase after inhibition in the first. If one looks at specific dilution factors, during the first 12 hrs, *A. salmonicida* was significantly different from the Control at the 1:2048 dilution where it grew better than the Control. At the 24-hour reading, *A. salmonicida* was only different from the Control at the 1:32 dilution point where it was inhibited below the levels of the Control. *Y. ruckeri* was only different than the control for the 1:8 dilution factor during the second 12-hour growth period, and it grew significantly less than Control.

Initially, it was thought that the microtiter broth dilution assay failed to measure significant antibacterial activity at all; however, the wrong question was being asked of the data. Early experiments involved calculating only antibacterial activity that reached the level of 50% growth reduction; this resulted in findings that were essentially hidden in the numbers. Once levels lower than 50% were analyzed, the antibacterial activity became apparent. These findings reinforce the necessity for further development of a highly sensitive test which takes into account *real world* conditions. Additionally, further research is needed into both the general and specific modes in which antimicrobial compounds found in tilapia mucus attack varying species of bacteria.

Table 4.1: Mean Comparison (via t-test) of antibacterial activity (in antibacterial units) against *A. salmonicida* and *Y. ruckeri* at 12 and 24 hours using three processing methods

Disease Name	Time (Hours)	Growth Reduction	Method 1 Mean[1]	Method 2 Mean[2]	Method 3 Mean[3]
A. salmonicida	12	10%	2.33^{A}	0.00^{B}	0.00^{B}
A. salmonicida	12	20%	1.67^{A}	0.00^{B}	0.00^{B}
A. salmonicida	12	30%	1.00^{A}	0.00^{B}	0.00^{B}
A. salmonicida	12	40%	1.00^{A}	0.00^{B}	0.00^{B}
A. salmonicida	12	50%	0.33^{A}	0.00^{A}	0.00^{A}
A. salmonicida	24	10%	3602.67^{A}	2004.33^{AB}	0.00^{B}
A. salmonicida	24	20%	2642.67^{A}	0.00^{B}	0.00^{B}
A. salmonicida	24	30%	2338.00^{A}	0.00^{B}	0.00^{B}
A. salmonicida	24	40%	77.33^{A}	0.00^{B}	0.00^{B}
A. salmonicida	24	50%	20.67^{A}	0.00^{B}	0.00^{B}
Y. ruckeri	12	10%	1654.00^{A}	0.00^{B}	0.00^{B}
Y. ruckeri	12	20%	1311.67^{A}	0.00^{B}	0.00^{B}
Y. ruckeri	12	30%	586.00^{A}	0.00^{B}	0.00^{B}
Y. ruckeri	12	40%	148.67^{A}	0.00^{B}	0.00^{B}
Y. ruckeri	12	50%	137.33^{A}	0.00^{B}	0.00^{B}
Y. ruckeri	24	10%	83.33^{A}	5.00^{B}	0.67^{B}
Y. ruckeri	24	20%	82.00^{A}	0.00^{B}	0.00^{B}
Y. ruckeri	24	30%	59.00^{A}	0.00^{B}	0.00^{B}
Y. ruckeri	24	40%	41.67^{A}	0.00^{B}	0.00^{B}
Y. ruckeri	24	50%	37.33^{A}	0.00^{B}	0.00^{B}

[1]Method 1: Microtiter Broth Dilution Assay/Minimal Sample Processing
[2]Method 2: Microtiter Broth Dilution Assay with PBS and Nanosep Centrifugal Filter for Retentate
[3]Method 3: Microtiter Broth Dilution Assay with PBS and Nanosep Centrifugal Filter for Filtrate

Means not connected by the same letter (A/B) are significantly different and are highlighted in yellow. Statistical analysis was completed using JMP software (Appendix B)

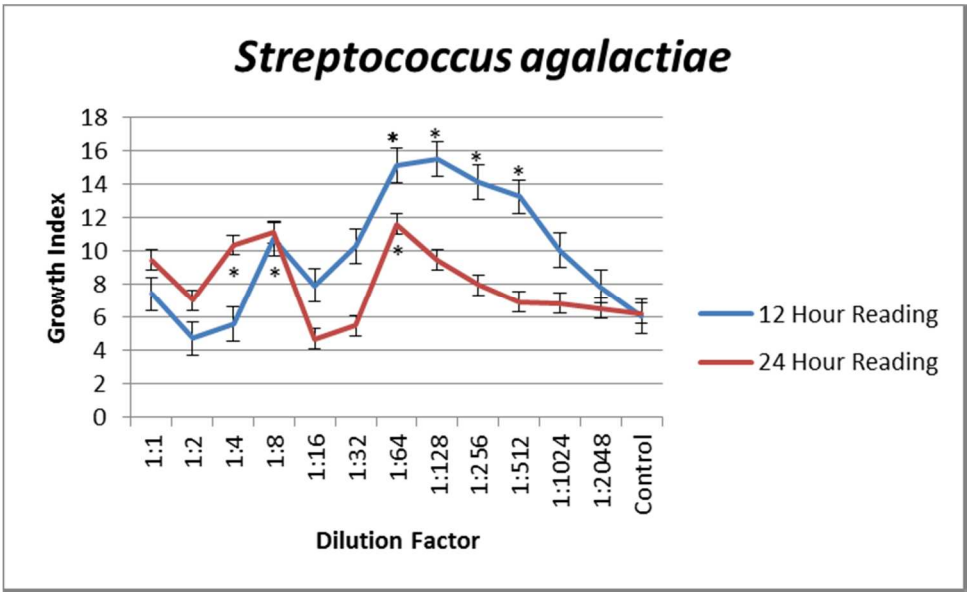
Table 4.2: Mean Comparison (via t-test) of antibacterial activity (in antibacterial units) against *S. agalactiae* and *S.iniae* at 12 and 24 hours using three processing methods

Disease Name	Time (Hours)	Growth Reduction	Method 1 Mean[1]	Method 2 Mean[2]	Method 3 Mean[3]
S. agalactiae	12	10%	23.00^{A}	0.00^{A}	0.00^{A}
S. agalactiae	12	20%	34.67^{A}	0.00^{B}	0.00^{B}
S. agalactiae	12	30%	29.00^{A}	0.00^{B}	0.00^{B}
S. agalactiae	12	40%	26.67^{A}	0.00^{A}	0.00^{A}
S. agalactiae	12	50%	29.33^{A}	0.00^{A}	0.00^{A}
S. agalactiae	24	10%	1.00^{A}	0.00^{A}	0.00^{A}
S. agalactiae	24	20%	0.33^{A}	0.00^{A}	0.00^{A}
S. agalactiae	24	30%	0.00^{A}	0.00^{A}	0.00^{A}
S. agalactiae	24	40%	0.00^{A}	0.00^{A}	0.00^{A}
S. agalactiae	24	50%	0.00^{A}	0.00^{A}	0.00^{A}
S. iniae	12	10%	2731.67^{A}	0.00^{B}	0.00^{B}
S. iniae	12	20%	2716.67^{A}	0.00^{B}	0.00^{B}
S. iniae	12	30%	0.00^{A}	0.00^{A}	0.00^{A}
S. iniae	12	40%	0.00^{A}	0.00^{A}	0.00^{A}
S. iniae	12	50%	0.00^{A}	0.00^{A}	0.00^{A}
S. iniae	24	10%	2732.33^{A}	0.00^{B}	0.00^{B}
S. iniae	24	20%	2728.00^{A}	0.00^{B}	0.00^{B}
S. iniae	24	30%	0.67^{A}	0.00^{A}	0.00^{A}
S. iniae	24	40%	0.00^{A}	0.00^{A}	0.00^{A}
S. iniae	24	50%	0.00^{A}	0.00^{A}	0.00^{A}

[1]Method 1: Microtiter Broth Dilution Assay/Minimal Sample Processing
[2]Method 2: Microtiter Broth Dilution Assay with PBS and Nanosep Centrifugal Filter for Retentate
[3]Method 3: Microtiter Broth Dilution Assay with PBS and Nanosep Centrifugal Filter for Filtrate

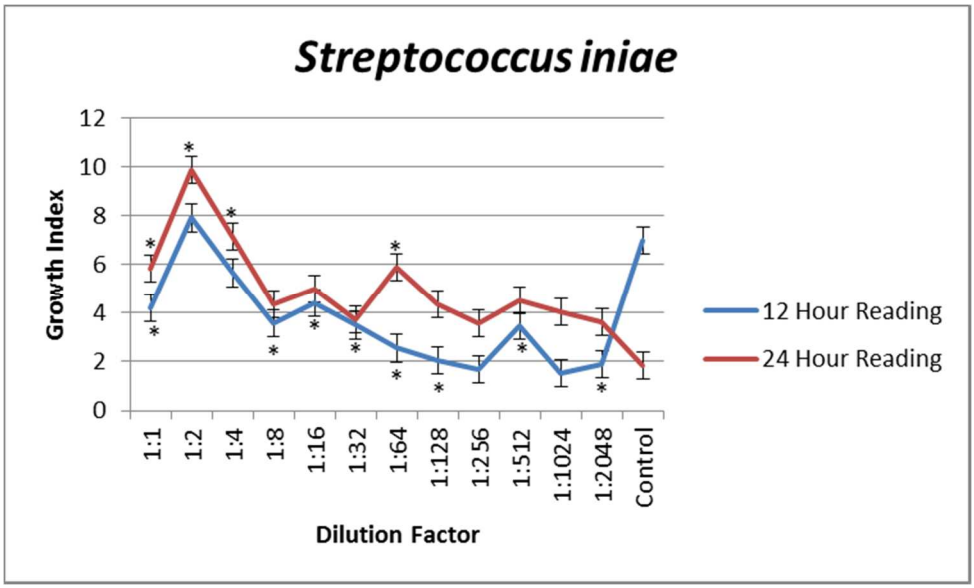
Means not connected by the same letter (A/B) are significantly different and are highlighted.
Statistical analysis was completed using JMP software (Appendix B)

Figure 4.1: *S. agalactiae* growth measured after 12 hours and 24 hours using the minimally processed sample microtiter broth plate assay with dilution factor as an isolated variable.



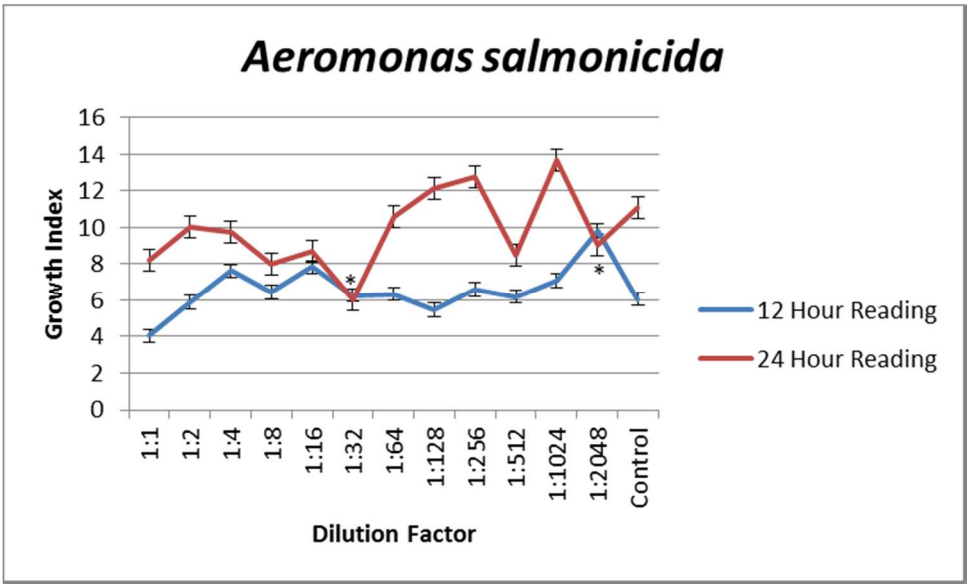
Growth Index is defined as a ratio of the plate reading to an average of the entire zero hour plate. Asterisk indicates statistical significance with a 95% confidence level

Figure 4.2: *S. iniae* growth measured after 12 hours and 24 hours using the minimally processed sample microtiter broth plate assay with dilution factor as an isolated variable.



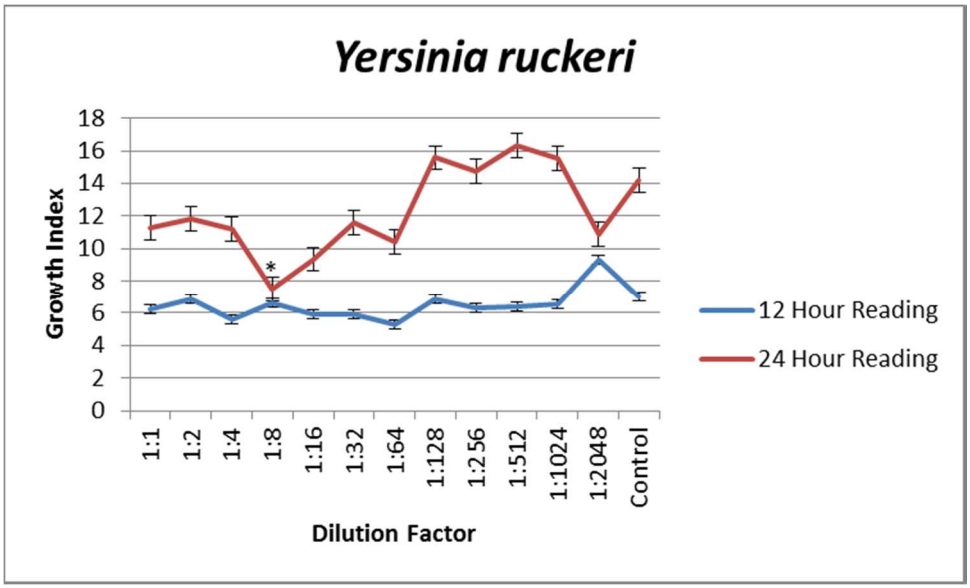
Growth Index is defined as a ratio of the plate reading to an average of the entire zero hour plate.
Asterisk indicates statistical significance with a 95% confidence level

Figure 4.3: *A. salmonicida* growth measured after 12 hours and 24 hours using the minimally processed sample microtiter broth plate assay with dilution factor as an isolated variable.



Growth Index is defined as a ratio of the plate reading to an average of the entire zero hour plate. Asterisk indicates statistical significance with a 95% confidence level

Figure 4.3: *A. salmonicida* growth measured after 12 hours and 24 hours using the minimally processed sample microtiter broth plate assay with dilution factor as an isolated variable.



Growth Index is defined as a ratio of the plate reading to an average of the entire zero hour plate. Asterisk indicates statistical significance with a 95% confidence level

APPENDIX A: RAW DATA

Table A.1: Zero-hour spectrophotometer reading of microtiter assay with minimal processing for *S. agalactiae* and *S. iniae*

0 Hr	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
S. agalactiae + sample dilution	0.042	0.041	0.041	0.04	0.04	0.04	0.04	0.04	0.041	0.041	0.04	0.04
S. agalactiae + sample dilution	0.042	0.041	0.04	0.04	0.039	0.039	0.039	0.041	0.04	0.04	0.042	0.04
S. agalactiae + sample dilution	0.042	0.041	0.041	0.04	0.039	0.039	0.039	0.041	0.041	0.042	0.039	0.04
S. iniae + sample dilution	0.043	0.043	0.041	0.041	0.04	0.041	0.04	0.042	0.041	0.04	0.043	0.04
S. iniae + sample dilution	0.043	0.042	0.041	0.041	0.04	0.041	0.04	0.041	0.041	0.04	0.04	0.04
S.iniae + sample dilution	0.042	0.042	0.042	0.04	0.04	0.04	0.04	0.04	0.041	0.04	0.039	0.039
S. agalactiae GROWTH CONTROL	0.042	0.039	0.039	0.039	0.039	0.04	0.039	0.04	0.04	0.04	0.04	0.04
S. iniae GROWTH CONTROL	0.041	0.039	0.043	0.045	0.04	0.04	0.04	0.041	0.041	0.041	0.039	0.041

Table A.2: Twelve-hour spectrophotometer reading of microtiter assay with minimal processing for *S. agalactiae* and *S. iniae*

12 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
S. agalactiae + sample dilution	0.322	0.212	0.37	0.278	0.52	0.316	0.818	0.69	0.562	0.437	0.369	0.272
S. agalactiae + sample dilution	0.345	0.1	0.076	0.387	0.306	0.695	0.511	0.599	0.64	0.6	0.454	0.319
S. agalactiae + sample dilution	0.244	0.266	0.241	0.654	0.149	0.255	0.531	0.62	0.536	0.594	0.411	0.375
S. iniae + sample dilution	0.15	0.239	0.249	0.199	0.275	0.229	0.114	0.081	0.071	0.183	0.067	0.056
S. iniae + sample dilution	0.169	0.393	0.217	0.172	0.203	0.095	0.116	0.077	0.074	0.089	0.063	0.061
S.iniae + sample dilution	0.2	0.341	0.23	0.07	0.065	0.106	0.084	0.096	0.062	0.156	0.059	0.117
S. agalactiae GROWTH CONTROL	0.238	0.408	0.341	0.354	0.446	0.443	0.409	0.409	0.477	0.606	0.649	0.412
S. iniae GROWTH CONTROL	0.047	0.044	0.052	0.054	0.124	0.058	0.06	0.07	0.055	0.05	0.055	0.047

Table A.3: Twenty-four-hour spectrophotometer reading of microtiter assay with minimal processing for *S. agalactiae* and *S. iniae*

24 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
S. agalactiae + sample dilution	0.4	0.308	0.586	0.396	0.356	0.077	0.349	0.329	0.281	0.273	0.28	0.273
S. agalactiae + sample dilution	0.392	0.246	0.309	0.5	0.114	0.4	0.515	0.405	0.327	0.285	0.283	0.26
S. agalactiae + sample dilution	0.37	0.311	0.379	0.472	0.105	0.198	0.563	0.428	0.37	0.291	0.279	0.271
S. iniae + sample dilution	0.284	0.297	0.418	0.245	0.271	0.245	0.059	0.115	0.07	0.21	0.064	0.084
S. iniae + sample dilution	0.284	0.497	0.314	0.167	0.234	0.076	0.274	0.128	0.113	0.166	0.133	0.07
S.iniae + sample dilution	0.172	0.458	0.309	0.11	0.095	0.111	0.18	0.128	0.071	0.139	0.123	0.13
S. agalactiae GROWTH CONTROL	0.26	0.263	0.255	0.259	0.286	0.269	0.272	0.282	0.258	0.252	0.241	0.247
S. iniae GROWTH CONTROL	0.09	0.065	0.076	0.092	0.069	0.086	0.068	0.083	0.111	0.08	0.066	0.075

Table A.4: Zero-hour spectrophotometer reading of microtiter assay with minimal processing for *A. salmonicida and Y. ruckeri*

0 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
A. salmon. + sample dilution	0.044	0.042	0.04	0.04	0.039	0.054	0.041	0.04	0.04	0.045	0.04	0.04
A. salmon. + sample dilution	0.043	0.041	0.04	0.039	0.039	0.04	0.04	0.041	0.04	0.043	0.04	0.04
A. salmon. + sample dilution	0.042	0.041	0.041	0.04	0.04	0.041	0.04	0.041	0.041	0.043	0.04	0.04
Y. ruckeri + sample dilution	0.044	0.041	0.042	0.04	0.041	0.04	0.041	0.042	0.041	0.04	0.04	0.041
Y. ruckeri + sample dilution	0.043	0.043	0.041	0.041	0.041	0.041	0.041	0.042	0.041	0.04	0.041	0.041
Y. ruckeri + sample dilution	0.047	0.045	0.043	0.299	0.042	0.042	0.039	0.041	0.039	0.04	0.04	0.04
A. salmonicida GROWTH CONTROL	0.04	0.04	0.041	0.04	0.04	0.04	0.04	0.04	0.04	0.044	0.04	0.04
Y. ruckeri GROWTH CONTROL	0.04	0.04	0.043	0.04	0.04	0.04	0.04	0.04	0.038	0.044	0.04	0.041

Table A.5: Twelve-hour spectrophotometer reading of microtiter assay with minimal processing for *A. salmonicida and Y. ruckeri*

12 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
A. salmon. + sample dilution	0.18	0.211	0.271	0.271	0.274	0.084	0.349	0.266	0.31	0.088	0.312	0.417
A. salmon. + sample dilution	0.163	0.243	0.311	0.285	0.321	0.295	0.23	0.211	0.265	0.349	0.295	0.414
A. salmon. + sample dilution	0.153	0.269	0.354	0.238	0.367	0.392	0.201	0.195	0.234	0.325	0.262	0.378
Y. ruckeri + sample dilution	0.195	0.269	0.268	0.259	0.293	0.222	0.161	0.247	0.171	0.21	0.255	0.345
Y. ruckeri + sample dilution	0.179	0.27	0.289	0.251	0.358	0.299	0.322	0.303	0.219	0.215	0.246	0.376
Y. ruckeri + sample dilution	0.236	0.276	0.122	0.332	0.138	0.173	0.09	0.282	0.314	0.317	0.241	0.414
A. salmonicida GROWTH CONTROL	0.347	0.296	0.274	0.228	0.23	0.257	0.234	0.258	0.242	0.255	0.315	0.355
Y. ruckeri GROWTH CONTROL	0.393	0.267	0.311	0.312	0.319	0.32	0.298	0.268	0.291	0.287	0.263	0.282

Table A.6: Twenty-four-hour spectrophotometer reading of microtiter assay with minimal processing for *A. salmonicida and Y. ruckeri*

24 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
A. salmon. + sample dilution	0.287	0.468	0.309	0.288	0.26	0.076	0.325	0.574	0.506	0.072	0.603	0.323
A. salmon. + sample dilution	0.456	0.359	0.482	0.326	0.344	0.339	0.464	0.486	0.571	0.458	0.533	0.378
A. salmon. + sample dilution	0.268	0.408	0.404	0.367	0.463	0.327	0.514	0.432	0.491	0.51	0.544	0.408
Y. ruckeri + sample dilution	0.473	0.424	0.39	0.35	0.377	0.309	0.468	0.666	0.542	0.684	0.664	0.508
Y. ruckeri + sample dilution	0.321	0.438	0.663	0.258	0.391	0.534	0.681	0.632	0.647	0.681	0.715	0.432
Y. ruckeri + sample dilution	0.589	0.59	0.323	0.314	0.381	0.586	0.127	0.617	0.627	0.639	0.53	0.394
A. salmonicida GROWTH CONTROL	0.591	0.674	0.672	0.625	0.623	0.675	0.638	0.644	0.609	0.41	0.718	0.411
Y. ruckeri GROWTH CONTROL	0.866	0.74	0.43	0.497	0.542	0.56	0.653	0.651	0.595	0.666	0.485	0.345

Table A.7: Zero-hour spectrophotometer reading of microtiter assay with sodium acetate for *S. agalactiae* and *S. iniae*

0 Hr	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
S. agalactiae + sample dilution	0.307	0.308	0.281	0.288	0.287	0.282	0.29	0.286	0.289	0.287	0.286	0.287
S. agalactiae + sample dilution	0.32	0.313	0.28	0.277	0.312	0.29	0.29	0.292	0.289	0.288	0.293	0.283
S. agalactiae + sample dilution	0.321	0.329	0.264	0.291	0.295	0.286	0.292	0.288	0.293	0.287	0.29	0.285
S. iniae + sample dilution	0.269	0.285	0.262	0.263	0.263	0.263	0.249	0.25	0.261	0.243	0.234	0.255
S. iniae + sample dilution	0.257	0.282	0.265	0.269	0.262	0.27	0.267	0.26	0.264	0.247	0.235	0.258
S.iniae + sample dilution	0.264	0.286	0.265	0.263	0.27	0.274	0.258	0.266	0.264	0.256	0.237	0.255
S. agalactiae GROWTH CONTROL	0.267	0.276	0.277	0.268	0.285	0.274	0.275	0.275	0.282	0.276	0.28	0.277
S. iniae GROWTH CONTROL	0.251	0.25	0.248	0.261	0.248	0.249	0.264	0.257	0.262	0.286	0.26	0.246

Table A.8: Twelve-hour spectrophotometer reading of microtiter assay with sodium acetate for *S. agalactiae* and *S. iniae*

12 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
S. agalactiae + sample dilution	0.744	0.619	0.504	0.538	0.536	0.518	0.541	0.548	0.491	0.464	0.515	0.543
S. agalactiae + sample dilution	0.774	0.47	0.471	0.372	0.762	0.478	0.465	0.443	0.441	0.448	0.451	0.453
S. agalactiae + sample dilution	0.739	0.609	0.408	0.483	0.48	0.44	0.417	0.421	0.407	0.424	0.397	0.438
S. iniae + sample dilution	0.343	0.301	0.281	0.269	0.272	0.261	0.253	0.26	0.263	0.254	0.235	0.244
S. iniae + sample dilution	0.335	0.268	0.281	0.279	0.271	0.285	0.265	0.267	0.271	0.261	0.241	0.257
S.iniae + sample dilution	0.342	0.279	0.28	0.278	0.277	0.275	0.257	0.259	0.263	0.258	0.244	0.25
S. agalactiae GROWTH CONTROL	0.255	0.363	0.189	0.4	0.412	0.394	0.387	0.381	0.402	0.397	0.397	0.401
S. iniae GROWTH CONTROL	0.276	0.259	0.252	0.269	0.258	0.257	0.278	0.25	0.262	0.293	0.261	0.255

Table A.9: Twenty-four-hour spectrophotometer reading of microtiter assay with sodium acetate for *S. agalactiae* and *S. iniae*

24 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
S. agalactiae + sample dilution	0.791	0.605	0.562	0.586	0.558	0.555	0.585	0.577	0.538	0.52	0.512	0.566
S. agalactiae + sample dilution	0.85	0.515	0.369	0.461	0.548	0.5	0.524	0.479	0.485	0.504	0.487	0.439
S. agalactiae + sample dilution	0.772	0.6	0.456	0.558	0.515	0.491	0.449	0.476	0.431	0.451	0.409	0.429
S. iniae + sample dilution	0.369	0.299	0.28	0.27	0.27	0.261	0.25	0.254	0.252	0.232	0.21	0.233
S. iniae + sample dilution	0.36	0.266	0.282	0.277	0.28	0.257	0.253	0.255	0.264	0.235	0.226	0.241
S.iniae + sample dilution	0.374	0.28	0.277	0.278	0.274	0.271	0.252	0.245	0.25	0.243	0.228	0.239
S. agalactiae GROWTH CONTROL	0.336	0.343	0.441	0.446	0.469	0.392	0.368	0.347	0.376	0.345	0.28	0.285
S. iniae GROWTH CONTROL	0.253	0.241	0.237	0.255	0.243	0.242	0.263	0.239	0.246	0.281	0.247	0.242

Table A.10: Zero-hour spectrophotometer reading of microtiter assay with sodium acetate for *A. salmonicida and Y. ruckeri*

0 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
A. salmon. + sample dilution	0.237	0.277	0.311	0.315	0.321	0.314	0.318	0.268	0.308	0.319	0.318	0.322
A. salmon. + sample dilution	0.427	0.251	0.313	0.301	0.314	0.313	0.319	0.319	0.314	0.326	0.33	0.327
A. salmon. + sample dilution	0.249	0.261	0.307	0.304	0.319	0.314	0.319	0.32	0.313	0.318	0.322	0.327
Y. ruckeri + sample dilution	0.268	0.248	0.259	0.261	0.269	0.275	0.271	0.274	0.274	0.276	0.275	0.261
Y. ruckeri + sample dilution	0.246	0.249	0.258	0.268	0.271	0.274	0.27	0.25	0.268	0.275	0.269	0.255
Y. ruckeri + sample dilution	0.248	0.261	0.271	0.267	0.267	0.274	0.275	0.275	0.274	0.281	0.269	0.27
A. salmonicida GROWTH CONTROL	0.242	0.233	0.275	0.291	0.314	0.312	0.336	0.313	0.306	0.293	0.305	0.319
Y. ruckeri GROWTH CONTROL	0.255	0.259	0.278	0.255	0.233	0.265	0.27	0.269	0.262	0.273	0.265	0.249

Table A.11: Twelve-hour spectrophotometer reading of microtiter assay with sodium acetate for *A. salmonicida and Y. ruckeri*

12 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
A. salmon. + sample dilution	0.264	0.263	0.24	0.241	0.221	0.2	0.184	0.234	0.173	0.215	0.199	0.195
A. salmon. + sample dilution	0.271	0.271	0.246	0.22	0.217	0.191	0.201	0.188	0.175	0.193	0.193	0.2
A. salmon. + sample dilution	0.279	0.271	0.242	0.221	0.213	0.21	0.186	0.192	0.183	0.183	0.194	0.18
Y. ruckeri + sample dilution	0.31	0.281	0.277	0.272	0.263	0.274	0.261	0.259	0.27	0.265	0.264	0.245
Y. ruckeri + sample dilution	0.291	0.276	0.275	0.277	0.272	0.258	0.263	0.254	0.286	0.264	0.273	0.248
Y. ruckeri + sample dilution	0.302	0.281	0.275	0.271	0.273	0.267	0.258	0.266	0.271	0.258	0.273	0.267
A. salmonicida GROWTH CONTROL	0.116	0.114	0.153	0.156	0.196	0.189	0.186	0.184	0.182	0.154	0.168	0.187
Y. ruckeri GROWTH CONTROL	0.228	0.246	0.256	0.233	0.225	0.261	0.254	0.259	0.247	0.244	0.243	0.242

Table A.12: Twenty-four-hour spectrophotometer reading of microtiter assay with sodium acetate for *A. salmonicida and Y. ruckeri*

24 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
A. salmon. + sample dilution	0.267	0.26	0.201	0.202	0.194	0.163	0.125	0.249	0.145	0.128	0.13	0.139
A. salmon. + sample dilution	0.272	0.27	0.208	0.184	0.2	0.142	0.134	0.135	0.138	0.131	0.147	0.12
A. salmon. + sample dilution	0.28	0.232	0.208	0.183	0.181	0.147	0.127	0.136	0.124	0.142	0.139	0.117
Y. ruckeri + sample dilution	0.311	0.282	0.284	0.281	0.274	0.281	0.259	0.264	0.265	0.255	0.26	0.252
Y. ruckeri + sample dilution	0.298	0.281	0.286	0.289	0.276	0.262	0.255	0.276	0.275	0.25	0.261	0.25
Y. ruckeri + sample dilution	0.312	0.281	0.281	0.28	0.279	0.268	0.257	0.263	0.259	0.256	0.266	0.266
A. salmonicida GROWTH CONTROL	0.093	0.084	0.099	0.102	0.129	0.127	0.131	0.125	0.12	0.105	0.104	0.115
Y. ruckeri GROWTH CONTROL	0.245	0.252	0.249	0.231	0.224	0.245	0.241	0.239	0.233	0.24	0.249	0.232

Table A.13: Zero-hour spectrophotometer readings of all diseases grown in sodium acetate (NaAc) or phosphate buffered saline (PBS).

0 Hr												
S. agalactiae + NaAc	0.057	0.056	0.058	0.055	0.056	0.056	0.057	0.057	0.056	0.056	0.055	0.058
S. iniae. + NaAc	0.053	0.053	0.05	0.053	0.053	0.053	0.054	0.053	0.052	0.053	0.053	0.053
A. salmon. + NaAc	0.286	0.574	0.564	0.562	0.696	0.742	0.722	0.648	0.61	0.609	0.421	0.292
Y. ruckeri + NaAc	0.284	0.468	0.476	0.4	0.362	0.34	0.274	0.349	0.32	0.745	0.287	0.329
S. agalactiae + PBS	0.044	0.044	0.044	0.044	0.044	0.044	0.044	0.045	0.044	0.045	0.045	0.045
S. iniae + PBS	0.044	0.044	0.045	0.044	0.044	0.045	0.045	0.044	0.044	0.043	0.044	0.046
A. salmonicida + PBS	0.045	0.044	0.044	0.043	0.041	0.044	0.046	0.044	0.045	0.044	0.045	0.046
Y. ruckeri + PBS	0.044	0.045	0.046	0.044	0.044	0.044	0.044	0.044	0.044	0.047	0.045	0.044

Table A.14: Twelve-hour spectrophotometer readings of all diseases grown in sodium acetate or phosphate buffered saline.

12 Hrs												
S. agalactiae + NaAc	0.052	0.053	0.052	0.053	0.052	0.053	0.053	0.052	0.052	0.052	0.053	0.049
S. iniae. + NaAc	0.056	0.053	0.053	0.053	0.053	0.052	0.052	0.052	0.052	0.053	0.053	0.053
A. salmon. + NaAc	0.244	0.177	0.181	0.188	0.177	0.187	0.16	0.159	0.154	0.175	0.162	0.2
Y. ruckeri + NaAc	0.262	0.363	0.354	0.289	0.264	0.295	0.298	0.207	0.168	0.214	0.206	0.266
S. agalactiae + PBS	0.045	0.045	0.045	0.045	0.045	0.046	0.045	0.045	0.045	0.046	0.044	0.044
S. iniae + PBS	0.045	0.045	0.045	0.045	0.045	0.046	0.045	0.044	0.045	0.045	0.044	0.044
A. salmonicida + PBS	0.045	0.045	0.044	0.045	0.044	0.044	0.045	0.044	0.044	0.044	0.044	0.045
Y. ruckeri + PBS	0.045	0.044	0.047	0.044	0.045	0.044	0.044	0.044	0.045	0.047	0.045	0.046

Table A.15: Difference between 12 h and 0 hr spectrophotometer readings of all diseases grown in sodium acetate or phosphate buffered saline.

12 Hrs – 0 Hrs												
S. agalactiae + NaAc	-0.005	-0.003	-0.006	-0.002	-0.004	-0.003	-0.004	-0.005	-0.004	-0.004	-0.002	-0.009
S. iniae. + NaAc	0.003	0	0.003	0	0	-0.001	-0.002	-0.001	0	0	0	0
A. salmon. + NaAc	-0.042	-0.397	-0.383	-0.374	-0.519	-0.555	-0.562	-0.489	-0.456	-0.434	-0.259	-0.092
Y. ruckeri + NaAc	-0.022	-0.105	-0.122	-0.111	-0.098	-0.045	0.024	-0.142	-0.152	-0.531	-0.081	-0.063
S. agalactiae + PBS	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0	0.001	0.001	-0.001	-0.001
S. iniae + PBS	0.001	0.001	0	0.001	0.001	0.001	0	0	0.001	0.002	0	-0.002
A. salmonicida + PBS	0	0.001	0	0.002	0.003	0	-0.001	0	-0.001	0	-0.001	-0.001
Y. ruckeri + PBS	0.001	-0.001	0.001	0	0.001	0	0	0	0.001	0	0	0.002

Table A.16: Zero-hour spectrophotometer reading of microtiter assay retentate sample with centrifugal filter for *S. agalactiae* and *S. iniae*

0 Hr	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
S. agalactiae + sample dilution	0.067	0.063	0.062	0.061	0.059	0.059	0.059	0.059	0.059	0.059	0.059	0.059
S. agalactiae + sample dilution	0.07	0.064	0.062	0.06	0.059	0.059	0.059	0.058	0.059	0.06	0.059	0.059
S. agalactiae + sample dilution	0.07	0.07	0.063	0.06	0.06	0.06	0.059	0.059	0.059	0.059	0.058	0.06
S. iniae + sample dilution	0.07	0.067	0.063	0.063	0.062	0.061	0.062	0.061	0.061	0.06	0.06	0.06
S. iniae + sample dilution	0.075	0.065	0.064	0.063	0.063	0.062	0.063	0.061	0.062	0.061	0.061	0.06
S.iniae + sample dilution	0.07	0.065	0.065	0.063	0.062	0.061	0.062	0.061	0.061	0.06	0.061	0.06
S. agalactiae GROWTH CONTROL	0.06	0.059	0.061	0.059	0.06	0.06	0.06	0.06	0.06	0.06	0.059	0.06
S. iniae GROWTH CONTROL	0.061	0.06	0.063	0.061	0.061	0.062	0.061	0.06	0.061	0.063	0.06	0.06

Table A.17: Twelve-hour spectrophotometer reading of microtiter assay retentate sample with centrifugal filter for *S. agalactiae* and *S. iniae*

12 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
S. agalactiae + sample dilution	0.726	0.639	0.64	0.631	0.645	0.644	0.653	0.651	0.645	0.638	0.629	0.664
S. agalactiae + sample dilution	0.754	0.656	0.625	0.649	0.626	0.637	0.636	0.609	0.646	0.661	0.666	0.64
S. agalactiae + sample dilution	0.643	0.653	0.627	0.64	0.635	0.643	0.623	0.613	0.637	0.624	0.628	0.668
S. iniae + sample dilution	0.491	0.518	0.522	0.547	0.521	0.523	0.48	0.481	0.462	0.489	0.466	0.507
S. iniae + sample dilution	0.502	0.5	0.536	0.537	0.492	0.491	0.517	0.475	0.474	0.477	0.473	0.534
S.iniae + sample dilution	0.506	0.512	0.502	0.557	0.526	0.469	0.493	0.485	0.473	0.468	0.453	0.548
S. agalactiae GROWTH CONTROL	0.627	0.64	0.647	0.578	0.643	0.64	0.665	0.628	0.653	0.653	0.653	0.667
S. iniae GROWTH CONTROL	0.551	0.486	0.467	0.39	0.488	0.456	0.451	0.467	0.386	0.423	0.4	0.506

Table A.18: Twenty-four-hour spectrophotometer reading of microtiter assay retentate sample with centrifugal filter for *S. agalactiae* and *S. iniae*

24 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
S. agalactiae + sample dilution	0.638	0.62	0.744	0.778	0.794	0.823	0.782	0.768	0.763	0.74	0.736	0.742
S. agalactiae + sample dilution	1.471	0.718	0.724	0.742	0.715	0.744	0.772	0.726	0.763	0.766	0.751	0.738
S. agalactiae + sample dilution	0.606	0.687	0.818	0.729	0.777	0.775	0.744	0.724	0.738	0.734	0.727	0.778
S. iniae + sample dilution	0.523	0.537	0.546	0.566	0.536	0.542	0.499	0.5	0.487	0.505	0.497	0.544
S. iniae + sample dilution	0.533	0.519	0.557	0.557	0.529	0.523	0.543	0.5	0.499	0.5	0.499	0.57
S.iniae + sample dilution	0.586	0.533	0.515	0.579	0.842	0.509	0.527	0.522	0.514	0.509	0.494	0.587
S. agalactiae GROWTH CONTROL	0.733	0.735	0.77	0.691	0.755	0.758	0.759	0.731	0.756	0.74	0.723	0.731
S. iniae GROWTH CONTROL	0.603	0.545	0.517	0.43	0.532	0.493	0.484	0.505	0.418	0.447	0.443	0.538

Table A.19: Zero-hour spectrophotometer reading of microtiter assay retentate sample with centrifugal filter for *A. salmonicida and Y. ruckeri*

0 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
A. salmon. + sample dilution	0.072	0.069	0.067	0.064	0.063	0.064	0.064	0.064	0.064	0.063	0.063	0.064
A. salmon. + sample dilution	0.071	0.069	0.067	0.065	0.064	0.063	0.064	0.063	0.064	0.064	0.064	0.064
A. salmon. + sample dilution	0.073	0.069	0.067	0.065	0.065	0.064	0.063	0.064	0.063	0.063	0.063	0.064
Y. ruckeri + sample dilution	0.072	0.07	0.067	0.066	0.066	0.065	0.065	0.066	0.065	0.066	0.066	0.066
Y. ruckeri + sample dilution	0.075	0.069	0.067	0.066	0.066	0.066	0.067	0.067	0.066	0.065	0.065	0.065
Y. ruckeri + sample dilution	0.071	0.07	0.067	0.066	0.066	0.065	0.066	0.066	0.065	0.065	0.065	0.065
A. salmonicida GROWTH CONTROL	0.063	0.063	0.063	0.064	0.063	0.064	0.064	0.063	0.064	0.064	0.064	0.064
Y. ruckeri GROWTH CONTROL	0.056	0.064	0.066	0.065	0.065	0.064	0.063	0.065	0.064	0.068	0.066	0.066

Table A.20: Twelve-hour spectrophotometer reading of microtiter assay retentate sample with centrifugal filter for *A. salmonicida and Y. ruckeri*

12 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
A. salmon. + sample dilution	0.371	0.336	0.325	0.324	0.298	0.322	0.305	0.286	0.279	0.291	0.291	0.287
A. salmon. + sample dilution	0.299	0.312	0.297	0.294	0.29	0.279	0.278	0.286	0.281	0.257	0.281	0.264
A. salmon. + sample dilution	0.334	0.301	0.288	0.294	0.296	0.292	0.271	0.283	0.284	0.24	0.276	0.294
Y. ruckeri + sample dilution	0.374	0.331	0.338	0.362	0.347	0.342	0.289	0.326	0.298	0.354	0.327	0.279
Y. ruckeri + sample dilution	0.327	0.343	0.329	0.335	0.335	0.34	0.331	0.327	0.326	0.35	0.316	0.317
Y. ruckeri + sample dilution	0.339	0.31	0.312	0.343	0.316	0.332	0.345	0.324	0.318	0.34	0.327	0.309
A. salmonicida GROWTH CONTROL	0.277	0.248	0.245	0.256	0.252	0.25	0.265	0.23	0.263	0.252	0.27	0.275
Y. ruckeri GROWTH CONTROL	0.292	0.336	0.347	0.34	0.331	0.357	0.351	0.331	0.321	0.309	0.308	0.303

Table A.21**:** Twenty-four-hour spectrophotometer reading of microtiter assay retentate sample with centrifugal filter for *A. salmonicida and Y. ruckeri*

24 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
A. salmon. + sample dilution	0.322	0.316	0.311	0.302	0.292	0.283	0.283	0.267	0.26	0.292	0.295	0.298
A. salmon. + sample dilution	0.332	0.298	0.973	0.31	0.291	0.291	0.288	0.316	0.307	0.294	0.326	0.268
A. salmon. + sample dilution	0.314	0.314	0.332	0.313	0.307	0.32	0.323	0.352	0.339	0.311	0.309	0.283
Y. ruckeri + sample dilution	0.348	0.307	0.352	0.341	0.322	0.331	0.36	0.36	0.369	0.345	0.3	0.248
Y. ruckeri + sample dilution	0.329	0.315	0.39	0.453	0.359	0.374	0.388	0.372	0.42	0.363	0.279	0.252
Y. ruckeri + sample dilution	0.315	0.295	0.345	0.371	0.348	0.341	0.374	0.361	0.331	0.329	0.236	0.245
A. salmonicida GROWTH CONTROL	0.271	0.27	0.289	0.305	0.306	0.298	0.278	0.268	0.324	0.3	0.289	0.242
Y. ruckeri GROWTH CONTROL	0.512	0.294	0.321	0.331	0.308	0.364	0.371	0.334	0.331	0.3	0.26	0.289

Table A.22: Twenty-four-hour spectrophotometer reading of microtiter assay filtrate sample with centrifugal filter for *S. agalactiae* and *S. iniae*

24 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
S. agalactiae + sample dilution	0.715	0.512	0.653	0.7	0.733	0.757	0.788	0.766	0.755	0.758	0.748	0.724
S. agalactiae + sample dilution	0.633	0.505	0.649	0.7	0.719	0.775	0.746	0.77	0.747	0.726	0.716	0.71
S. agalactiae + sample dilution	0.556	0.518	0.56	0.566	0.531	0.534	0.512	0.514	0.532	0.518	0.499	0.49
S. iniae + sample dilution	0.564	0.493	0.534	0.637	0.756	0.871	0.806	0.732	0.781	0.721	0.745	0.737
S. iniae + sample dilution	0.526	0.543	0.558	0.568	0.541	0.514	0.508	0.53	0.509	0.506	0.498	0.478
S.iniae + sample dilution	0.556	0.518	0.593	0.555	0.56	0.555	0.51	0.553	0.46	0.495	0.509	0.472
S. agalactiae GROWTH CONTROL	0.649	0.566	0.571	0.593	0.673	0.469	0.436	0.515	0.547	0.454	0.567	0.443
S. iniae GROWTH CONTROL	0.566	0.543	0.665	0.701	0.738	0.721	0.713	0.749	0.728	0.734	0.726	0.73

Table A.23: Zero-hour spectrophotometer reading of microtiter assay filtrate sample with centrifugal filter for *A. salmonicida and Y. ruckeri*

0 Hr	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
A. salmon. + sample dilution	0.067	0.064	0.067	0.063	0.063	0.063	0.064	0.063	0.063	0.063	0.066	0.063
A. salmon. + sample dilution	0.066	0.067	0.065	0.063	0.064	0.064	0.063	0.063	0.063	0.064	0.063	0.064
A. salmon. + sample dilution	0.065	0.064	0.064	0.063	0.063	0.064	0.063	0.063	0.063	0.063	0.063	0.065
Y. ruckeri + sample dilution	0.071	0.067	0.074	0.065	0.065	0.065	0.066	0.066	0.065	0.065	0.066	0.064
Y. ruckeri + sample dilution	0.069	0.067	0.067	0.066	0.066	0.068	0.068	0.067	0.067	0.065	0.065	0.068
Y. ruckeri + sample dilution	0.07	0.066	0.066	0.066	0.065	0.066	0.067	0.066	0.065	0.065	0.065	0.066
A. salmonicida GROWTH CONTROL	0.067	0.065	0.065	0.064	0.063	0.065	0.064	0.064	0.064	0.063	0.064	0.062
Y. ruckeri GROWTH CONTROL	0.069	0.066	0.067	0.065	0.065	0.065	0.065	0.065	0.065	0.067	0.065	0.066

Table A.24: Twelve-hour spectrophotometer reading of microtiter assay filtrate sample with centrifugal filter for *A. salmonicida and Y. ruckeri*

12 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
A. salmon. + sample dilution	0.348	0.29	0.325	0.274	0.327	0.293	0.298	0.285	0.287	0.284	0.264	0.288
A. salmon. + sample dilution	0.293	0.263	0.279	0.263	0.289	0.283	0.269	0.281	0.261	0.252	0.236	0.264
A. salmon. + sample dilution	0.282	0.247	0.26	0.248	0.286	0.286	0.287	0.288	0.286	0.276	0.283	0.279
Y. ruckeri + sample dilution	0.348	0.339	0.373	0.294	0.318	0.33	0.299	0.283	0.272	0.291	0.302	0.314
Y. ruckeri + sample dilution	0.337	0.304	0.329	0.294	0.322	0.329	0.264	0.307	0.305	0.331	0.3	0.329
Y. ruckeri + sample dilution	0.342	0.318	0.282	0.273	0.298	0.301	0.272	0.28	0.329	0.313	0.315	0.337
A. salmonicida GROWTH CONTROL	0.293	0.211	0.241	0.249	0.247	0.243	0.238	0.237	0.26	0.281	0.273	0.271
Y. ruckeri GROWTH CONTROL	0.339	0.3	0.329	0.28	0.301	0.297	0.294	0.303	0.306	0.299	0.307	0.304

Table A.25: Twenty-four-hour spectrophotometer reading of microtiter assay filtrate sample with centrifugal filter for *A. salmonicida and Y. ruckeri*

24 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
A. salmon. + sample dilution	0.322	0.3	0.3	0.264	0.275	0.269	0.28	0.27	0.275	0.287	0.263	0.279
A. salmon. + sample dilution	0.278	0.276	0.312	0.267	0.288	0.289	0.289	0.308	0.292	0.277	0.298	0.295
A. salmon. + sample dilution	0.336	0.273	0.33	0.27	0.295	0.311	0.304	0.314	0.302	0.305	0.292	0.322
Y. ruckeri + sample dilution	0.316	0.302	0.377	0.322	0.331	0.353	0.37	0.366	0.363	0.331	0.35	0.26
Y. ruckeri + sample dilution	0.31	0.336	0.378	0.435	0.396	0.388	0.37	0.389	0.423	0.347	0.348	0.281
Y. ruckeri + sample dilution	0.313	0.31	0.33	0.352	0.364	0.374	0.379	0.368	0.346	0.35	0.301	0.299
A. salmonicida GROWTH CONTROL	0.322	0.309	0.325	0.331	0.371	0.317	0.317	0.311	0.34	0.332	0.304	0.252
Y. ruckeri GROWTH CONTROL	0.315	0.32	0.339	0.377	0.373	0.412	0.436	0.374	0.385	0.349	0.278	0.294

Table A.26: Zero-hour spectrophotometer reading of microtiter assay filtrate sample with centrifugal filter for *S. agalactiae* and *S. iniae*

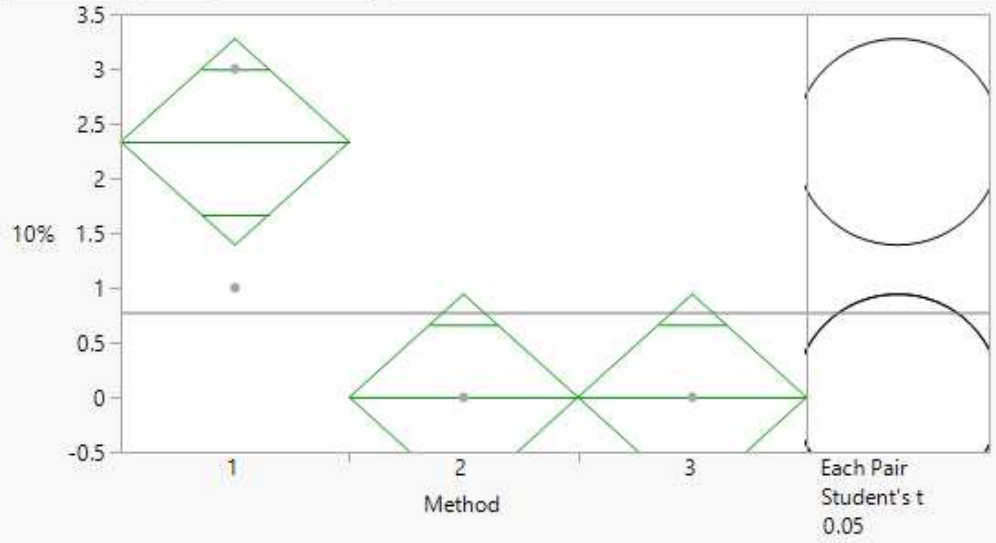
0 Hr	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
S. agalactiae + sample dilution	0.06	0.059	0.059	0.058	0.059	0.058	0.058	0.058	0.059	0.06	0.059	0.059
S. agalactiae + sample dilution	0.061	0.061	0.06	0.058	0.059	0.059	0.058	0.059	0.059	0.059	0.053	0.059
S. agalactiae + sample dilution	0.061	0.062	0.062	0.061	0.059	0.061	0.061	0.06	0.059	0.056	0.053	0.059
S. iniae + sample dilution	0.061	0.059	0.059	0.059	0.058	0.058	0.058	0.058	0.058	0.058	0.058	0.059
S. iniae + sample dilution	0.061	0.083	0.061	0.061	0.058	0.061	0.062	0.06	0.06	0.059	0.06	0.06
S.iniae + sample dilution	0.068	0.063	0.062	0.061	0.057	0.062	0.061	0.061	0.06	0.059	0.06	0.061
S. agalactiae GROWTH CONTROL	0.061	0.059	0.063	0.06	0.06	0.059	0.06	0.06	0.06	0.064	0.061	0.059
S. iniae GROWTH CONTROL	0.063	0.059	0.059	0.059	0.059	0.059	0.059	0.059	0.059	0.058	0.06	0.059

Table A.27: Twelve-hour spectrophotometer reading of microtiter assay filtrate sample with centrifugal filter for *S. agalactiae* and *S. iniae*

12 Hrs	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
S. agalactiae + sample dilution	0.741	0.659	0.669	0.633	0.642	0.64	0.663	0.656	0.664	0.653	0.656	0.628
S. agalactiae + sample dilution	0.745	0.686	0.674	0.655	0.645	0.687	0.653	0.682	0.672	0.65	0.648	0.628
S. agalactiae + sample dilution	0.753	0.499	0.547	0.548	0.51	0.505	0.475	0.485	0.507	0.497	0.474	0.467
S. iniae + sample dilution	0.542	0.609	0.583	0.652	0.639	0.707	0.609	0.616	0.657	0.61	0.651	0.646
S. iniae + sample dilution	0.493	0.521	0.538	0.545	0.517	0.49	0.48	0.505	0.489	0.483	0.474	0.447
S.iniae + sample dilution	0.512	0.499	0.581	0.541	0.538	0.527	0.476	0.509	0.419	0.439	0.462	0.432
S. agalactiae GROWTH CONTROL	0.796	0.539	0.54	0.576	0.602	0.398	0.375	0.455	0.477	0.395	0.498	0.391
S. iniae GROWTH CONTROL	0.514	0.654	0.655	0.688	0.679	0.653	0.663	0.67	0.655	0.657	0.64	0.637

APPENDIX B: Statistical Analysis

Oneway Analysis of 10% By Method



Oneway Anova

Summary of Fit

Rsquare	0.803279
Adj Rsquare	0.737705
Root Mean Square Error	0.666667
Mean of Response	0.777778
Observations (or Sum Wgts)	9

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Method	2	10.888889	5.44444	12.2500	0.0076*
Error	6	2.666667	0.44444		
C. Total	8	13.555556			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	3	2.33333	0.38490	1.392	3.2752
2	3	0.00000	0.38490	-0.942	0.9418
3	3	0.00000	0.38490	-0.942	0.9418

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for each pair using Student's t

Confidence Quantile

t	Alpha
2.44691	0.05

LSD Threshold Matrix

Abs(Dif)-LSD

	1	2	3
1	-1.3319	1.0014	1.0014
2	1.0014	-1.3319	-1.3319
3	1.0014	-1.3319	-1.3319

Positive values show pairs of means that are significantly different.

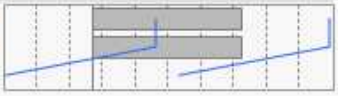
Connecting Letters Report

Level		Mean
1	A	2.3333333
2	B	0.0000000
3	B	0.0000000

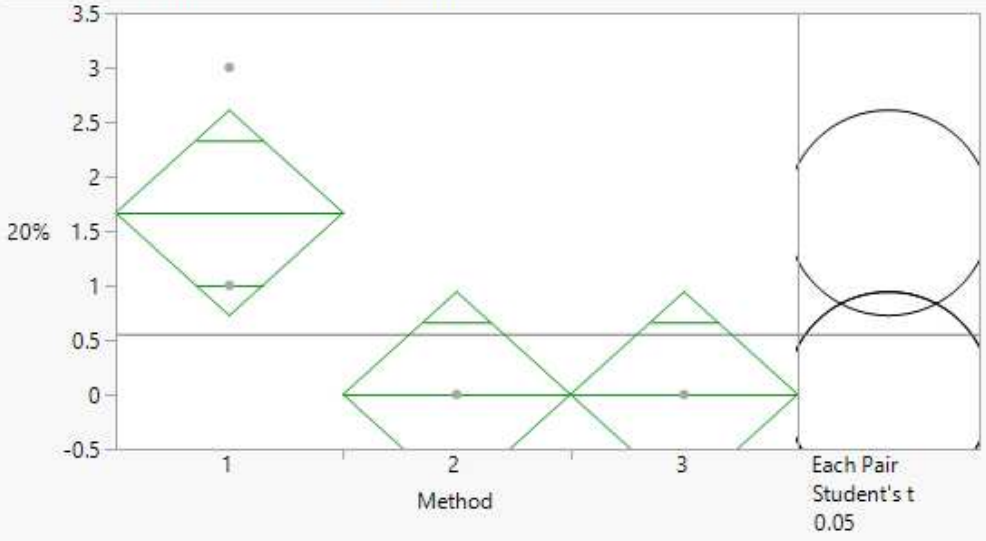
Levels not connected by same letter are significantly different.

Ordered Differences Report

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
1	2	2.333333	0.5443311	1.00140	3.665263	0.0052*
1	3	2.333333	0.5443311	1.00140	3.665263	0.0052*
3	2	0.000000	0.5443311	-1.33193	1.331930	1.0000



Oneway Analysis of 20% By Method



Oneway Anova

Summary of Fit

Rsquare	0.675676
Adj Rsquare	0.567568
Root Mean Square Error	0.666667
Mean of Response	0.555556
Observations (or Sum Wgts)	9

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Method	2	5.5555556	2.77778	6.2500	0.0341*
Error	6	2.6666667	0.44444		
C. Total	8	8.2222222			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	3	1.66667	0.38490	0.7248	2.6085
2	3	0.00000	0.38490	-0.9418	0.9418
3	3	0.00000	0.38490	-0.9418	0.9418

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for each pair using Student's t

Confidence Quantile

t	Alpha
2.44691	0.05

LSD Threshold Matrix

Abs(Dif)-LSD

	1	2	3
1	-1.3319	0.3347	0.3347
2	0.3347	-1.3319	-1.3319
3	0.3347	-1.3319	-1.3319

Positive values show pairs of means that are significantly different.

Connecting Letters Report

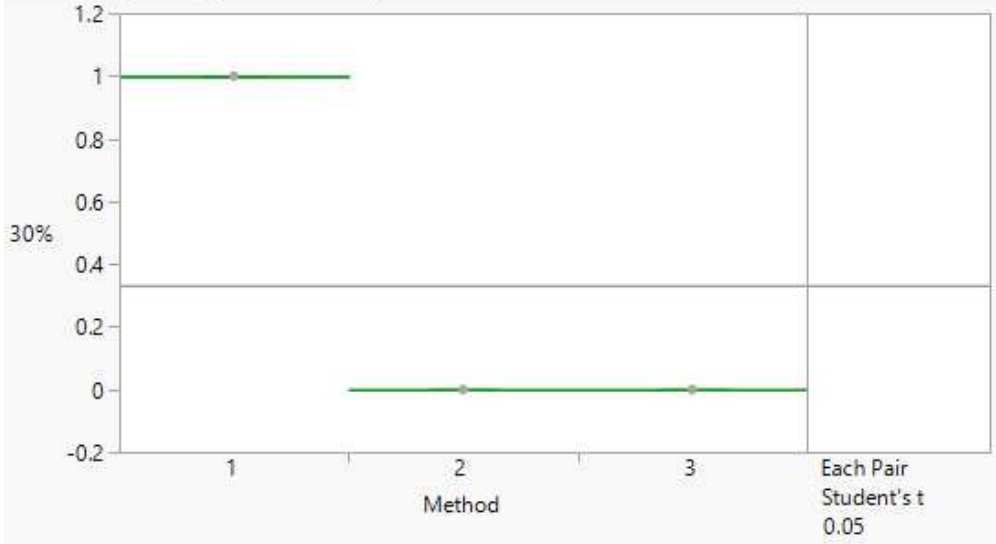
Level		Mean
1	A	1.6666667
2	B	0.0000000
3	B	0.0000000

Levels not connected by same letter are significantly different.

Ordered Differences Report

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
1	2	1.666667	0.5443311	0.33474	2.998597	0.0222*
1	3	1.666667	0.5443311	0.33474	2.998597	0.0222*
3	2	0.000000	0.5443311	-1.33193	1.331930	1.0000

Oneway Analysis of 30% By Method



Oneway Anova

Summary of Fit

Rsquare	1
Adj Rsquare	1
Root Mean Square Error	8.603e-9
Mean of Response	0.333333
Observations (or Sum Wgts)	9

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Method	2	2.0000000	1.00000	1.35e+16	<.0001*
Error	6	4.4409e-16	7.4e-17		
C. Total	8	2.0000000			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	3	1.00000	4.9671e-9	1.000	1.0000
2	3	0.00000	4.9671e-9	-1.2e-8	1.22e-8
3	3	0.00000	4.9671e-9	-1.2e-8	1.22e-8

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for each pair using Student's t

Confidence Quantile

t	Alpha
2.44691	0.05

LSD Threshold Matrix

Abs(Dif)-LSD

	1	2	3
1	-0.0000	1.0000	1.0000
2	1.0000	-0.0000	-0.0000
3	1.0000	-0.0000	-0.0000

Positive values show pairs of means that are significantly different.

Connecting Letters Report

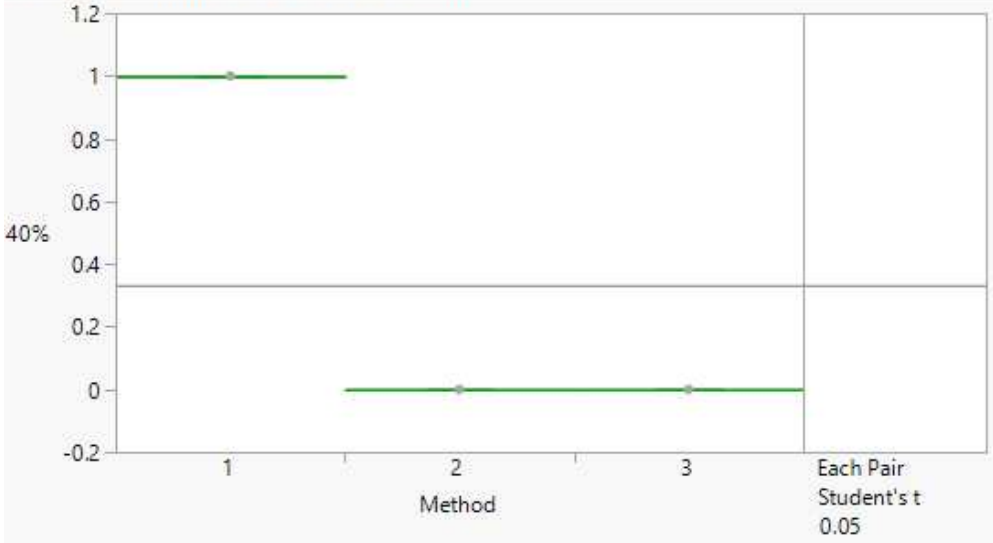
Level		Mean
1	A	1.0000000
2	B	0.0000000
3	B	0.0000000

Levels not connected by same letter are significantly different.

Ordered Differences Report

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
1	2	1.000000	7.0245e-9	1.00000	1.000000	<.0001*
1	3	1.000000	7.0245e-9	1.00000	1.000000	<.0001*
3	2	0.000000	7.0245e-9	-1.719e-8	1.7188e-8	1.0000

Oneway Analysis of 40% By Method



Oneway Anova

Summary of Fit

Rsquare	1
Adj Rsquare	1
Root Mean Square Error	8.603e-9
Mean of Response	0.333333
Observations (or Sum Wgts)	9

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Method	2	2.0000000	1.00000	1.35e+16	<.0001*
Error	6	4.4409e-16	7.4e-17		
C. Total	8	2.0000000			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	3	1.00000	4.9671e-9	1.000	1.0000
2	3	0.00000	4.9671e-9	-1.2e-8	1.22e-8
3	3	0.00000	4.9671e-9	-1.2e-8	1.22e-8

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for each pair using Student's t

Confidence Quantile

t	Alpha
2.44691	0.05

LSD Threshold Matrix

Abs(Dif)-LSD

	1	2	3
1	-0.0000	1.0000	1.0000
2	1.0000	-0.0000	-0.0000
3	1.0000	-0.0000	-0.0000

Positive values show pairs of means that are significantly different.

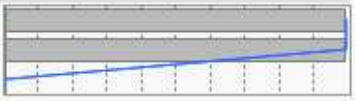
Connecting Letters Report

Level		Mean
1	A	1.0000000
2	B	0.0000000
3	B	0.0000000

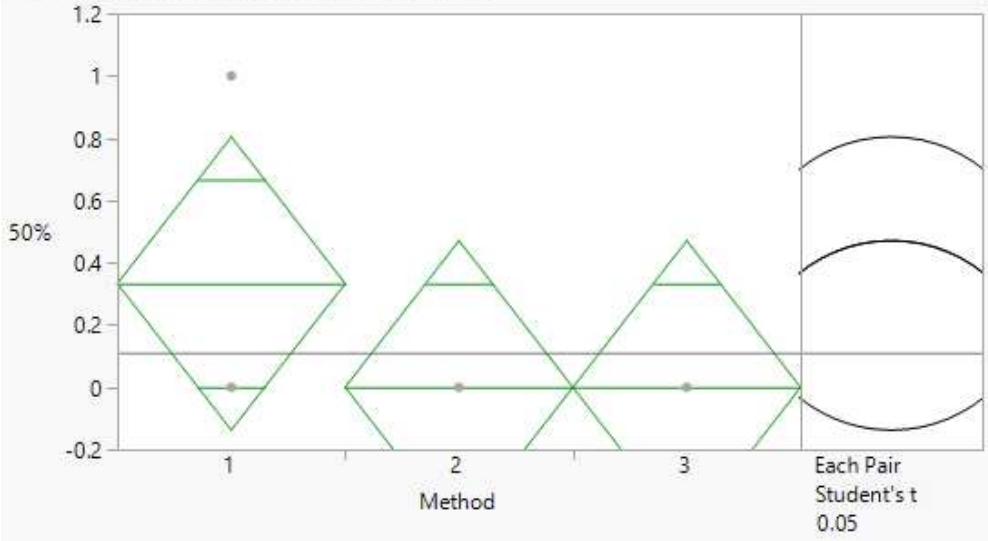
Levels not connected by same letter are significantly different.

Ordered Differences Report

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
1	2	1.000000	7.0245e-9	1.00000	1.000000	<.0001*
1	3	1.000000	7.0245e-9	1.00000	1.000000	<.0001*
3	2	0.000000	7.0245e-9	-1.719e-8	1.7188e-8	1.0000



Oneway Analysis of 50% By Method



Oneway Anova

Summary of Fit

Rsquare	0.25
Adj Rsquare	-2.2e-16
Root Mean Square Error	0.333333
Mean of Response	0.111111
Observations (or Sum Wgts)	9

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Method	2	0.22222222	0.111111	1.0000	0.4219
Error	6	0.66666667	0.111111		
C. Total	8	0.88888889			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	3	0.333333	0.19245	-0.1376	0.80424
2	3	0.000000	0.19245	-0.4709	0.47091
3	3	0.000000	0.19245	-0.4709	0.47091

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for each pair using Student's t

Confidence Quantile

t	Alpha
2.44691	0.05

LSD Threshold Matrix

Abs(Dif)-LSD

	1	2	3
1	-0.66597	-0.33263	-0.33263
2	-0.33263	-0.66597	-0.66597
3	-0.33263	-0.66597	-0.66597

Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level		Mean
1	A	0.33333333
2	A	0.00000000
3	A	0.00000000

Levels not connected by same letter are significantly different.

Ordered Differences Report

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
1	2	0.3333333	0.2721655	-0.332632	0.9992984	0.2666
1	3	0.3333333	0.2721655	-0.332632	0.9992984	0.2666
3	2	0.0000000	0.2721655	-0.665965	0.6659651	1.0000

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