Exploration of the Anti-biofilm Effects of Fish Antimicrobial Peptides Against Common Aquaculture Pathogens

by

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A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 8, 2020

Keywords: antimicrobial peptide, biofilm, natural-killer lysin, Piscidins

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Abstract

Aquaculture's rapid growth and commercialization has been accompanied by increased spread of fish-related diseases that threaten aquaculture's production capabilities. Diseases caused by Gram-negative bacteria such as, *Aeromonas hydrophila* and *Flavobacterium columnare*, are particularly problematic and are responsible for tremendous losses in fish. These bacterial fish pathogens exist not only in a free-living state but also occur readily as biofilms. Biofilms are microbial communities incased in a self-produced organic matrix and are much more resistant to conventional antimicrobial agents than their free-living counterparts. The prevalence of these pathogenic bacteria as biofilms in the aquaculture setting has encouraged the exploration of alternative anti-biofilm agents. The innate immune system of fish and other organisms produce peptides that are effective in combatting a wide range of pathogens. These peptides are collectively known as antimicrobial peptides (AMPs) and they may prove to be useful anti-biofilm agents due to their broad-spectrum capabilities. These peptides have been characterized among many different fish species and their effect on different Gram-negative bacteria has been evaluated. However, there exists a gap of knowledge in their anti-biofilm capabilities. The current study has assessed the activity of different teleost AMPs against *Aeromonas hydrophila*, *Aeromonas veronii*, *Flavobacterium columnare* and *Escherichia coli*. The NK-lysins (NK-1, NK-2, etc.) inhibited the growth of *E. coli* and *A. veronii* and were particularly effective against biofilm formation in these two pathogens. The NK-lysins tended to show an expected dose response in that higher concentrations showed greater inhibition of *E. coli* and *A. veronii*. The Piscidins inhibited *E. coli* and *F. columnare* total growth and showed effectiveness against their respective biofilm formation. Unlike the NK-lysins, the Piscidins inhibited biofilm formation of *E. coli* and *F. columnare* at the lowest tested concentration. The

positive control melittin demonstrated significant total and biofilm inhibition towards all four bacteria; some of the AMPs in this test showed results comparable to melittin. These results suggest that several of the AMPs might serve as effective therapeutic agents against bacteria in aquaculture settings due to their ability to inhibit both free-living and biofilm-encased bacteria and are worthy of further study. Future studies should examine mechanisms of action regarding biofilm inhibition, while also observing activity under more natural conditions.

Acknowledgments

I would like to express sincere appreciation to my committee members: Dr. Eric Peatman, Chairman of the Advisory Committee, for his guidance, support, invaluable insight, and the opportunity to pursue a Master's degree at Auburn University; Dr. Miles Lange for forming the framework of this project, his expertise in fish immunology, and allowing me to conduct experiments in his laboratory; and Dr. Ben Beck for being the catalyst of my interest in fisheries, providing guidance and support, and allowing me use of USDA-ARS Aquatic Animal Health Research Laboratory facilities.

I am extremely grateful for the kindness, support, and knowledge imparted to me by the scientists of the USDA-ARS Aquatic Animal Health Research Laboratory: Dr. Craig Shoemaker; Dr. Julio Garcia; Dr. Ben LaFrentz; Dr. John Shelley; Dr. Dunhua Zhang; and Dr. De-Hai Xu. I am honored to have worked closely with and learned from Matt Paulson, Damian Downing, Troy Bader, Lee Collins, and Michael Smallwood. I would also like to extend my sincere thanks to Patt Hodnett and Paige Mumma for their kindness and help during my time at USDA-ARS. I am also extremely grateful to Dr. Jesse Chappell, Dr. Terry Hanson, Dr. Allen Torbert, Dr. Brett Runion, Barry Dorman, and Robert Icenogle for their practical knowledge and unrelenting support.

I would also like to extend my deepest gratitude to my fellow graduate students: Aaron Fewell; Cody Stites; John Lewis; João Reis; Allen Pattillo; Emmanuel Ayipio; Noli Jocson; Jill Malecki; Jenni Dorick; and others for their assistance, suggestions, and constant moral support.

I am extremely grateful for my good friend, Willkell Boler, for his relentless encouragement and advice. I would also like to extend my deepest gratitude to my many Aunts, Uncles, and cousins for their enthusiasm towards my endeavors. I am especially grateful to my

mother, Judy Prior, and sister, Beth Prior, for their unwavering support and helpful advice. Lastly, I cannot begin to express my thanks to my father, Dr. Stephen Prior, who showed profound belief in my abilities and who was a constant source of encouragement and knowledge.

This thesis is dedicated to the tragic loss of two special people last year: My Uncle, Peter Cauterucci, who imparted his love of fishing and music to me; and my Grandmother, Ayako Hayashida Prior, who inspired and supported my creativity through her relentless passion in the arts.

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List of Abbreviations

3-D	Three Dimensional
AMP	Antimicrobial Peptide
CBD	Calgary Biofilm Device
DiSC35	3,3dipropylthiacarbocyanine
DSC	Differential Scanning Calorimetry
EDTA	Ethylene Diamine Tetra-Acetic Acid
EPS	Extracellular Polymeric Substances
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*
MAS	Motile Aeromonad Septicemia
MBEC	Minimum Biofilm Eradication Concentration
MBIC	Minimum Biofilm Inhibitory Concentration
MIC	Minimum Inhibitory Concentration
MOS	Mannan Oligosacchrides
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide
NK	Natural Killer
NMR	Nuclear Magnetic Resonance
OCD	Oriented Circular Dichroism
pppGpp	Guanosine pentaphosphate
ppGpp	Guanosine Tetraphosphate
SAPLIP	Saposin-like Proteins

Chapter One: Literature Review

With the addition of 83 million people per year, growth of the global human population is on pace to exceed 9 billion people by the middle of the twenty-first century [1]. To sustain the health and livelihood of this growing population, sustainable means of worldwide food acquisition are needed. Aquaculture, which is the culture of aquatic animals and plants, provides a safe and sustainable source of high protein food that can be utilized globally [2, 3]. Since the 1980s, aquaculture has been responsible for the continuous growth in the supply of fish for human consumption and this demand will likely only increase in the future [3, 4].

Diseases caused by bacteria, viruses and parasites are ever-present during aquaculture production. If left unchecked, diseases could significantly hinder the growth and development of the aquaculture industry. Diseases are directly responsible for a decrease in overall production, with survivors often being of lesser food quality. Bacterial-induced diseases prove to be particularly detrimental in aquaculture, especially those caused by *Aeromonas* spp. and *Flavobacterium columnare*. Both *Aeromonas spp.* and *F. columnare* have the capability to form biofilms, which are sessile communities of bacteria encased in a self-produced organic matrix. These biofilms confer many benefits to the bacteria housed within, particularly increased resistance to antibiotics. Antibiotic resistance is already present in many non-biofilm bacterial populations due to extensive antibiotic use in animals and humans which has contributed to the selection, persistence and spread of antibiotic-resistant bacteria within the environment [5]. The formation of bacterial biofilms by antibiotic-resistant bacteria pose a serious problem for many industries, including aquaculture.

Over-reliance on traditional drug and chemical treatments, especially antibiotics, has led to the emergence and spread of antibiotic-resistant pathogens. This coupled with the ability of

bacteria to form biofilms means production-based infections will continually become harder to treat. Due to their potent activity against a plethora of pathogens, antimicrobial peptides (AMPs) have recently garnered attention as potential candidates for future disease treatment [6]. This review will give insight on the role of diseases and bacterial biofilms within aquaculture along with the potential of AMPs for disease control, specifically to combat infections caused by *Aeromonas* spp. and *F. columnare*.

1.0 Aquaculture

The earliest known record of aquaculture can be traced back to 475 B.C in China, where the culture of common carp (*Cyprinus carpio*) as a food product for humans flourished [7]. Aquaculture has since seen continuous expansion worldwide and as of the 21st century continues to outpace the growth of other major food production sectors [4]. In the 1980s and 90s, aquaculture recorded double digit annual growth rates of 11.3 and 10.0 percent, respectively. Global aquaculture production of food fish reached 80 million tons in 2016, with finfish representing over half at 54.1 million tons. Due to continued growth in aquaculture, fish have become much more readily available for human consumption and the demand for fish continues to grow.

The growing global demand for fish shows no sign of stopping and is predicted to escalate throughout the 21st century [4]. In 2016, global fish production of both wild-caught and aquaculture fish hit an all-time high of 171 million tons, with 88 percent going directly to human consumption. Wild-caught fisheries remained somewhat static and in 2016, 37 countries were producing more fish via aquaculture than wild-caught fisheries (Figure 1). Thus, aquaculture will likely be responsible for sustaining future global fish demand.

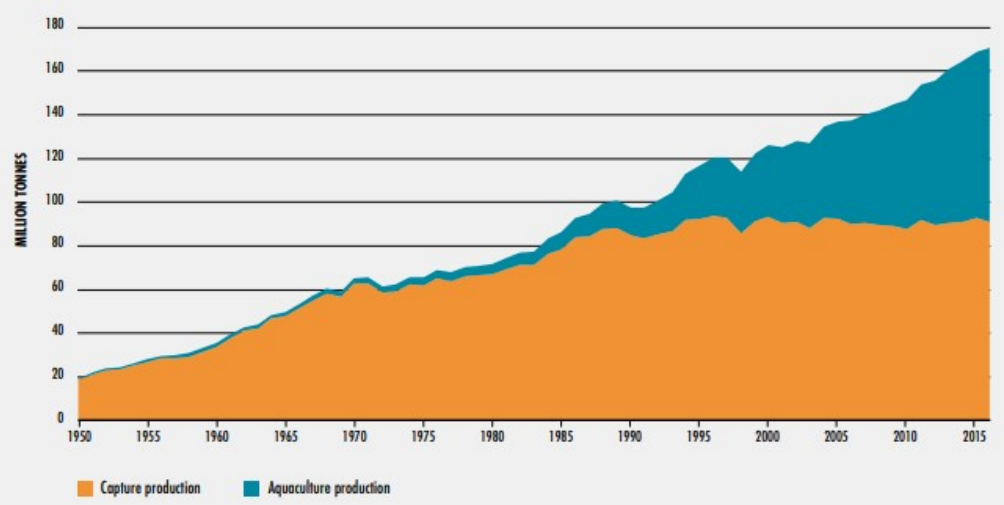

Figure 1: Comparison of wild capture and aquaculture production from 1950 to 2015. Adapted from FAO [4].

The growing demand for fish, coupled with the continued expansion of aquaculture, has made fish products one of the most heavily traded food products in the world, with 35 percent of global fish production directly entering international trade in 2016 [4]. Therefore, fish products are of great economic value. In 2016, the 110.2 million tons of product produced by aquaculture had a first-sale value estimated at 243.5 billion U.S. dollars (USD) [4]. Of the total 2016 production, 80.0 million tons were food fish with an estimated value of 231.6 billion USD. The growing economic importance of aquaculture has also brought about new employment opportunities, especially within developing countries in Africa and Asia. The economic growth and increased importance of aquaculture has resulted in increased diversity of species cultured, including higher valued species to fulfill niche markets [2, 4].

As of 2016, there were 369 commercially farmed finfishes within the field of aquaculture [4]. However, there exists a "core" of finfish species that are predominantly farmed at global and national levels. This "core" consists of 27 species of finfish that accounted for 90 percent of total finfish production in 2016. The top species were grass carp (*Ctenopharyngodon idellus*), silver

carp (*Hypophthalmichthys molitrix*), common carp (*Cyprinus carpio*), and Nile tilapia (*Oreochromis niloticus*). Combined, they comprised 29 percent of total 2016 finfish production. Popular fish in the western culture market, such as Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), contributed four and two percent, respectively, to total finfish production in 2016.

1.1 Aquaculture in the United States

Over the last 40 years, aquaculture within the United States (U.S.) has become a relatively established and diverse industry, producing a variety of fish, crustaceans, mollusks, and plants. In 2014, combined aquaculture production within the U.S. was 275 million tons valued at 1.3 billion USD [8]. In the past, freshwater aquaculture production was dominant over marine but since 2009 freshwater aquaculture production has slightly declined. Even with substantial growth of shellfish production within the U.S., finfish production remains dominant.

The "core" species of fish cultured within the U.S. are Ictalurid catfish (*Ictalurus spp*), rainbow trout, Atlantic salmon, hybrid striped bass (*Morone saxatilis* x *Morone chrysops*) and tilapia [8]. The freshwater species, catfish and rainbow trout, form the backbone of finfish aquaculture in the U.S. The bulk of the catfish industry is located in the southern states of Mississippi, Arkansas, Alabama and Louisiana, while over 50 percent of U.S. rainbow trout production occurs in Idaho [8, 9].

Catfish is the predominant finfish currently cultured in the U.S., and the industry saw most of its growth during the mid 1980s to early 2000s. Between 1982 and 2002, pond area dedicated to channel catfish culture doubled and production increased by six-fold [9]. In 2003, channel catfish production reached 300,000 tons, this represented 64 percent of the total North American aquaculture production and carried a total value of 384 million USD [8, 9]. However,

pressure from lower-priced imports of farmed basa catfish (primarily *Pangasius hypophthalmus*) from Asia has been a primary factor responsible for the continued decline in U.S. catfish production. In 2014, U.S. channel catfish production had fallen to less than half of 2003 values [8].

The increase in lower priced freshwater finfish imports (due to lower feed prices and operating costs versus U.S. production) and the fast growth of marine aquaculture has caused a substantial decrease in freshwater finfish production within the U.S., especially for channel catfish [8]. However, freshwater finfish production still remains the dominant sector of aquaculture within the U.S. and will be invaluable in supporting the future supply of fish for human consumption within the U.S. Therefore, research and support for U.S. based farmers, which will enhance production, is still needed to help keep the U.S. freshwater aquaculture industry alive. Continued research on treatments for diseases that plague channel catfish, rainbow trout and hybrid striped bass are especially important. New insights on diseases will allow farmers within the industry to efficiently combat disease, thus reducing losses and increasing production and profit.

2.0 Disease in Aquaculture

The tremendous growth of aquaculture globally has also facilitated the spread and increased abundance of many fish diseases [10]. Large-scale aquaculture production typically requires high-density animal populations, which provides an extremely favorable environment for the emergence and proliferation of fish pathogens. This poses a major problem for farmers, as fish loss results in direct loss of profits [11]. This also hinders aquaculture's ability to readily contribute to global food supply. Therefore, disease is a major obstacle for the continued success

of aquaculture and will continue to be as the intensification and commercialization of aquaculture continues.

Aquaculture faces a diverse array of pathogens, including viruses, bacteria, fungi, and parasites. Bacterial infections are known to be particularly problematic. *Aeromonas hydrophila* and *F.columnare* are two examples of bacterial fish pathogens that cause losses during aquaculture production cycles. The Gram-negative *A. hydrophila* is prevalent in a wide range of aquatic environments and has been isolated from many bodies of fresh water and organs of healthy fish [12, 13]. The occurrence of *A. hydrophila* typically poses no serious problems to fish populations under normal conditions [14]. However, when fish are exposed to environmental and/or infection by a primary pathogen, *A. hydrophila* has the potential to become pathogenic and is therefore usually considered a secondary pathogen [15]. As a pathogen, *A. hydrophila* is responsible for a condition known as motile aeromonad septicemia (MAS), which can cause the following symptoms in fish: dermal necrosis, ulcerations, petechial hemorrhaging or hyperemia, ocular disease, hepatic and renal necrosis, and hemorrhagic septicemia [16]. The emergence of virulent strains of *A. hydrophila*, most notably where the U.S. catfish industry lost over three million pounds of fish in 2009, has revealed that this bacterium has the ability to act as a primary pathogen to fish [17, 18]. The development of resistance to multiple antibiotics has also been demonstrated among many *A. hydrophila* isolates [19]. Thus, the ability of *A. hydrophila* to become virulent and resistant to multiple antibiotics means this bacterium will continue to be a major problem for freshwater aquaculture.

The Gram-negative bacterium *F. columnare* is the causative agent of columnaris disease which affects both wild and cultured fish worldwide [20]. In fish, columnaris disease is typically characterized by the presence of external greyish white or yellow areas of erosion surrounded by

a red hyperemic zone that commonly form on gills and dorsal fins. The bacterium can also cause systemic infections and has been isolated from the internal organs of fish. The Ictalurid family of fish, which includes channel catfish, demonstrate susceptibility to columnaris which routinely causes significant economic losses within aquaculture [21]. Populations of catfish reared in ponds can reach mortality rates of 60 percent during columnaris outbreaks. Columnaris has been extremely detrimental to the U.S. channel catfish and rainbow trout industries, solely causing the channel catfish industry up to 30 million dollars in losses on an annual basis [20, 22]. This disease can be especially devastating during early developmental stages of channel catfish and rainbow trout [22]. Columnaris outbreaks are often correlated with adverse environmental conditions such as high fish densities, high levels of ammonia and/or high organic load [23, 24]. Since *F. columnare* is ubiquitous in aquatic environments and acts primarily as a facultative pathogen, columnaris disease will continue to be an ever-present problem in the aquaculture industry.

Both *A. hydrophila* and *F. columnare* can cause significant damage to cultured fish populations. Their pathogenicity will only increase as they continue to develop resistance to antibiotics commonly used within aquaculture [25]. Both can also readily form biofilms, which are communities of bacteria encased in organic matrices that often display high antibiotic resistance, sometimes up to 10,000-fold more than free-living bacteria [26, 27]. Since both *A. hydrophila* and *F. columnare* routinely exist in aquatic environments as biofilms displaying the ability to develop antibiotic resistance and highly virulent forms, they will continue to threaten the health of cultured fish and subsequently hinder the success of aquaculture.

3.0 Bacterial Biofilms

Biofilms can be broadly defined as ‘aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface’ [28]. Bacterial biofilms and bacterial infections of fish within aquaculture go hand in hand. Many bacteria that are pathogenic to fish establish biofilm communities to better survive their environments. As a “bacterial community” they work together to gather resources and display a high resistance and tolerance to antimicrobial agents [29]. Often, these communities are established directly on cultured fish resulting in chronic infections and subsequent death if left untreated [30]. Once established, biofilms are extremely hard to eliminate. Therefore, biofilms pose a major problem to the aquatic industry due to their unique ability to remain present in even the most hostile environments.

3.1 General Physiology

Biofilms are communities of microorganisms that can consist of single or multiple species and can occur in almost any type of environment. They are one of the most widely established and successful modes of life on earth and are major drivers of biogeochemical cycling processes of many elements found in water, soil, sediment, and subsurface environments [31]. Biofilms can either be attached to an abiotic/biotic surface or formed in flocs, which refers to mobile biofilms that lack any surface attachment [32]. Given their wide distribution, biofilms are often responsible for persistent infections in animals and plants, contamination of medical devices and implants, biofouling of water and corrosion of surfaces [33, 34]. Biofilms are generated by bacteria, archaea, fungi, and microalgae. However, bacterial biofilms pose a major threat to the aquaculture industry and are the focus for this review.

Bacterial biofilms are often sessile communities which are emergent forms of bacterial life, in which communal life differs drastically from bacteria living in a free-floating, planktonic state [29]. Bacterial biofilms display some key communal emergent properties, such as social cooperation and resource capture [32, 33]. Biofilms differ from bacterial planktonic cells in terms of gene expression and cellular physiology. Bacterial cells housed in these communities often display increased resistance to antibiotics, biocides and host immunity [34]. The self-produced extracellular hydrated matrix of bacterial biofilms is fundamental in the establishment/maintenance of these properties and is a defining characteristic of bacterial biofilms [29]. Since bacterial biofilms can form and thrive on several surfaces and are often found in wet environments, they provide a way in which pathogenic bacteria can remain ever-present in the purely aquatic setting present in aquaculture. Thus, biofilms can act as a reservoir for pathogenic bacteria and allow for routine exposure of these bacteria to cultured fish, resulting in chronic infections. The biofilm state is generally regarded as a virulence factor because the planktonic cells are activated into a pathogenic biofilm state.

3.2 Biofilm Formation

A general knowledge of the process of biofilm formation is helpful in understanding how biofilms interact with surfaces and in which formation stages they may be more vulnerable. This is useful knowledge when combating biofilms. Formation of bacterial biofilms can be described by a series of five sequential developmental stages based on *Pseudomonas aeruginosa* biofilm formation [31, 35]. *Pseudomonas* has been well-studied and is therefore a good model system; however, all biofilms likely are formed using this general strategy. Stages one and two involve initial association of the bacterial cells with a surface followed by EPS production and adhesion. Stages three and four are characterized by biofilm maturation; biofilm cells aggregate into

microcolonies, the bacterial biofilm's EPS architecture is finalized, and maximal growth is achieved. In stage five, biofilm cells disperse and revert to their planktonic lifestyle. Figure 2 visually depicts the above process. Knowing how bacterial biofilms form can help give valuable insight on establishing measures to prevent biofilm formation and ways to induce biofilm dispersal within aquaculture and other industries.

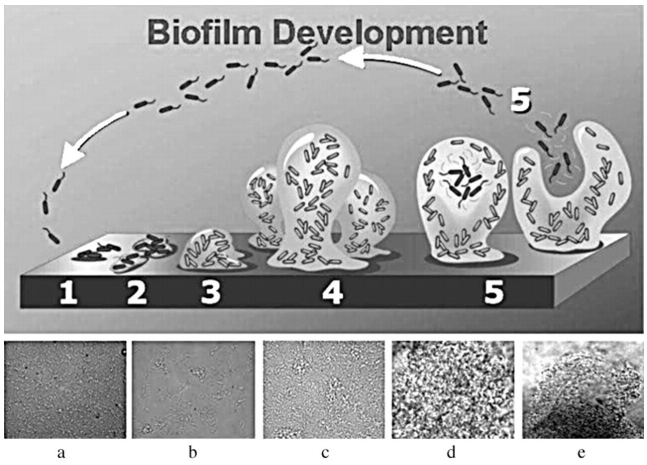

Figure 2: A generalized set of developmental stages of *Pseudomonas aeruginosa* biofilm formation. **Stage One:** Initial attachment of bacterial cells to a surface. **Stage Two:** Production of extracellular polymeric substances resulting in the establishment of a more "irreversible" adhesion to the surface. **Stage Three:** Initial development of the complex biofilm matrix architecture. **Stage Four:** The biofilm matrix architecture is fully matured. **Stage Five:** Bacterial cells begin to disperse from the biofilm, returning to their planktonic way of life. The bottom panels (a-e) depict each of the five stages and are represented by a photomicrograph of *P. aeruginosa* when grown under continuous-flow conditions on a glass substratum. Adapted from Stoodley et al. [31].

3.3 Biofilm Matrix

The EPS biofilm matrix is arguably the most important feature of biofilms since most of the enhanced survival mechanisms employed by biofilms are a result of the matrix structure.

Therefore, it is important to understand the many features of the matrix, especially if the goal is to combat biofilms. The more knowledge one has about matrix capabilities, the better able one can develop new treatment methods that target and/or bypass the matrix. The matrix represents most of the total biomass of biofilms, typically accounting for over 90 percent of total biomass [29]. The matrix is where the individual cells of the biofilm reside (they are embedded throughout) and is the glue that holds this community together. Water is the primary component of the matrix and can account for up to 97 percent of the matrix composition. The remainder of the matrix consists of an assortment of biopolymers which are collectively referred to as extracellular polymeric substances (EPS) and includes polysaccharides, proteins, eDNA, amyloids, cellulose, fimbriae, pili and flagella [36]. The EPS provides the scaffold of the three-dimensional structure of the biofilm. This is where cells and other matrix components of the biofilm attach.

The bacterial biofilm EPS matrix displays an extremely complex and differentiated architecture. Observational studies have shown that bacterial biofilms are composed of heterogeneous matrix-enclosed microcolonies of sessile bacteria that are separated by open water channels and pores that facilitate liquid exchange [37]. These channels and pores provide a sort of circulation system for the biofilm, allowing for the optimization of nutrient and waste-product exchange within these communities via the infusion of fluid to the biofilm from the bulk phase. The efficient nutrient exchange of biofilms, conferred by their inner channel system, allows them to survive with minimal nutrients [32, 33]. Thus, bacteria within biofilms can better survive in unfavorable and hostile environments.

Not only does the biofilm EPS matrix provide a circulation system for nutrients, it also allows the biofilm to capture nutrients from the surrounding environment [29]. Acquiring

nutrients is fundamental to the survival of any organism, and bacterial biofilms have an efficient capture strategy for nutrients exceeding that of free-living bacterial cells. Sorption, which refers to the combined processes of both adsorption and absorption, is a passive property of the EPS matrix and modulates the exchange of nutrients, gases and other molecules between the biofilm and external environments [38]. This property is what drives the efficient acquisition of nutrients for biofilms. Once nutrients and other substances are sequestered into the biofilm, they bind to binding sites found on biofilm cells and the EPS matrix. The anionic and cationic nature of these binding sites allow for a wide range of substances to be trapped and accumulated within the biofilm for potential consumption by their cells. The sorption of the biofilm is not substance specific and many toxins can accumulate within biofilms including erythromycin, ethylsuccinate, acetaminophen, acidic pharmaceuticals, steroidal hormones, 4-nonylphenol compounds, benzene, toluene, and xylene [39, 40]. This makes biofilms both a sink and source for many toxins. Calcium, iron, manganese, and other essential metal ions accumulate in the biofilm via sorption and provide stabilization to the EPS biofilm matrix by the bridging of carboxyl groups across EPS molecules [41]. A wide range of other suspended and dissolved solids (e.g., nanoparticles, clay, silicate, graphite particles, granular activated carbon, charcoal and carbon cloth) are also captured by biofilms and resuspended in the EPS matrix [42, 43]. The EPS matrix's efficiency in sequestering this wide variety of substances from the external environment enables biofilms to grow in oligotrophic, low nutrient environments [44]. The EPS matrix is the foundation for the form and function of biofilms. It provides many things: structure for the whole biofilm community, circulation of nutrients throughout the biofilm community and efficient sequestering of nutrients and other substances from almost any external environment. This

makes bacterial biofilms a much more capable and dangerous foe for animal rearing industries, such as aquaculture, when compared to free-living bacteria.

3.4 Biofilms in Aquaculture

Biofilms are widely prevalent in the aquaculture setting due to their abilities to readily form in aquatic environments. There are both pros and cons to the formation of biofilms in aquaculture. Biofilms are an essential component for the development of efficient biofilters for the processing of fish waste in aquaculture facilities [45]. Many sectors of aquaculture have implemented the use of recirculating systems for the culture of fish. These systems rely on biofilms present in biofilters to control levels of toxic organics, especially ammonia and nitrite. The nitrifying bacteria of these biofilms are responsible for the nitrification process, which oxidizes ammonia secreted by fish to nitrite and ultimately to nitrate. This enables fish to survive within recirculating systems, making these systems an environment conducive for fish growth. While vital for the control of ammonia and nitrite, biofilms do pose serious problems for the aquaculture industry. A common phenomenon, known as biofouling, is caused by the formation of biofilms on surfaces of many things including pipes, netting and cages [29]. Bio-fouling causes reduced flow of water via blockage which results in less efficient removal of waste from tanks and cages commonly used in aquaculture, ultimately decreasing the overall quality of living conditions for fish [46]. Biofilms have also been shown to be a reservoir for bacteria pathogenic to fish [47]. Both *A. hydrophila* and *F. columnare* have been observed to form biofilms in aquaculture settings [27]. One study isolated *A. hydrophila* from biofilms that were present on the surfaces of the following materials at commercial aquaculture facilities: fiberglass, aluminum, PVC, plastic, rubber, cement and stainless steel [47]. Recently, virulent *A. hydrophila* (vAh) has been isolated from biofilms taken from catfish ponds that had previously experienced

outbreaks of vAh, suggesting that biofilms can act as reservoir for *A. hyrdrophlia* [48]. A study conducted by Welker et al. [49], observed that *F. columnare* can be obtained from biofilms present in fish holding tanks proceeding an outbreak of columnaris. As shown by Cai et al. [50], *F. columnare* can readily form biofilms in aquatic environments under both static and flow conditions, while also retaining its virulence in the biofilm state. Biofilms offer pathogenic bacteria, such as *A. hydrophila* and *F. columnare*, places of permanent and temporary refuge within a variety of aquaculture settings [27].

4.0 Antimicrobial Peptides (AMPs)

AMPs are molecules produced by the immune systems of many organisms across the animal and plant kingdoms [51]. These peptides are recognized as ancient weapons that accompanied many multi-cellular organisms through evolution and their effectiveness against pathogens has remained intact [52]. Their conserved evolution coupled with their continued effectiveness, proves that AMPs still hold tremendous value in the fight against bacterial infections. Their value has the potential to be very high in the aquaculture industry due to the increasing establishment of antibiotic-resistant bacteria, both in the planktonic and biofilm forms [53]. AMPs may prove to be a valuable ally for the aquaculture industry in the development of new methods of treatment for bacteria induced infections of fish [54].

AMPs share many features and only a limited number of structural variations exist [55]. With that said, the activities and targets of AMPs differ considerably. Antimicrobial peptides that exhibit bactericidal activity typically target the “bacterial Achilles heel” which refers to the cellular membrane of bacteria, specifically the component known as peptidoglycan [56]. While the predominant mode of action for AMPs is the disruption of the bacterial cell membrane integrity, other AMP modes of action have been characterized that include the targeting of key

cellular functions such as DNA and protein synthesis, protein folding, enzymatic activity and cell wall synthesis [57, 58]. The proven effectiveness of AMPs against a wide range of bacterial species makes them a major source of knowledge in the development of manmade methods to combat bacterial infections. AMPs could serve as a therapy against common bacterial infections.

4.1 Structural Features

Even though AMPs are widely distributed across many different organisms, all AMPs still share several common structural features that distinctly characterize them [55]. Typically, AMPs are produced by ribosomes and exist initially as inactive pro-polypeptides [59]. Once they are processed into their active forms, they range from 12 to 50 protein residues in size. Most AMPs are cationic and amphipathic in nature, meaning that they carry a net positive charge and have areas within their structure that are polar and non-polar [60]. Thus, cationic AMPs will be the focus of this review. The net positive charge of AMPs ranges from +2 to +7 and is due to an excess of the basic amino acids arginine, lysine and/or histidine found in their peptide structure [61]. This means that AMPs are more attracted to the negatively charged cytoplasmic membranes of bacteria rather than the positively charged membranes of mammals or other multicellular organisms [62]. Typically, at least 50 percent of the amino acids found in AMPs are hydrophobic, which allows them to undergo interactions with the fatty acyl chains of bacterial membranes [61, 62]. The three-dimensional (3-D) structures of AMPs can be readily determined via two-dimensional NMR methods due to their short peptide lengths. The structures of AMPs obtained by NMR in conjunction with sequence analysis of AMPs has resulted in the classification of AMPs into three general groups [63]. Also, the existence of larger proteins with antimicrobial activity has been acknowledged and are considered to have significant functional overlap with their smaller AMP counterparts [64]. There are several antimicrobial proteins

characterized within fish, namely natural killer (NK)-lysins. Therefore, the three general structure groups of AMPs along with antimicrobial proteins will be covered (Figure 3).

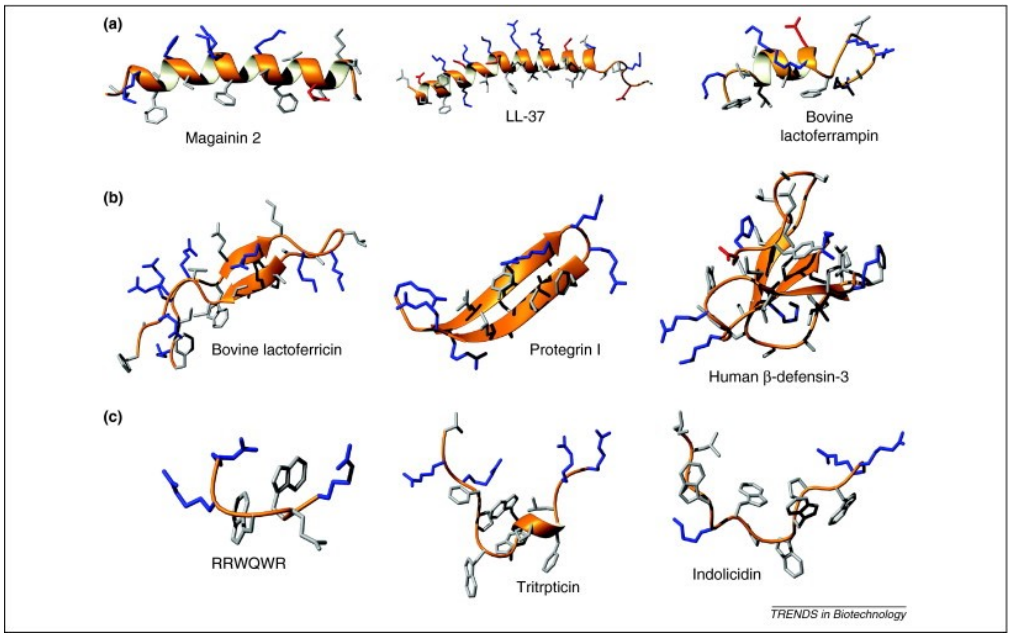

Figure 3: Representative AMPs from each of the three structural AMP classes along with their 3-D structures. **(a)** alpha-helical peptides, (b) beta-sheet peptides and **(c)** extended peptides. Positively charged side chains are blue, negatively charged side chains are red and the remaining side chains are grey. 3-D structures displayed were obtained by solution NMR spectroscopy in the presence of detergent micelles. IDs for the AMPs: IDs: magainin 2, 2MAG; LL-37, 2K6O; bovine lactoferricin, 1LFC; protegrin 1, 1PG1; human -defensin-3, 1KJ5; tritrpticin, 1D6X; indolicidin, 1G89. Adapted from Nguyen et al. [64].

Alpha-helical AMPs

Antimicrobial peptides designated as having an alpha helical secondary structure are among the most heavily studied AMPs and approximately 1/3 of all AMPs identified have an alpha helical structure [65, 66]. Typically, they range from 12 to 40 amino acids in length, with a

large number consisting of the helix-stabilizing amino acids lysine, leucine and alanine. They tend to remain unstructured in aqueous solutions and take up their characteristic amphipathic-helical conformation when exposed to membrane-mimetic environments. Some of the most well-known AMPs display an alpha helical structure such as magainin, cecropin, melittin, and Piscidins which are known to be produced by different species of amphibians, insects, fish, and mammals [67, 68]. Melittin is a cationic linear peptide composed of 26 amino acid residues and is derived from European honeybee (*Apis mellifera*) venom [69]. This AMP is known to readily interact with prokaryotic and eukaryotic cellular membranes, causing severe membrane disruption and subsequent cellular lysis. Melittin also has demonstrated potent anti-biofilm activity towards Gram-negative and Gram-positive bacterial biofilms [70]. Due to its well-established killing abilities against a wide array of organisms, several studies exploring the antibacterial and anti-biofilm effects of AMPs have used melittin as a positive control [71, 72]. Piscidins are of interest due to their extensive presence in fish, which means they may have potential for use within the aquaculture industry [6, 73]. They were originally isolated from striped bass (*M. saxatilis*), white bass (*M. chrysops*), and hybrid striped bass (*M. chrysops, x M. saxatilis*) [74]. Since their original isolation, Piscidins have since been found in a wide range of teleost fish taxa, including the families Moronidae, Sciaenidae, Siganidae, Belontidae, Cichlidae, Percichthyidae, Latidae, Sparidae, Sygnathidae, and Latridae [75]. The Piscidins are unique to teleost fish and are the only type of AMPs that are not found in other vertebrates [76]. Outside of the Piscidins, all other AMPs identified from fish share similar structure and functions to that of other vertebrate AMPs. Thus, Piscidins may be of importance in the development of AMP-based treatments and diagnostic tools for the assessment of fish population health due to their wide presence in fish.

Beta sheet AMPs

Antimicrobial peptides that display a beta sheet secondary structure make up another large portion of the known AMPs and are characterized by having two to ten cysteine residues that form one to five disulfide bonds [77]. The beta sheet AMPs can be further split up into two subgroups: the defensin and beta hairpin peptides [64]. The defensins are evolutionary conserved across a wide range of plants, fungi, invertebrates and vertebrates and are characterized by having six to eight cysteine residues arranged in defined positions [78]. Many of the beta sheet AMPs are known to act upon the microbial membrane via formation of pores [79]. A specific group of beta sheet AMPs, known as defensins, show a widespread presence within fish, specifically in the form of beta-defensins [88]. Beta-defensins are characterized by three disulfide bridges that are conserved across several species despite variations in overall amino acid sequences [81]. Beta-defensins have displayed antimicrobial actions against bacteria, viruses, fungi and parasites and show functions in a wide range of other biological processes (i.e., cell activation, proliferation, regulation of cytokine/chemokine production, migration, differentiation, angiogenesis, wound healing, embryo development and reproduction) [82, 83]. They have been characterized in olive flounder (*Paralichthys olivaceus*), rainbow trout, Atlantic salmon and channel catfish [84-86]. Thus, beta-defensins may have an application in the development of new treatments towards bacterial biofilms and other pathogens.

Extended AMPs

Extended AMPs are characterized by not folding into regular secondary structures and typically having high numbers of the amino acids tryptophan, arginine, proline, glycine and histidine [64, 77]. Due to their unusual amino acid composition their structural conformations are highly variable. Most of the extended AMPs do not act upon the microbial cell membrane,

instead they typically enter the bacterial cell and disrupt intracellular activities [57, 64]. These extended AMPs have yet to be readily identified within species of fish and have not been isolated from any major fish species reared for aquaculture [87, 88].

Protein-derived antimicrobial fragments and Proteins

Antimicrobial proteins are often cationic in nature and share many of the antimicrobial effects of AMPs [64]. What differentiates antimicrobial proteins from AMPs is that they have a greater amount of amino acid residues in their structure (over 50). A well-documented class of these proteins are NK-lysins that range from 74 to 78 amino acid residues in size and are known to display activity against a wide variety of pathogens from bacteria [89] to parasites [90]. NK-lysins are thought to be effector proteins of cytotoxic T and NK cells due to their expression in CD2+, CD4+ and CD8+ cells. NK-lysins fall under the wider family of saposin-like proteins (SAPLIP) due to similar amino acid homology, which also includes human granulysins [91, 92]. SAPLIP, which includes human granulysins and cytoplasmic granules of *Entamoeba histolytica*, are known for their membrane-binding and membrane disruption activities [92, 93]. This suggests that NK-lysins are ancient, highly conserved antimicrobial proteins that serve as an important defense mechanism for many hosts [94]. NK-lysins have been characterized in many fish species important to aquaculture such as channel catfish [95], common carp [96], and Atlantic salmon [97]. Therefore, they contain valuable insights and potential application towards the battle against bacterial biofilm infections within aquaculture.

4.2 Mechanisms of Action

The execution of AMPs' bactericidal effects and the speed at which they do so varies among AMPs. For some, such as the alpha helical AMPs, targeted cells are killed so quickly that characterizing the steps involved are extremely challenging [98]. In comparison, others have kill

times ranging from 15 to 90 minutes, which include magainin 2, cecropin P1, PR-39 and SMAP29 [99-101]. All AMPs share one crucial step when it comes to AMP cell-mediated killing, which is the initial interaction with the outer and/or inner membranes of bacteria [102]. Electrostatic forces occurring between the positively charged AMPs and negatively charged bacterial membranes is what drives this intense interaction [103]. This step also commonly serves as the "killing" step for many AMPs, it is here that many of them exert their antimicrobial effects via disruption of the bacterial membrane [57, 102]. Some AMPs interact with the bacterial membrane so they can translocate into the cytoplasm of bacterial cells where their intracellular targets are located (i.e., macromolecular synthesis in cells, heat shock proteins, cell division etc.) [104, 105]. It has also been proposed that AMPs likely rely on multiple modes of action and that individual AMPs elicit a unique response from bacteria. Antimicrobial peptides that demonstrate a multi-modal action are referred to as having a multi-hit mechanism [106]. This mechanism is thought to contribute to the overall efficiency of AMPs and to help with evasion of AMP resistance development by bacteria [107]. AMP-mediated membrane disruption occurs by a variety of mechanisms and five primary models have been proposed to describe these mechanisms: the barrel-stave, toroidal-pore, carpet, sinking-raft and molecular electroporation models [102]. Each of these models causes a leaking of ions and metabolites, depolarization of the transmembrane potential which results in membrane dysfunction (i.e., impaired osmotic regulation and inhibition of respiration), and membrane rupture coupled with rapid lysis of microbial cells [57, 103]. These five membrane disruption models along with AMP modes of intracellular killing will be covered in this section (Figure 4).

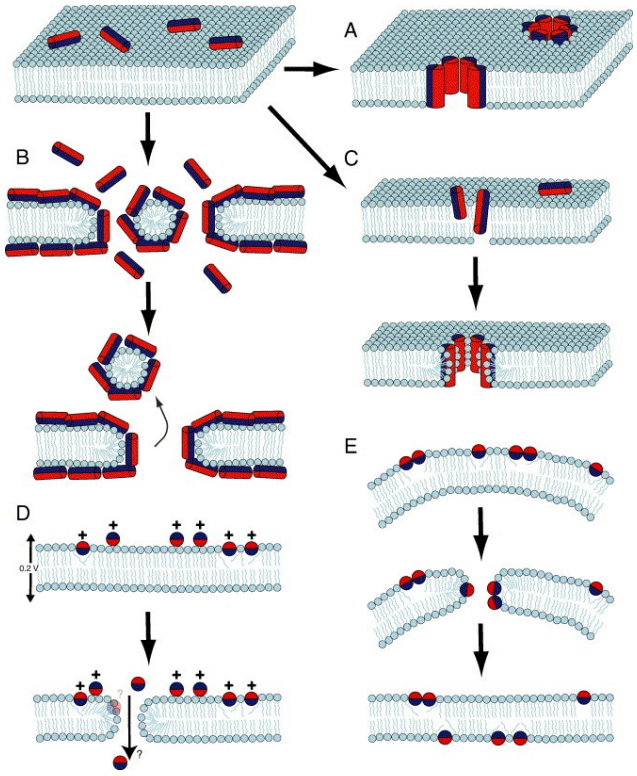

Figure 4: Visual representations of the primary models proposed to explain the different mechanisms of AMP induced membrane disruption: **(A)** Barrel-stave model, (B) Carpet model **(C)**, Toroidal pore model **(D)** Molecular electroporation model and **(E)** Sinking raft model. Adapted from Chan et al. [102].

Barrel-stave Model

The barrel-stave model involves AMPs that have an alpha helical conformation [63]. The peptides first associate with the bacterial membrane and bind to it in a helical fashion [57, 108]. They then proceed to form a bundle within the bacterial membrane that resembles a barrel with the helical peptides acting as the barrel staves. Once this barrel of peptides is formed within the hydrophobic core of the bacterial membrane, it acts as a transmembrane pore [63, 102]. This pore gradually increases in size as more peptides are recruited and mass leakage of cell contents ensues, resulting in eventual cell death.

Toroidal-pore Model

The toroidal-pore model resembles the barrel-stave model in regards to a formation of a transmembrane pore. The primary difference between the two is that the peptides involved in the toroidal-pore model are associated with the lipid head groups of the microbial membrane's bilayer throughout the entire process, even when they are perpendicularly embedded into the lipid bilayer [108, 109]. During toroidal pore formation, AMPs insert themselves into the membrane and cause the lipid heads of the membrane's bilayer to bend inwards towards the pore. This results in the pore being lined by both the AMPs and lipid head groups, which serve the purpose of screening and masking the charges of the AMPs. Magainins, protegrins and melittin are AMPs that induce toroidal-pore formation.

Carpet Model

The carpet model is characterized by peptides lining the bacterial membrane in a parallel fashion, "carpeting" the membrane [102]. The peptides first bind to the phospholipid head groups of the bacterial membrane and will remain bound to them throughout the whole process. The peptides then align themselves so as their hydrophilic residues face the phospholipid head groups [63]. Eventually, the peptides reorient towards the hydrophobic core of the bacterial membrane and cause disruption of the bilayer curvature. Toroidal transient holes are formed by the peptides which opens up access to the membrane to even more peptides [57]. This results in disintegration of the bacterial membrane and the formation of micelles [110]. Micelles are thought to form due to the detergent-like actions of the peptides during the process [111].

Sinking-raft Model

The sinking-raft model is characterized by the sinking of AMPs into a membrane to form transient transmembrane pores [112]. Specifically, AMPs begin to bind to the membrane which

causes a mass imbalance and a subsequent increase in the curvature of the membrane [102]. The AMPs then begin to self-associate and sink into the membrane which results in AMPs residing on both sides of the bilayer and creation of transient transmembrane pores. Delta-lysin, which is an alpha helical AMP of bacterial origin, is known to disrupt membranes following this model [112].

Molecular Electroporation Model

The process of electroporation applies short electrical pulses to induce the formation of temporary pores in cellular membranes [113]. Some AMPs employ a form of molecular electroporation to produce pores in bacterial membranes [102]. This occurs when an electrical field is established due to the association of AMPs with the bacterial membrane [114]. This electrical field forms an electrical potential which is generated across the bacterial membrane and when the electrical potential reaches a voltage of 0.2 it is thought that transmembrane pores are generated.

Modes of Intracellular Killing

Certain AMPs do not simply rely on membrane disruption to kill bacterial cells but also have killing mechanisms that focus on targets located in the bacterial cytoplasm. These AMPs typically target key cellular functions of bacterial cells which include DNA/RNA synthesis, protein synthesis, protein folding, enzymatic activity, and/or cell wall synthesis. The AMPs PR-39, PR-26, indolicidin and microcin 25 are known to induce cell filamentation in bacterial strains within the genera *Salmonella*, *Escherichia* and *Shigella* [115, 116]. When cell filamentation is induced, bacterial cells are unable to undergo cell division [117]. The AMPs drosocin and pyrrhocoricin have both been shown to target DnaK, which is an intracellular bacterial heat shock protein that functions as a cellular chaperone for protein folding [118, 119]. Specifically,

when droscin and pyrrhocoricin are bound to DnaK, the opening and closing of the multi-helical lid over the peptide-binding pocket of DnaK ceases which permanently closes the cavity. This results in the inhibition of the ATPase and chaperone-assisted protein folding actions of DnaK. It is apparent that some AMPs have specific targets other than solely the bacterial membrane. This shows the wide diversity of AMP action and proves that they are not a "one trick pony". Thus, AMPs have the potential to be applied in a wide variety of treatment situations due to their broad spectrum and variety of killing mechanisms.

4.3 Anti-biofilm AMPs

Some AMPs have been isolated that exhibit excellent activity towards bacterial biofilms but poor activity against their planktonic counterparts [120]. This has resulted in a sub-classification of AMPs that demonstrate specific anti-biofilm effects [121]. For example, the Gram-negative bacteria, *Burkholderia cenocepacia*, displays extreme resistance to most AMPs when in a planktonic state but as a biofilm it has been shown to be greatly susceptible to AMPs [122]. The AMPs within this class have been documented to exhibit anti-biofilm actions against some of the most resistant bacteria termed as ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae*) [123].

Several AMPs are known to specifically target the bacterial pro-biofilm signaling nucleotides; guanosine tetraphosphate (ppGpp) and pentaphosphate ((p)ppGpp) (collectively known as (p)ppGpp) [121, 123]. Both nucleotides are upregulated in many bacterial species when they are faced with stressful conditions [124]. Their upregulation induces bacteria to shift nutrients from growth and division to biofilm formation processes [124, 125]. Many bacterial species require these signaling nucleotides for biofilm formation and mutants that are unable to

produce them have been shown to be unable to form biofilms [123, 126]. The AMPs IDR1018 and DJK-5 both target (p)ppGpp and preferentially bind to it, resulting in the depletion of (p)ppGpp and eliminating biofilm formation [123]. This has been demonstrated in, *in vivo* and *in vitro* laboratory experiments [127, 128].

4.3.1 *In Vitro* Methods for Determining Antibiofilm Effects of AMPs

Determining the mechanism of action (MOA) for AMPs is vital for truly understanding the AMP activity landscape [121]. While the sequences of many AMPs exist in several databases (i.e., AMP Database with over 3000 entries [129] and DRAMP with over 17000 entries [130]), the MOA is only known for a small fraction of them. Much more work needs to be done on the MOAs of AMPs, as sequences only show part of the AMP picture. Characterization of AMP MOAs allows for a better idea of how AMPs can be applied as treatments. It also gives valuable insight into how different AMPs exploit microbes, which may shed light on microbial weaknesses that can be exploited by the development of new antimicrobial treatments. Through the use of a wide variety of assays, the MOA of AMPs can be pinpointed. Below, several assays are explored that are commonly used to determine the anti-biofilm effects of AMPs. Typically, AMPs are tested first to see if they display any major biofilm inhibition and/or eradication effects. The assays commonly used to determine biofilm inhibition/eradication will be covered first. Once an AMP is determined to have a major biofilm inhibition and/or eradication effect, more assays are employed to determine the AMP's MOA. These assays will be covered second.

4.3.1.1 Minimum Biofilm Inhibitory Concentration Assays

The minimum biofilm inhibitory concentration (MBIC) refers to the lowest concentration of antimicrobial agent needed to inhibit biofilm formation [131]. For an AMP to be considered anti-biofilm, the MBIC should be below the minimum inhibitory concentration (MIC), with a

distinct structure activity relationship compared to the direct killing antimicrobial activity. The MIC refers to the lowest concentration of an antimicrobial agent needed to prevent growth of planktonic bacteria.

The well-known biofilm microtiter plate assay can be used in determining the MIBC of an AMP. This assay is a static biofilm system used to monitor microbial attachment to an abiotic surface and has been used primarily for the study of bacterial biofilms [132]. The standard protocol for this assay is derived from a protocol published by Christensen et al. [133] and became popularized in the mid-1990s [134]. This assay can be used in the following way for AMP MIBC determination: 1) 96well microtiter plates with growth media are inoculated with bacteria, 2) the AMP being tested is added at varying concentrations to observe biofilm prevention, 3) plates are allowed to incubate (typically for at least 24 hours), 4) After incubation, the planktonic growth is removed and crystal violet is used for cell staining, 5) the crystal violet is then dissolved in ethanol or glacial acetic acid, and 6) the optical density of the solubilized crystal violet is measured using a microplate reader for semi-quantitative biomass assessment.

Although the biofilm microtiter plate assay was used in this thesis, several additional susceptibility assays that can be used to assess the MBIC of an AMP are briefly outlined below. The biofilm microtiter plate assay can be modified to assess survival of viable biofilm cells after antimicrobial treatment by using other dyes [MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) and resazurin] that are converted to other products in the presence of live cells [131]. The Calgary biofilm device (CBD) can be used to test if an antimicrobial agent acts on a uniform biofilm and allows for clear distinction between effects on planktonic and biofilm cells. The flow cell system is a sophisticated technique that can capture the

antibiofilm activities of antimicrobial agents in real time [131, 135] via plating of the output flow [123].

4.4 AMPs and Aquaculture

Reactive disease treatments employed in aquaculture commonly revolve around the heavy use of chemicals and drugs [6]. As previously mentioned, the prevalence of pathogens resistant to these common treatments is increasing throughout the aquaculture industry [5]. This is especially a problem with certain bacterial species, as several species have developed antibiotic-resistant strains. Antibiotic-resistant bacteria coupled with their ability to form "fortresses" known as biofilms makes them an extremely formidable foe in the aquaculture industry. Thus, the application of AMPs towards disease problems in aquaculture have garnered attention due to their wide diversity, broad-spectrum killing abilities and proven effectiveness evident by their conservation through the evolution of many organisms.

Antimicrobial peptides serve as a fundamental line of defense against pathogens for many organisms, including fish. Within fish, they form an important pillar of the innate immune system response against infections by pathogens. Several groups of AMPs have been identified to be produced by fish; these include Piscidins, beta-defensins, hepcidins, cathelicidins and the antimicrobial protein NK-lysin. Their production within fish are induced by a wide range of stimuli, including Gram-positive and Gram-negative bacteria [136], viruses [137], and parasites [138]. This final section will explore the potential application of AMPs in the fight against disease within aquaculture.

The synergistic effect that certain AMPs exhibit with conventional antibiotics has long been recognized [139]. Recently, this synergistic relationship has gained more attention as a potential method to overcome bacterial resistance mechanisms and restore once-potent

antibiotics that currently show diminished effectiveness [121, 140]. Several *in vitro* studies have demonstrated the joint effectiveness of AMPs and conventional antibiotics against antibiotic-resistant bacteria [141, 142]. Recently, a hepcidin homologue isolated from starry flounder (*Platichthys stellatus*), which is a species relevant to aquaculture, was shown to have synergistic activity with kanamycin and ampicillin against several bacterial species belonging to the genus *Vibrio* [143]. The joint activity between AMPs and antibiotics has also been demonstrated with *in vivo* infection models [144]. Studies have also demonstrated that anti-biofilm AMPs can synergize with conventional antibiotics to prevent and eradicate bacterial biofilms *in vitro* [145]. A potential way to exploit this synergistic relationship within aquaculture as a treatment would be to supplement antibiotic feeds with AMPs. Several studies have indicated the innate immune systems of common carp [146], orange-spotted grouper (*Epinephelus coioides*) [147], largemouth bass (*Micropterus salmoides*) [148], and Nile tilapia [149] were enhanced upon the incorporation of AMPs into their diets. Since many antibiotic-based treatments in aquaculture are administered to diseased fish via antibiotic-supplemented feeds, AMPs and antibiotics could be included together in feeds to harness their demonstrated powerful synergistic relationship. This combination could be particularly useful in dealing with infection outbreaks caused by antibiotic-resistant strains of bacteria, such as *A. hydrophila* and *F. columnare*. AMPs could serve as a sort of adjuvant for traditional antibiotics to better combat resistant bacteria and their biofilms. This could prove to be an effective duo for use within aquaculture as antibiotic-resistant bacteria and bacterial biofilms are both prevalent within the aquaculture industry.

Along the same line of thinking, AMPs could also possibly be applied as an adjuvant to help facilitate the effectiveness of protein antigen vaccines. Vaccines are becoming an increasingly important prophylactic measure within aquaculture, and several have been

developed against pathogens that affect fish, including *A. salmonicida* [150] and *F. columnare* [151]. Typically, vaccines are composed of live attenuated pathogens, whole inactivated organisms, inactivated toxins or subunit protein antigens [152]. Subunit protein antigen vaccines are a safer alternative to others but require the use of adjuvants to elicit an effective immune response [153]. Commonly used adjuvants, such as those containing oils and aluminum, often induce a biased immune response and potentially harmful inflammation [154, 155]. Antimicrobial peptides that are inducers of proinflammatory cytokines could potentially be used as safer alternatives to these other adjuvants for subunit protein antigen vaccines for fish and other animals [155, 156]. As demonstrated in cattle, the inclusion of the AMP indolicidin as an adjuvant in a subunit protein antigen vaccine conferred a potent and long-lasting antigen-specific humoral and cell-mediated immune response. Antimicrobial peptides isolated from fish could work in conjunction with other agents to form safe and effective adjuvant complexes for future vaccines [53].

Antimicrobial peptides could also be used as direct feed additives to enhance the immune systems of fish. As previously mentioned, several studies have shown the positive immune and growth effects conferred to fish fed with diets supplemented with AMPs. These studies have administered AMPs into fish feed using several different methods. AMPs have been added directly to feeds in their pure peptide form [157]. Transgenic organisms that have been genetically modified to express certain AMPs have also been used to deliver AMPs to fish. Transgenic *Artemia*, which were made to express the *Epinephelus coioides* AMP epinecidin-1, were feed to Nile tilapia fry and adult zebra fish (*Danio rerio*) [149, 158]. Feeding of these modified *Artemia* resulted in an elevated induction of several immune related genes in both the tilapia and zebra fish. Several studies have incorporated genetically modified bacteria that

express specific AMPs into fish diets. *Escherichia coli* has been used as an expression system for the production of the grouper AMP epinecidin-1 and subsequent feeding of these *E. coli* to zebra fish resulted in an increase in expression of immune-related genes along with increased resistance to the pathogenic, Gram-negative bacterium *V. vulnificus* [159]. The Gram-positive bacterium *Bacillales subtilis* has been used to express lactoferrin, which is an antimicrobial protein produced by cattle [160]. The lactoferrin producing *B. subtilis* was feed to tilapia fry and conferred a greater resistance to *Edwardsiella tarda*. As demonstrated by previous studies, fish feed supplemented with AMPs can be used to boost the immune system of fish.

Inducing the up-regulation of AMP levels within fish to help bolster their immune response to combat disease is a potential indirect application of AMPs. Immunostimulants would have to be administered, likely via feed, to the fish to enhance their AMP production. Prebiotics are examples of such immunostimulants that have been shown to increase AMP expression in fish upon dietary supplementation [161, 162]. Mannan oligosacchrides (MOS) are prebiotics that have been used as feed additives in channel catfish [163], rainbow trout [164], Japanese flounder (*Paralichthys olivaceus*) [165], and common carp [166] feed studies. MOS is derived from the cell wall of *Saccharomyces cerevisiae*, and Alltech Inc. has licensed an official MOS product for inclusion in animal diets [161]. Two studies have observed the expression levels of AMPs in fish during MOS supplementation. Increased transcript levels of the AMP dicentracin were observed in the head kidney of European seabass upon the inclusion of three percent dietary MOS [161]. MOS diets were also shown to increase expression levels of the AMP Piscidin in the gills, head kidney, spleen and intestines of the greater amberjack [162]. Therefore, the modulation of fish diets with immunosimulants, such as MOS, could be used to increase the endogenous expression of AMPs in fish to better enable them to combat bacterial and other pathogenic infections.

This review has focused on bacterial biofilms and AMPs in context to each other and aquaculture. Bacterial biofilms are prevalent worldwide in a variety of environments and exist to allow bacteria to survive adverse conditions. The Gram-negative, fish pathogens *A. hydrophila* and *F. columnare* both readily form biofilms within the aquaculture industry, causing persistent infections that are becoming harder to treat with conventional antibiotics. However, AMPs have showed promise as being new antimicrobial agents that are effective in combating bacterial biofilms. Therefore, AMPs may hold some value in the fight against bacterial biofilms present in aquaculture.

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Chapter Two: Exploration of the Anti-biofilm Effects of Fish Antimicrobial Peptides Against Common Aquaculture Pathogens

ABSTRACT

Disease is a constraint to the aquaculture industry and certain bacterial pathogens can exist in a biofilm state that improves their resistance to antibiotics. Fish-derived antimicrobial peptides (AMPs)have demonstrated potent antimicrobial properties. Therefore, this work examined several of these AMPs for their ability to inhibit growth and biofilm formation of several bacterial pathogens. A standard spectrophotometric microtiter dish assay was used to measure total *in vitro* growth and biofilm formation of several Gram-negative bacterial fish pathogens incubated in the presence of 14 AMPs at varying concentrations. Results from these 14 AMPs were compared to an untreated control as well as the AMP melittin. The NK-lysin AMPs inhibited growth of *Escherichia coli* and *Aeromonas veronii* and were particularly effective against biofilm formation of these two pathogens. These NK-lysin AMPs tended to show an expected dose response in that higher concentrations showed greater inhibition for *E. coli* and *A. veronii*. The piscidin AMPs inhibited *E. coli* and *Flavobacterium columnare* total growth and showed effectiveness against their respective biofilm formation. Unlike the NK-lysin AMPs, the piscidin AMPs inhibited biofilm formation of *E. coli* and *F. columnare* at the lowest tested concentration. The positive control melittin demonstrated significant total and biofilm inhibition towards all four bacteria; some of the AMPs in this test showed results comparable to melittin. Results from this study suggest that several of these peptides might serve as effective agents against bacteria in aquaculture settings due to their ability to inhibit both free-living and biofilm encased bacteria and are worthy of further study. Future studies should examine mechanisms of action in regard to biofilm inhibition while also observing activity under more natural conditions.

1. Introduction

Due to growing demand for fish-related products and deficiencies in wild-caught fish populations, aquaculture is currently one of the fastest growing sectors of agriculture globally [1, 2]. However, the rapid intensification and commercialization of aquaculture has led to conditions conducive to disease outbreak among fish, making disease one of the primary economic constraints in aquaculture production [3, 4]. For example, disease outbreaks caused by bacteria, such as *Aeromonas hydrophila* and *Flavobacterium columnare*, which are the causative agents of motile *Aeromonas* septicemia (MAS) and columnaris disease, respectively, cause the U.S. catfish industry losses that can exceed 150 million USD annually [5, 6]. Both fish pathogens are prevalent in freshwater aquatic environments, where they can persist without a fish host [7, 8] and can often exist as biofilms [9, 10].

Biofilms are sessile, surface-attached microbial communities surrounded by a self-produced matrix of extracellular polymeric substances (EPS) hat can readily form on both abiotic and biotic surfaces under a wide range of environmental conditions [11, 12]. Compared to planktonic or free-living lifestyle, biofilms offer many benefits to bacteria including increased efficiency in nutrient acquisition and improved resistance to antibiotic agents. The wide range of environmental conditions tolerated by biofilms allows them to become problematic in several different industries, including aquaculture, where they are recognized as natural reservoirs for bacteria [13].

Aquaculture systems provide an ideal habitat for the formation of biofilms from fish pathogens, such as *A. hydrophila* and *F. columnare*, due to the proximity of fish and numerous surfaces for attachment [14]. In aquaculture systems, these Gram-negative bacteria have been found to readily form biofilms on surfaces ranging from the mucosal surface of fish [15] to the

lining of in-pond raceways [10]. Recent work has shown that the *in vitro* presence of fish mucus enhances *F. columnare* biofilm growth and upregulates genes associated with binding, which demonstrates that the fish mucosal surface supports biofilm growth [16, 17]. Since biofilms readily form on fish and other surfaces, they allow fish pathogens to persist within aquaculture facilities and potentially cause continuous infection outbreaks. Not only are *A. hydrophila* and *F. columnare* capable of forming biofilms but some identified isolates also have developed widespread antibiotic-resistance [18-20]. Therefore, due to the prevalence of antibiotic resistance displayed in *A. hydrophila* and *F. columnare* and their abilities to form biofilms in the aquaculture industry, the exploration of new anti-biofilm agents is needed. Antimicrobial peptides (AMPs) produced by the innate immune systems of fish and other organisms have promising potential as anti-biofilm agents due to their demonstrated broad-spectrum killing ability [21].

AMPs, innate immune system effector molecules, are recognized for their broad-spectrum potency against a wide range of pathogens. These peptides have accompanied many multi-cellular organisms through evolution, and their effectiveness against pathogens has remained intact. Over 90 AMPs have been identified in teleost fish and are classified based on structure, which include the natural killer (NK)-lysins identified in rainbow trout (*Oncorhynchus mykiss*) and Moronid Piscidins [22].

The NK-lysins typically range from 74 to 78 amino acid residues in size and are known to display activity against bacteria, fungi, viruses, parasites, and tumor cells [23, 24]. They fall under the wider family of saposin-like proteins (SAPLIP), which also includes human granulysins, due to similar amino acid homology [25, 26]. These types of lysins have been identified in several fish species including channel catfish (*Ictalurus punctatus*) [27], Japanese

flounder (*Paralichthys olivaceus*) [28], zebrafish (*Danio rerio*) [29], Nile tilapia (*Oreochromis niloticus*) [30], common carp (*Cyprinus carpio*) [31], Atlantic salmon (*Salmo salar*) [32], and yellow catfish (*Pelteobagrus fulvidraco*) [33]. Fish-derived NKlysins have demonstrated *in vitro* antibacterial activity against several Gram-negative bacteria including *Escherichia coli* [31, 34], *Vibrio harveyi* [34, 35], *A. hydrophila* [31], and *Edwardsiella tarda* [31, 35].

The Piscidins range from 18-46 amino acid residues in length and are only found in teleost fish. They were originally identified from striped bass (*Morone saxatilis*), white bass (*M. chrysops*), and hybrid striped bass (*M. chrysops, x M. saxatilis*) and have since been found in a wide range of teleost fish taxa, including the families Moronidae, Sciaenidae, Siganidae, Belontidae, Cichlidae, Percichthyidae, Latidae, Sparidae, Sygnathidae, and Latridae [36, 37]. Piscidins show broad-spectrum activity against bacteria, viruses, fungi and parasites [38- 40] and have displayed antibacterial activity *in vitro* towards Gram-negative and positive bacteria such as *Photobacterium damsela* [41]*, A. hydrophila* [42, 43], and *Streptococcus iniae* [37]. While the antibacterial activities of NK lysins and Piscidins have been demonstrated, their activity towards bacterial biofilms has not been well documented; however, their antibacterial activity suggests that they may have similar anti-biofilm effects.

The purpose of this study was to assess the *in vitro* antibacterial and anti-biofilm activities of synthetic AMPs against different Gram-negative bacterial fish pathogens, *A. veronii, A. hydrophila* and *F. columnare*. A total of 14 AMPs were used in this study and primarily consisted of NK-lysins and Piscidins derived from fish. To determine each AMP's activity against biofilms, these bacteria were grown in the presence of varying concentrations of AMPs, and their subsequent total and biofilm growth were quantified. Exploring the anti-biofilm capabilities of AMPs is not only useful for identifying novel anti-biofilm agents but also

provides new insights in how these multi-faceted peptides contribute to an effective innate immune system response in fish and other organisms.

2. Materials and methods

2.1 Antimicrobial peptides

The antimicrobial peptides used to conduct these studies were supplied by Dr. Brian Shepherd (USDA-ARS) and Dr. Scott Salger (Barton College). Rainbow trout (*Oncorhynchus mykiss*) antimicrobial peptides included: natural killer lysins (NK-lysin 1, NK-lysin 2, NK-lysin 3, NK-lysin 4); natural killer-lysin like (NKL-like-a, NKL-like-b), and Onchorhyncin II (OII). Synthesis of all NK-lysins were done via LifeTein Corp.; all peptides were amidated and acylated. Moronid antimicrobial peptides included: Striped bass (*Morone saxatilis*) piscidin one (SB P1), white bass (*Morone chrysops*) piscidin one (WB P1), hybrid striped bass (*Morone saxatilis x Morone chrysops*) piscidin three (SBWB P3), striped bass (*Morone saxatilis*) piscidin four (SB P4), and white bass (*Morone chrysops*) piscidin five (WB P5). Piscidin peptides were synthesized at Yale University School of Medicine Small Scale Peptide Synthesis according to previously described methods [37]. Olive flounder (*Paralichthys olivaceus*) NK (OFNK) and porcine natural killer lysin (PNK), were also used. Synthetic melittin was purchased at ≥97% (HPLC) purity from Sigma-Aldrich (Cat No. M4171, St. Louis, MO). The air-dried peptides were reconstituted in either 1x PBS (rainbow trout peptides, OFNK, PNK and melittin) or DI water (bass peptides) and stored at -20°C until needed.

2.2 Bacterial strains and culture conditions

The bacteria used in this study were an isolate of *Aeromonas veronii* identified from an outbreak among largemouth bass (*Micropterus salmoides*) (Stuttgart, AR), virulent *Aeromonas hydrophila* ALG-15-097 [44], *Flavobacterium columnare* LV-359-01 [17, 45], and *Escherichia*

coli DH5α. All bacterial strains were cultured from glycerol stocks stored at -80°C. *E. coli*, *A. veronii* and, *A. hydrophila* were streaked onto tryptic soy agar (TSA, Difco™ Tryptic Soy Agar) plates and incubated at 28°C for 24 h. However, *F. columnare* was streaked onto modified Shieh [46] agar plates and was incubated at 28°C for 48 h. After incubation, a single colony from each bacterium was selected and used to inoculate 10 mL of broth media, and these were referred to as starter cultures. The broth used for the culture of *E. coli*, *A. veronii* and *A. hydrophila* was cation-adjusted Mueller Hinton Broth (MHB, BBL™ Mueller Hinton II Broth), while modified Shieh broth was used for *F. columnare.* The starter cultures of each bacterium were cultured overnight at 28°C and shaken at a speed of 150 rpm.

2.3 Total bacterial growth and biofilm assays

Quantification of *E. coli*, *A. veronii*, *A. hydrophlia* and *F. columnare* biofilm growth was conducted using previously described methods [47, 48], but with modifications. Starter broth cultures of each bacterium were diluted 1:10 into fresh media prior to inoculation into sterile 96-well clear round-bottom polypropylene non-treated microtiter plates (Corning®, Corning, NY). Due to the cationic nature typically demonstrated by AMPs, polypropylene was used to minimize the interaction of the peptides with the plate material [49]. Each designated replicate well used in the 96-well plate received 150 µL of the 1:10 bacterium dilutions. Every 96-well plate had five replicate wells as untreated controls. Another five replicate wells of each 96-well plate received melittin at a concentration of 117 µM per well; these were the positive controls. The AMP treatments consisted of four concentrations (10, 25, 50 and 100 µM). Each AMP concentration treatment consisted of five replicate wells; each well directly received its designated AMP concentration via pipetting. Empty wells were filled with 150 µL of DI water. The 96-well plates were wrapped in Parafilm™ M film (Bemis Company, Oshkosh, WI) to prevent evaporative loss

and incubated at 28°C for 24 h. After incubation, optic density (OD) at 550 nm was measured using an Epoch™ 2 Microplate Spectrophotometer (BioTek, Winooski, VT) to quantify total bacterial growth. The supernatant was gently removed with a pipette from each well. Using a pipette, each well was washed once with 300 µL of DI water to remove any residual planktonic cells. The biofilm was then stained with 185 µL of a 0.1% solution of crystal violet (CV) in DI water prior to incubation at room temperature for 15 m. Immediately afterwards, the dye was gently removed from each well with a pipette, and the wells were then washed three times with 300 µL of DI water. Plates were then blotted vigorously on a stack of paper towels to remove excess cells and dye, flipped over, and allowed to air dry at room temperature overnight. After drying, the CV dye bound to biofilm cells was solubilized with 200 µL of 30% glacial acetic acid per well. Plates were then incubated at room temperature for 15 m. After incubation, the solubilized biofilm biomass was transferred to new flat-bottom polystyrene 96-well plates, and their OD was measured at 550 nm for quantification.

2.4 *Statistical analysis*

Data was submitted to analysis of variance (ANOVA), and Tukey's Honest Significant Differences (HSD) post-hoc test was conducted using R software version 3.1.3 (https://www.R-project.org/). A *p*-value of less than 0.05 was considered statistically significant.

3. Results

Synthetic fish NK-lysins and Piscidins were assessed for their *in vitro* activity towards the total and biofilm growth of the following Gram-negative bacteria: the *E. coli* DH5α strain, an *A. veronii* isolate, the the virulent *A. hydrophila* ALG-15-097 strain, and the *F. columnare* LV-359-01strain. The *Aeromonas* spps and *F. columnare* used in this study have been isolated from MAS and columnaris outbreaks, respectively. These represent some pathogenic bacterial species

that fish commonly come into contact within aquaculture settings. Each bacterial strain was cultured in the presence of different concentrations of synthetic fish NK-lysins and Piscidins. After 24 hours, the optical density of each replicate well was measured to assess the total growth, followed by the quantification of biofilm formation.

Throughout the results section, peptide responses are compared to controls run on the same 96-well plate. Designated control values appear on the left in each table. A negative control lacking (no AMP treatment) and a positive control (melittin) were used for each experimental plate. Melittin was selected due to its broad anti-bacterial activity against Gram-negative bacteria [50-52].

3.1 Activity of NK-Lysins and Piscidins against E. coli DH5α

To assess the overall activity of different fish AMPs against the growth of different Gram-negative bacteria, we first evaluated their effect against the laboratory strain of *E. coli* DH5α. Optical density spectrophotometry measured the relative absorbance of total growth and demonstrated no significant reduction in DH5 alpha *E. coli* total growth at the 10 μM concentration (Table 1) among the NK-lysins. However, both NKL-like-a and OII showed slight increases. The peptides NKL-like-a, OII, OFNK, and PNK also showed significantly reduced biofilm growth at the 10 μM concentration.

The peptides NK-lysin 3, NKL-like-a, OFNK and PNK all showed significantly reduced bacterial growth at 25 μM, while OII continued to show growth at this concentration. The peptides NK-lysin 1 through 4 showed significantly reduced total growth at the 50 μM concentration.

Table 1. The effect of rainbow trout (*Oncorhynchus mykiss*) antimicrobial peptides (AMPs) on the total and biofilm growth of the *Escherichia coli* DH5α strain.

Bacterial Growth[a]	AMPs[b]					
	Control	Melittin 117 µM	NK-1 10 µM	NK-2 10 µM	NK-3 10 µM	NK-4 10 µM
Total	1.48±0.10	**0.31±0.01**[c]	1.47±0.01	1.47±0.01	1.46±0.02	1.48±0.01
Biofilm	0.14±0.04	**0.06±0.004**	0.12±0.02	0.13±0.02	0.14±0.04	0.12±0.03
			NK-1 25 µM	NK-2 25 µM	NK-3 25 µM	NK-4 25 µM
			1.48±0.01	1.49±0.01	**1.42±0.03**	1.42±0.09
			0.10±0.02	**0.09±0.01**	0.12±0.02	0.12±0.02
			NK-1 50 µM	NK-2 50 µM	NK-3 50 µM	NK-4 50 µM
Total	1.48±0.01	**0.30±0.01**	**1.41±0.01**	**1.34±0.01**	**1.27±0.07**	**1.31±0.02**
Biofilm	1.23±0.29	**0.08±0.004**	**0.64±0.14**	**0.18±0.03**	**0.22±0.02**	**0.21±0.06**
			NKL-A 10 µM	NKL-B 10 µM		
Total	1.44±0.01	**0.32±0.01**	*1.47±0.01*	1.44±0.02		
Biofilm	1.23±0.29	**0.09±0.01**	**0.34±0.04**	0.77±0.16		
			NKL-A 25 µM	NKL-B 25 µM		
			1.39±0.01	1.43±0.02		
			0.37±0.05	1.30±1.38		
			OII 10 µM	OFNK 10 µM	PNK 10 µM	
Total	1.51±0.01	**0.29±0.01**	*1.55±0.01*	1.50±0.01	1.46±0.06	
Biofilm	0.83±0.32	**0.11±0.04**	**0.36±0.10**	**0.19±0.05**	**0.15±0.02**	
			OII 25 µM	OFNK 25 µM	PNK 25 µM	
			1.56±0.02	**1.46±0.01**	**1.42±0.07**	
			0.43±0.08	**0.18±0.03**	**0.12±0.01**	

[a] Muller Hinton broth was used alone or supplemented with different AMPs at 37°C under static conditions for 24 hr. Absorbances were measured at OD_{550} (biofilms after crystal violet staining). Data are represented as mean absorbance ± SD for each treatment group.
[b] The AMPs NK= natural killer lysin, NKL= natural killer-like lysin, OII= Onchorhyncin II, OFNK= olive flounder (*Paralichthys olivaceus*) natural killer lysin and PNK= porcine natural killer lysin.
[c] Bolded indicate significantly lower values and italicized indicate significantly higher values ($p<0.05$) when compared to the media-only control according to a generalized linear model of a Tukey's HSD post hoc test.

These four peptides, along with NK-lysin 1and 2, reduced biofilm growth at 25 µM. The peptides NK-lysin 1 through 4 showed reduced biofilm growth at the 50 µM concentration.

The evaluation of Piscidins showed that SB P4 and WB P5 reduced DH5 alpha *E. coli* total growth at the 10 µM concentration, while SB P1 and SBWB P3 showed no response (Table 2). All of the above peptides showed significantly reduced total growth at 25 µM, while peptide WB P1 actually increased total growth at both 10 and 25 µM. All the Pisidins reduced biofilm growth at 10 and 25 µM concentrations.

Table 2. The effect of striped bass (*Morone saxatilis*), white bass (*Morone chrysops*) and hybrid striped bass (*Morone saxatilis* x *Morone chrysops*) antimicrobial peptides (AMPs) on the total and biofilm growth of the *Escherichia coli* DH5α strain.

Bacterial Growth[a]	AMPs[b]				
	Control	Melittin 117 µM	SB P1 10 µM	WB P1 10 µM	SBWB P3 10 µM
Total	1.39±0.001	**0.31±0.02**[c]	1.17±0.53	*1.51±0.01*	1.37±0.07
Biofilm	0.47±0.04	**0.08±0.004**	**0.11±0.04**	**0.13±0.01**	**0.13±0.01**
			SB P1 25 µM	WB P1 25 µM	SBWB P3 25 µM
			0.52±0.36	*1.44±0.01*	**0.19±0.02**
			0.09±0.01	**0.12±0.01**	**0.10±0.01**
			SB P4 10 µM	WB P5 10 µM	
Total	1.52±0.02	**0.30±0.01**	**1.26±0.11**	**1.16±0.15**	
Biofilm	1.01±0.24	**0.09±0.01**	**0.10±0.02**	**0.17±0.01**	
			SB P4 25 µM	WB P5 25 µM	
			0.52±0.03	**0.35±0.01**	
			0.09±0.01	**0.19±0.02**	

[a] Muller Hinton broth was used alone or supplemented with different AMPs at 37°C under static conditions for 24 hr. Absorbances were measured at OD_{550} (biofilms after crystal violet staining). Data are represented as mean absorbance ± SD for each treatment group.
[b] The AMPs SB= striped bass piscidin, WB= white bass piscidin, SB/WB= hybrid striped bass [37].
[c] Bolded indicate significantly lower values and italicized indicate significantly higher values ($p<0.05$) when compared to the media-only control according to a generalized linear model of a Tukey's HSD post hoc test.

3.2 Activity of NK-Lysins and Piscidins against A. veronii

At the 10 µM concentration, the only rainbow trout peptide to show reduced total growth of *A. veronii* was NK-lysin 3 (Table 3). The peptides NK-lysin 2, NK-lysin 3, NK-lysin 4 and PNK showed reduced total growth at 25 µM. At 50 µM, NK-lysin 3 showed a significant reduction in total growth while NK-lysins 1, 2 and 4 did not. The peptide NKL-like-b showed

Table 3. The effect of rainbow trout (*Oncorhynchus mykiss*) antimicrobial peptides (AMPs) on the total and biofilm growth of an *Aeromonas veronii* isolate.

Bacterial Growth[a]	AMPs[b]					
	Control	Melittin 117 µM	NK-1 10 µM	NK-2 10 µM	NK-3 10 µM	NK-4 10 µM
Total	1.80±0.002	**0.29±0.01**[c]	1.80±0.004	1.78±0.01	**1.75±0.02**	1.79±0.01
Biofilm	1.31±0.27	**0.08±0.01**	**0.91±0.25**	**0.95±0.20**	**0.82±0.03**	**0.95±0.15**
			NK-1 25 µM	NK-2 25 µM	NK-3 25 µM	NK-4 25 µM
			1.79±0.005	**1.73±0.02**	**1.69±0.05**	**1.74±0.02**
			1.07±0.06	**0.93±0.14**	**0.98±0.14**	1.23±0.24
			NK-1 50 µM	NK-2 50 µM	NK-3 50 µM	NK-4 50 µM
Total	1.50±0.01	**0.28±0.01**	1.53±0.02	1.46±0.03	**1.27±0.07**	1.52±0.01
Biofilm	1.42±0.22	**0.08±0.01**	**1.06±0.10**	**0.93±0.10**	**1.22±0.20**	1.27±0.15
			NKLA 10 µM	NKLB 10 µM		
Total	1.69±0.01	**0.29±0.03**	1.68±0.02	1.67±0.02		
Biofilm	1.37±0.11	**0.08±0.01**	**1.11±0.11**	**0.88±0.01**		
			NKLA 25 µM	NKLB 25 µM		
			1.69±0.02	1.68±0.03		
			1.43±0.10	**1.07±0.14**		
			NKLA 50 µM	NKLB 50 µM		
			1.74±0.01	1.69±0.03		
			1.05±0.22	**1.08±0.11**		
			NKLA 100 µM	NKLB 100 µM		
			1.74±0.01	1.71±0.03		
			1.42±0.14	1.38±0.10		
			OII 10 µM	OFNK 10 µM	PNK 10 µM	
Total	1.72±0.01	**0.28±0.02**	1.72±0.014	1.72±0.02	1.69±0.02	
Biofilm	1.26±0.30	**0.08±0.01**	**0.82±0.17**	**0.86±0.14**	0.98±0.16	
			OII 25 µM	OFNK 25 µM	PNK 25 µM	
			1.72±0.01	1.73±0.02	**1.68±0.02**	
			1.25±0.22	**0.82±0.06**	0.95±0.06	

[a] Muller Hinton broth was used alone or supplemented with different AMPs at 28°C under static conditions for 24 hr. Absorbances were measured at OD_{550} (biofilms after crystal violet staining). Data are represented as mean absorbance ± SD for each treatment group.

[b] The AMPs NK= natural killer lysin, NKL= natural killer-like lysin, OII= Onchorhyncin II, OFNK= olive flounder natural killer lysin and PNK= porcine natural killer lysin.

[c] Bolded indicate significantly lower values and italicized indicate significantly higher values ($p<0.05$) when compared to the media-only control according to a generalized linear model of a Tukey's HSD post hoc test.

no effect on total growth, while NKL-like-a showed increased growth at 50 and 100 μM.

The biofilm growth of *A. veronii* showed highly variable responses with the rainbow trout peptides (Table 3). For example, NK-lysins 1-4 reduced biofilm growth at 10 μM, but only NK-lysin 2 and 3 showed reduced growth at 25 μM. At 50 μM, NK-lysins 1, 2 and 3 significantly reduced biofilm growth. Further, NKL-like-a showed reduced biofilm growth at 10 and 50 μM but had no effect at 25 or 100 μM. The peptide NKL-like-b reduced biofilm growth at 10, 25, and 50 μM, but not at 100 μM. The olive flounder NK-lysin reduced biofilm growth at 10 and 25 μM, while OII only showed biofilm reduction at 10 μM and PNK did not show reduction at either concentration.

The observed activity of the bass peptides showed that WB P1 and WB P5 reduced *A. veronii* total growth at the 10 μM concentration, while SB P1, SBWB P3 and SB P4 showed no response (Table 4). While the values of WB P1 and WB P5 at 10 μM are statistically significant, the WB P1 effect is numerically small. At the 25 μM concentration, SB P1, WB P1, SB P4 and WB P5 all demonstrated a statistically significant reduction in total growth, while SBWB P3 did not. However, while the total growth values of SB P1 and WB P1 at 25 μM are statistically significant, they show little numerical significance. At 50 and 100 μM concentrations, the three peptides SB P1, WB P1, and SBWB P3 did not show any significant activity towards total growth (Table 4).

Of the bass peptides at the 10 μM concentration, only SB P4 showed a significant reduction in *A. veronii* biofilm growth (Table 4). In contrast, SB P4 at the 25 μM concentration demonstrated a significant increase in biofilm growth, while the other hybrid bass peptides showed no significant activity. At concentrations of 50 and 100 μM, both SB P1 and WB P1

Table 4. The effect of striped bass (*Morone saxatilis*), white bass (*Morone chrysops*) and hybrid striped bass (*Morone saxatilis* x *Morone chrysops*) antimicrobial peptides (AMPs) on the total and biofilm growth of an *Aeromonas veronii* isolate.

Bacterial Growth[a]	AMPs[b]				
	Control	Melittin 117 µM	SB P1 10 µM	WB P1 10 µM	SBWB P3 10 µM
Total	1.82±0.01	**0.27±0.02**[c]	1.80±0.01	**1.79±0.01**	1.80±0.01
Biofilm	0.79±0.12	**0.08±0.01**	0.71±0.07	0.79±0.18	0.90±0.12
			SB P1 25 µM	WB P1 25 µM	SBWB P3 25 µM
			1.80±0.01	**1.80±0.01**	1.81±0.01
			0.77±0.13	0.91±0.14	0.90±0.07
			SB P1 50 µM	WB P1 50 µM	SBWB P3 50 µM
Total	1.76±0.01	**0.28±0.02**	1.80±0.01	1.81±0.01	1.75±0.04
Biofilm	1.19±0.08	**0.08±0.01**	**0.79±0.06**	**0.81±0.16**	1.17±0.28
			SB P1 100 µM	WB P1 100 µM	SBWB P3 100 µM
			1.75±0.07	1.72±0.09	1.31±0.65
			0.79±0.11	**0.93±0.14**	1.34±0.67
			SB P4 10 µM	WB P5 10 µM	
Total	1.77±0.01	**0.29±0.01**	1.76±0.02	**1.72±0.02**	
Biofilm	1.15±0.08	**0.08±0.005**	**0.91±0.17**	1.12±0.31	
			SB P4 25 µM	WB P5 25 µM	
			1.73±0.02	**1.69±0.01**	
			1.89±0.17	1.24±0.14	

[a] Muller Hinton broth was used alone or supplemented with different AMPs at 28°C under static conditions for 24 hr. Absorbances were measured at OD_{550} (biofilms after crystal violet staining). Data are represented as mean absorbance ± SD for each treatment group.
[b] The AMPs SB P= striped bass piscidin, WB P= white bass piscidin, SBWB P= striped bass white bass piscidin [37].
[c] Bolded indicate significantly lower values and italicized indicate significantly higher values ($p<0.05$) when compared to the media-only control according to a generalized linear model of a Tukey's HSD post hoc test.

showed significant reductions in *A. veronii* biofilm growth, while the other hybrid bass peptides showed no significant activity (Table 4).

3.3 Activity of NK-Lysins and Piscidins against A. hydrophila

None of the rainbow trout, nor OFNK and PNK peptides, showed any significant reduction in ALG total growth at the 10 µM concentration (Table 5); however, NKL-like-b showed a significant increase in total growth at 10 µM. Similarly, NKL-like-b showed a significant increase in total growth at the 25 µM concentration, while the other peptides showed

Table 5. The effect of rainbow trout (*Oncorhynchus mykiss*) antimicrobial peptides (AMPs) on the total and biofilm growth of the *Aeromonas hydrophila* ALG-15-097.

Bacterial Growth[a]	AMPs[b]					
	Control	Melittin 117 µM	NK-1 10 µM	NK-2 10 µM	NK-3 10 µM	NK-4 10 µM
Total	1.75±0.01	**0.32±0.04**[c]	1.75±0.01	1.72±0.01	1.74±0.01	1.75±0.01
Biofilm	0.96±0.16	**0.12±0.08**	0.69±0.32	0.81±0.27	0.81±0.30	0.74±0.29
			NK-1 25 µM	NK-2 25 µM	NK-3 25 µM	NK-4 25 µM
			1.76±0.01	1.72±0.01	1.74±0.01	1.76±0.01
			0.90±0.33	0.97±0.30	0.85±0.28	0.78±0.27
			NK-1 50 µM	NK-2 50 µM	NK-3 50 µM	NK-4 50 µM
Total	1.78±0.01	**0.32±0.04**	1.77±0.01	**1.74±0.01**	1.75±0.02	1.77±0.02
Biofilm	0.90±0.27	**0.12±0.05**	0.95±0.31	0.90±0.30	0.91±0.30	1.14±0.13
			NKLA 10 µM	NKLB 10 µM		
Total	1.73±0.01	**0.59±0.01**	1.76±0.01	*1.78±0.01*		
Biofilm	1.33±0.11	**0.36±0.46**	**0.93±0.18**	1.06±0.38		
			NKLA 25 µM	NKLB 25 µM		
			1.77±0.02	*1.79±0.01*		
			1.21±0.10	1.13±0.24		
			NKLA 50 µM	NKLB 50 µM		
			1.71±0.01	1.74±0.01		
			1.15±0.23	1.05±0.12		
			NKLA 100 µM	NKLB 100 µM		
			1.70±0.03	1.74±0.01		
			1.46±0.12	1.30±0.14		
			OII 10 µM	OFNK 10 µM	PNK 10 µM	
Total	1.79±0.01	**0.41±0.03**	1.80±0.01	1.80±0.01	1.80±0.01	
Biofilm	1.32±0.23	**0.08±0.02**	1.19±0.27	**0.91±0.22**	**0.72±0.15**	
			OII 25 µM	OFNK 25 µM	PNK 25 µM	
			1.80±0.01	1.79±0.01	1.78±0.01	
			1.26±0.07	**0.74±0.08**	**0.77±0.04**	
			OII 50 µM	OFNK 50 µM	PNK 50 µM	
Total	1.73±0.01	**0.43±0.02**	1.73±0.01	**1.70±0.01**	**1.64±0.01**	
Biofilm	1.33±0.11	**0.08±0.02**	*1.56±0.18*	**0.91±0.31**	1.23±0.27	
			OII 100 µM	OFNK 100 µM	PNK 100 µM	
			1.75±0.01	**1.70±0.02**	**1.49±0.09**	
			1.62±0.10	1.19±0.14	1.53±0.22	

[a] Muller Hinton broth was used alone or supplemented with different AMPs at 28°C under static conditions for 24 hr. Absorbances were measured at OD_{550} (biofilms after crystal violet staining). Data are represented as mean absorbance ± SD for each treatment group.

[b] The AMPs NK= natural killer lysin, NKL= natural killer-like lysin, OII= Onchorhyncin II, OFNK= olive flounder natural killer lysin and PNK= porcine natural killer lysin.

[c] Bolded indicate significantly lower values and italicized indicate significantly higher values ($p<0.05$) when compared to the media-only control according to a generalized linear model of a Tukey's HSD post hoc test.

no significant response towards total growth. The NK-lysin 2 rainbow trout peptide, along with OFNK and PNK, showed significant reductions in total growth at the 50 μM concentration. Both OFNK and PNK, along with OII, significantly reduced ALG total growth at the 100 μM concentration (Table 5).

The OFNK, PNK and NKL-like-b peptides demonstrated reduced ALG biofilm growth at the 10 μM concentration, while the remaining rainbow trout peptides show no significant activity at 10 μM (Table 5). Only the non-rainbow trout peptides, OFNK and PNK, showed significantly reduced biofilm growth at the 25 μM concentration. At the 50 μM concentration, only OFNK continued to show significant biofilm reduction. In contrast, OII showed a significant increase in biofilm growth at 50 μM. The rainbow trout peptides, along with OFNK and PNK, showed no significant reduction in biofilm growth at the concentration of 100 μM; OII continued to significantly increase biofilm growth at this concentration (Table 5).

Examination of the bass peptides (Table 6) showed that only WB P1 significantly reduced ALG total growth at the 10 μM concentration. The WB P1 peptide continued to significantly reduce total growth at the 25 μM concentration, as did WB P5 (Table 6). At the 50 μM concentration, only SB P4 and WB P5 reduced total growth significantly. Both SB P1 and SBWB P3 showed significant reduction in total growth at the 100 μM concentration. The only significant activity demonstrated towards ALG biofilm growth by the hybrid striped bass peptides was by SB P4 and WB P5, both of which decreased biofilm growth the at 10 μM concentration (Table 6).

3.4 Activity of NK-Lysins and Piscidins against F. columnare

The rainbow trout, OFNK, and PNK peptides showed no significant reduction of LV total growth at the 10 μM concentration (Table 7). However, NK-lysin 2 demonstrated an increase in

Table 6. The effect of striped bass (*Morone saxatilis*), white bass (*Morone chrysops*) and hybrid striped bass (*Morone saxatilis* x *Morone chrysops*) antimicrobial peptides (AMPs) on the total and biofilm growth of the *Aeromonas hydrophila* ALG-15-097.

Bacterial Growth[a]	AMPs[b]				
	Control	Melittin 117 µM	SB P1 10 µM	WB P1 10 µM	SBWB P3 10 µM
Total	1.77±0.01	**0.59±0.01**[c]	1.75±0.01	**1.75±0.01**	1.76±0.01
Biofilm	1.11±0.24	**0.08±0.02**	0.96±0.21	0.98±0.21	1.00±0.15
			SB P1 25 µM	WB P1 25 µM	SBWB P3 25 µM
			1.75±0.02	**1.75±0.01**	1.75±0.02
			0.89±0.22	0.81±0.16	1.10±0.11
			SB P1 50 µM	WB P1 50 µM	SBWB P3 50 µM
Total	1.77±0.01	**0.32±0.03**	1.78±0.01	1.79±0.01	1.77±0.05
Biofilm	1.67±0.36	**0.93±0.01**	1.16±0.23	1.26±0.27	1.47±0.44
			SB P1 100 µM	WB P1 100 µM	SBWB P3 100 µM
			1.70±0.04	1.67±0.10	**1.57±0.06**
			1.75±0.37	1.73±0.38	1.19±0.15
			SB P4 10 µM	WB P5 10 µM	
Total	1.76±0.01	**0.43±0.03**	1.75±0.01	1.76±0.01	
Biofilm	1.50±0.08	**0.10±0.01**	**1.07±0.13**	**1.10±0.20**	
			SB P4 25 µM	WB P5 25 µM	
			1.75±0.02	**1.72±0.01**	
			1.62±0.07	1.71±0.07	
			SB P4 50 µM	WB P5 50 µM	
			1.68±0.01	**1.72±0.01**	
			1.71±0.42	1.40±0.31	

[a] Muller Hinton broth was used alone or supplemented with different AMPs at 28°C under static conditions for 24 hr. Absorbances were measured at OD_{550} (biofilms after crystal violet staining). Data are represented as mean absorbance ± SD for each treatment group.
[b] The AMPs SB P= striped bass piscidin, WB P= white bass piscidin, SBWB P= striped bass white bass piscidin [37].
[c] Bolded indicate significantly lower values and italicized indicate significantly higher values ($p<0.05$) when compared to the media-only control according to a generalized linear model of a Tukey's HSD post hoc test.

total growth at 10 µM. Similarly, NK-lysin 2 and NK-lysin 3 both showed an increase in total growth at the 25 µM concentration. None of the rainbow trout, OFNK, or PNK peptides demonstrated significant total growth reduction at 25 µM. At the 50 and 100 µM concentrations,

only NK-lysin 3 and NK-lysin 4 demonstrated significant activity towards total growth; both resulted in a reduction in planktonic growth at those concentrations (Table 7).

The rainbow trout peptides NK-lysin 2, NK-lysin 3 and NK-lysin 4, along with the OFNK and PNK peptides, showed significant reduction of LV biofilm growth at the 10 μM concentration (Table 7). At the 25 μM concentration, only NK-lysin 4 demonstrated significantly reduced biofilm growth, while NKL-like-a showed a significant increase in biofilm growth at 25 μM. Both NK-lysin 3 and NK-lysin 4, along with PNK, showed a significant reduction in biofilm growth at the 50 μM concentration. The peptide NK-lysin 2 was observed to have a significant increase in biofilm growth at 50 μM. Similarly, at the 100 μM concentration, both NK-lysin 2 and OFNK significantly increased biofilm growth, while NK-lysin 3 and NK-lysin 4 showed a reduction (Table 7).

The bass peptides SBWB P3, SB P4, and WB P5 significantly increased LV total growth at the 10 μM concentration (Table 8). None of the bass Piscidins reduced total growth significantly at 10 μM. At the 25 μM concentration, WB P1 showed a significant reduction in total growth, while SBWB P3, SB P4, and WB P5 increased total growth significantly. The peptides SB P1, WB P1, and SBWB P3 significantly reduced total growth at the 50 μM concentration. A similar effect was observed at the 100 μM concentration, where SB P1 and SBWB P3 both reduced total growth. The Piscidins all showed a pronounced reduction of LV biofilm growth at the 10 μM concentration (Table 8). This trend was continued at the 25 μM concentration, where SB P1, SBWB P3, SB P4, and WB P5 reduced biofilm growth significantly. Both SB P1 and WB P1 showed significant biofilm reduction at the 50 μM concentration. At the 100 μM concentration, SB P1, WB P1, and SBWB P3 demonstrated significant reductions in LV biofilm growth (Table 8).

Table 7. The effect of rainbow trout (*Oncorhynchus mykiss*) antimicrobial peptides (AMPs) on the total and biofilm growth of the *Flavobacterium columnare* LV-359-01.

Bacterial Growth[a]	AMPs[b]					
	Control	Melittin 117 µM	NK-1 10 µM	NK-2 10 µM	NK-3 10 µM	NK-4 10 µM
Total	0.55±0.01	**0.42±0.06**[c]	0.56±0.01	*0.64±0.02*	0.62±0.02	0.55±0.05
Biofilm	0.98±0.07	**0.08±0.01**	0.84±0.13	**0.73±0.06**	**0.75±0.09**	**0.74±0.06**
			NK-1 25 µM	NK-2 25 µM	NK-3 25 µM	NK-4 25 µM
			0.58±0.05	*0.70±0.04*	*0.64±0.04*	0.45±0.05
			0.93±0.06	0.85±0.14	0.82±0.05	**0.73±0.13**
			NK-1 50 µM	NK-2 50 µM	NK-3 50 µM	NK-4 50 µM
Total	0.99±0.004	**0.44±0.05**	0.99±0.01	1.05±0.03	**0.86±0.05**	**0.85±0.06**
Biofilm	0.41±0.01	**0.08±0.01**	0.35±0.07	*0.59±0.15*	**0.26±0.06**	**0.22±0.02**
			NK-1 100 µM	NK-2 100 µM	NK-3 100 µM	NK-4 100 µM
			0.99±0.01	0.99±0.11	**0.73±0.06**	**0.68±0.03**
			0.40±0.01	*0.63±0.10*	**0.19±0.02**	**0.21±0.04**
			NKLA 10 µM	NKLB 10 µM		
Total	0.93±0.01	**0.44±0.04**	0.88±0.01	0.88±0.02		
Biofilm	1.05±0.12	**0.11±0.01**	1.07±0.06	1.01±0.11		
			NKLA 25 µM	NKLB 25 µM		
			0.90±0.01	0.88±0.04		
			1.37±0.09	1.14±0.08		
			NKLA 50 µM	NKLB 50 µM		
	1.01±0.04	**0.42±0.06**	1.03±0.02	1.02±0.01		
	0.28±0.02	**0.08±0.01**	0.51±0.05	0.27±0.04		
			NKLA 100 µM	NKLB 100 µM		
			0.97±0.16	0.97±0.01		
			0.65±0.45	0.30±0.02		
			OII 10 µM	OFNK 10 µM	PNK 10 µM	
Total	0.93±0.01	**0.44±0.04**	0.89±0.01	0.88±0.01	0.88±0.01	
Biofilm	1.05±0.12	**0.11±0.01**	0.89±0.21	**0.87±0.07**	**0.80±0.13**	
			OII 25 µM	OFNK 25 µM	PNK 25 µM	
			0.90±0.01	0.89±0.01	0.90±0.01	
			0.99±0.07	0.96±0.06	0.94±0.15	
				OFNK 50 µM	PNK 50 µM	
Total	1.01±0.01	**0.42±0.06**		0.97±0.02	1.05±0.07	
Biofilm	0.28±0.02	**0.08±0.01**		0.38±0.05	**0.23±0.05**	
				OFNK 100 µM	PNK 100 µM	
				0.98±0.03	1.09±0.04	
				0.39±0.10	0.25±0.02	

[a] Modified Shieh broth was used alone or supplemented with different AMPs at 28°C under static conditions for 24 hr. Absorbances were measured at OD_{550} (biofilms after crystal violet staining). Data are represented as mean absorbance ± SD for each treatment group.

[b] The AMPs NK= natural killer lysin, NKL= natural killer-like lysin, OII= Onchorhyncin II, OFNK= olive flounder natural killer lysin and PNK= porcine natural killer lysin.

[c] Bolded indicate significantly lower values and italicized indicate significantly higher values ($p<0.05$) when compared to the media-only control according to a generalized linear model of a Tukey's HSD post hoc test.

Table 8. The effect of striped bass (*Morone saxatilis*), white bass (*Morone chrysops*) and hybrid striped bass (*Morone saxatilis* x *Morone chrysops*) antimicrobial peptides (AMPs) on the total and biofilm growth of the *Flavobacterium columnare* LV-359-01.

Bacterial Growth[a]	AMPs[b]						
	Control	Melittin 117 µM	SB P1 10 µM	WB P1 10 µM	SBWB P3 10 µM	SB P4 10 µM	WB P5 10 µM
Total	0.49±0.01	**0.40±0.02**[c]	0.46±0.02	0.46±0.04	*0.57±0.03*	*0.75±0.06*	*0.68±0.05*
Biofilm	0.98±0.07	**0.09±0.01**	**0.80±0.10**	**0.77±0.14**	**0.65±0.11**	**0.31±0.05**	**0.42±0.11**
			SB P1 25 µM	WB P1 25 µM	SBWB P3 25 µM	SB P4 25 µM	WB P5 25 µM
			0.45±0.03	**0.41±0.02**	*0.56±0.03*	*0.75±0.03*	*1.02±0.03*
			0.83±0.07	0.82±0.16	**0.46±0.04**	**0.09±0.03**	**0.25±0.04**
			SB P1 50 µM	WB P1 50 µM	SBWB P3 50 µM		
Total	1.06±0.02	**0.41±0.03**	**0.80±0.04**	**0.77±0.03**	**0.64±0.02**		
Biofilm	1.02±0.16	**0.28±0.39**	**0.42±0.19**	**0.38±0.17**	0.86±0.06		
			SB P1 100 µM	WB P1 100 µM	SBWB P3 100 µM		
			0.47±0.02	0.61±0.03	**0.44±0.02**		
			0.57±0.22	**0.57±0.19**	**0.35±0.10**		

[a] Modified Shieh broth was used alone or supplemented with different AMPs at 28°C under static conditions for 24 hr. Absorbances were measured at OD_{550} (biofilms after crystal violet staining). Data are represented as mean absorbance ± SD for each treatment group.
[b] The AMPs SB P= striped bass piscidin, WB P= white bass piscidin, SBWB P= striped bass white bass piscidin [37].
[c] Bolded indicate significantly lower values and italicized indicate significantly higher values ($p<0.05$) when compared to the media-only control according to a generalized linear model of a Tukey's HSD post hoc test.

4. Discussion

Several bacterial pathogens that affect different fish species among the aquaculture industry have demonstrated their ability to effectively form biofilms [13, 53]. Biofilms are potential reservoirs for pathogenic bacteria and their prevalence within aquaculture can cause recurrent outbreaks in cultured fish [9, 10]. The high antibiotic resistance of biofilms and the increased occurrence of antibiotic resistance among strains of common bacterial fish pathogens, shows that the exploration of alternative anti-biofilm agents is required.

The innate immune systems of fish produce peptides, known as AMPs, which have the potential for potent broad-spectrum activity towards a range of pathogens. The current study tested several AMPs against the total and biofilm growth of *E. coli* and the fish pathogens *A. veronii*, *A. hydropila* and *F. columnare*. The positive control, melittin, demonstrated significant inhibitory activity against the total and biofilm growth of all bacteria used in this study (Tables 1-8). The NK-lysins used showed significant inhibition towards *E. coli* (Tables 1 and 2) and *A.*

veronii (Tables 3 and 4) total and biofilm growth, while the Piscidins showed significant inhibition towards *E. coli* (Tables 1 and 2) and *F. columnare* (Tables 7 and 8).

Melittin demonstrated significant *in vitro* total and biofilm inhibition towards *A. veronii*, *A. hydropila*, *E. coli*, and *F. columnare* (Tables 1-8). Melittin is an α-helical, amphipathic, AMP derived from the venom of the European honeybee (*Apis mellifera*) that consists of 26 amino acid residues [52]. This AMP is known to readily destabilize cell membranes of bacteria, fungi and animal cells. Due to melittin's established killing ability towards a wide range of cells, previous AMP activity studies have also used it as a positive control [54-56]. Like our results, several other studies have observed melittin's potency towards the total and biofilm growth of *E. coli* and other Gram-negative bacteria [50, 51]. These studies focused on melittin's total and biofilm growth inhibition activity towards *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus.* A recent study by Galdiero et al. showed that melittin was active towards the total and biofilm growth of *Aeromonas caviae*, which is a bacterium that can also cause MAS in fish [57, 58]. This observation combined with the observations from our results (Table 3, 4, 5, and 6), suggest that melittin is active against the motile members of the *Aeromonas* genus and could be a useful tool in combatting *Aeromonas* spp. biofilms in the aquaculture setting. The activity of melittin against *F. columnare* had not previously been observed but was not unwarranted in light of its inhibitory activity against several other Gram-negative bacteria.

The NK-lysins used in this current study displayed significant *in vitro* inhibition of *E. coli* (Tables 1 and 2) and *A. veronii* (Tables 3 and 4) total and biofilm growth. Fish NK-lysins have demonstrated *in vitro* inhibitory effects towards the total growth of a wide range of Gram-negative bacteria, including *A. hydrophila* [31], *A. caviae* [33], *Edwardsiella ictaluri* [33], *Edwardsiella tarda* [28, 31, 35], *E. coli* [33, 34, 59], *Klebsiella pneumonia* [28], *Photobacterium damselae* [28], *Pseudomonas aeruginosa* [28], *P. fluorescens* [34], *Serratia marcescens* [28], *Vibrio anguillarum*[34], and *V. harveyi* [34, 35]. Similar to our data, NK-lysins from Japanese

flounder (*Paralichthys olivaceus*) [28], *Larimichthys crocea* [59], and large yellow croaker (*Larimichthys crocea*) [60] did not show inhibitory activity towards *A. hydrophila* total growth. Our data also shows that the NK-lysins tested exhibited inhibitory activity towards *E. coli* total growth, which is like the findings of other studies. The inhibitory activity displayed by the NK-lysins used in our study against *A. veronii* had not been previously observed. A gap in the knowledge of NK-lysins derived from fish and other animals regarding their anti-biofilm activity exists. The antimicrobial activity assessment of NK-lysins have primarily focused on their total growth inhibition activities [61-63]. The current study sheds light on the anti-biofilm activities of NK-lysins towards Gram-negative bacteria. Further studies assessing the anti-biofilm action of NK-lysins towards other bacteria is needed to fully characterize their activity.

The Piscidins derived from different bass species exhibited significant *in vitro* inhibition towards *E. coli* and *F. columnare* total and biofilm growth across a range of different concentrations. The piscidin family of AMPs includes piscidin, epinecidin, pleurocidin, and moronecidin [22, 64]. These AMPs are unique to fish and have demonstrated antibacterial activity towards a wide range of Gram-negative bacteria. Previous studies have also observed similar *E. coli* total growth inhibition by Piscidins derived from hybrid striped bass [37, 65], mandarin fish (*Siniperca chuatsi*) [64], orange-spotted grouper (*Epinephelus coioides*) [66, 67], icefish (*Chionodraco hamatus*) [68], Malabar grouper (*Epinephelus malabaricus*) [69], and winter flounder (*Pleuronectes americanus*) [56]. The lack of piscidin total growth activity against *A. hydrophila* has also been previously observed in a piscidin from the freshwater finfish *S. chuatsi*, perhaps indicating a conserved function among teleost fish [64]. The total growth inhibition activities of the bass Piscidins used in the current study towards *F. columnare* has not been previously observed. The anti-biofilm activity of these bass Piscidins towards *E. coli* and *F. columnare* was potent, particularly the SB P4 peptide. Specifically, SB P4 proved to have the strongest inhibition towards *E. coli* and *F. columnare* when dosed at a concentration of 25 µM

(Tables 2 and 8). Piscidins' *in vitro* anti-biofilm activity towards bacteria has not been explored as thoroughly as their total growth inhibition activity. Several studies testing the anti-biofilm effect of a piscidin derived from winter flounder (*P. americanus*), found that it demonstrated biofilm inhibition towards *E. coli*, *P. aeruginosa,* and drug resistant *Staphylococcus aureus* [54, 56]. Another study observed potent anti-biofilm activity from a piscidin from witch flounder (*Glyptocephalus cynoglossus*) against *P. aeruginosa* biofilms [52]. Our results support the observation that Piscidins have anti-biofilm activity towards *E. coli* and suggests that they may be potent biofilm inhibitors of *F. columnare*.

Several of the AMPs tested showed inhibitory activity towards bacterial biofilm growth but did not display activity towards total growth. For example, some Piscidins only demonstrated anti-biofilm activity against *A. veronii* (Table 4) and *F. columnare* (Table 8) while showing little inhibition of total growth. This observation of peptides exerting anti-biofilm actions but lacking similar action against total growth has been noted previously among other AMPs [21, 70]. The phenomena has brought about a subclass of AMPs referred to as anti-biofilm peptides due to their anti-biofilm activity and lack of action against bacterial total growth. The distinction between anti-biofilm peptides versus other AMPs has been confirmed by structure-activity relationship studies that have shown no direct overlap between anti-biofilm and antimicrobial (vs. bacterial total growth) activities [71]. Therefore, some of the Piscidins could be anti-biofilm peptides, although further studies are needed to confirm these observations.

Future *in vitro* studies regarding AMPs derived from fish and other organisms should look to mimic the conditions found *in vivo* to truly understand their activity. The nutrient dense broths used in this study and others are useful for preliminary assessments of peptide activity, but further *in vitro* work should use growth conditions that resembles the natural environment in which these peptides would be found. Several AMP studies have observed that assessing peptide activity in nutrient limiting conditions often leads to an increase in antimicrobial activity when

compared to assessment in high nutrient conditions [70]. Dorschner et al. observed that bacteria grown in mammalian ionic conditions responded by altering their gene expression profile which subsequently resulted in higher susceptibility to AMPs [72]. This could explain why some of the AMPs tested in this study did not display significant inhibitory activity. This could also explain why the presence of several NK-lysins and Piscidins increased bacterial growth (Tables 7 and 8). These two behavioral observations could be due to the *in vitro* conditions present in this study. Further studies should assess these AMPs in conditions that mimic the environments in which fish pathogens, such as *A. hydrophila* and *F. columnare*, would be found. The fish mucosal environment has been shown to readily promote the growth of *F. columnare* biofilms. The presence of *in vitro* fish mucus has been demonstrated to promote the biofilm formation of *F. columnare* while also altering its gene expression. Fish mucus has also been suggested to be a nutrient source for *F. columnare* [73-75]. Therefore, assessing the activity of the AMPs from this study against bacteria grown in fish mucus may provide results that are more likely to be observed in the natural environment.

The current knowledge pertaining to AMPs has shown that these peptides are multi-faceted in terms of their role in the innate immune systems of fish and other organisms. They are no longer seen as simply broad-spectrum antimicrobials that act upon free-living cells. Many AMPs are now known to have immunomodulatory, anti-inflammatory and anti-biofilm activities. To capture their broad range immune-related actions, they have also begun to be referred to as host defense peptides [70]. The results obtained from the current study supports this idea by revealing the potent *in vitro* anti-biofilm activity of several NK-lysins and Piscidins that have not previously been observed. Our results also support existing literature to melittin's potent anti-biofilm activity while giving new insight into its effects toward several Gram-negative fish pathogens. These peptides could serve as effective agents against bacteria in the aquaculture setting due to their ability to inhibit both free-living and biofilm-encased bacteria. Future studies

should look to assess their mechanisms of action in regards to biofilm inhibition while also observing their activity in conditions similar to where they would occur naturally.

Acknowledgments

We would like to acknowledge Dr. Scott A. Salger, Assistant Professor of Biology, Barton College for providing the bass AMPs. We would also like to acknowledge Dr. Brian Shepherd, Research Physiologist, U.S. Dairy Forage Research Center USDA-ARS, for providing the rainbow trout, OFNK, and PNK AMPs.

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Appendix I. Raw data on the effect of rainbow trout (*Oncorhynchus mykiss*) antimicrobial peptides (AMPs) on the total and biofilm growth of the *Escherichia coli* DH5α strain.

		AMPs[a]				
Growth	**Control**	**Melittin**	**NK-1**	**NK-2**	**NK-3**	**NK-4**
		117 µM	**10 µM**	**10 µM**	**10 µM**	**10 µM**
	1.495	0.296	1.488	1.487	1.485	1.500
	1.487	0.315	1.477	1.471	1.467	1.493
Total	1.478	0.316	1.466	1.468	1.460	1.474
	1.473	0.309	1.471	1.474	1.441	1.472
	1.470	0.313	1.472	1.468	1.451	1.470
	0.124	0.072	0.146	0.120	0.191	0.113
	0.197	0.062	0.119	0.145	0.138	0.095
Biofilm	0.120	0.066	0.100	0.139	0.116	0.169
	0.169	0.063	0.131	0.145	0.097	0.104
	0.114	0.062	0.098	0.098	0.159	0.101
Growth	**Control**	**Melittin**	**NK-1**	**NK-2**	**NK-3**	**NK-4**
		117 µM	**25 µM**	**25 µM**	**25 µM**	**25 µM**
	1.495	0.296	1.481	1.492	1.441	1.360
	1.487	0.315	1.473	1.488	1.370	1.289
Total	1.478	0.316	1.476	1.489	1.423	1.468
	1.473	0.309	1.486	1.486	1.417	1.498
	1.470	0.313	1.492	1.498	1.429	1.506
	0.124	0.072	0.122	0.100	0.143	0.134
	0.197	0.062	0.097	0.099	0.110	0.135
Biofilm	0.120	0.066	0.113	0.095	0.144	0.147
	0.169	0.063	0.102	0.078	0.101	0.103
	0.114	0.062	0.077	0.088	0.109	0.106
Growth	**Control**	**Melittin**	**NK-1 50**	**NK-2 50**	**NK-3 50**	**NK-4 50**
		117 µM	**50 µM**	**50 µM**	**50 µM**	**50 µM**
	1.495	0.300	1.420	1.351	1.342	1.344
	1.487	0.312	1.393	1.345	1.260	1.298
Total	1.478	0.297	1.406	1.336	1.254	1.305
	1.473	0.316	1.418	1.348	1.325	1.295
	1.470	0.295	1.403	1.326	1.158	1.286
	0.906	0.078	0.439	0.168	0.232	0.167
	1.098	0.078	0.613	0.157	0.187	0.189
Biofilm	1.102	0.075	0.610	0.223	0.233	0.176
	1.655	0.085	0.775	0.152	0.233	0.218
	1.396	0.085	0.774	0.187	0.237	0.314
Growth	**Control**	**Melittin**	**NKLA**	**NKLB**	**NKLA**	**NKLB**
		117 µM	**10 µM**	**10 µM**	**25 µM**	**25 µM**
	1.437	0.333	1.484	1.463	1.383	1.432

	1.452	0.309	1.469	1.443	1.387	1.423	
Total	1.440	0.314	1.468	1.430	1.391	1.420	
	1.437	0.338	1.454	1.436	1.393	1.426	
	1.429	0.303	1.465	1.421	1.409	1.457	
	0.906	0.090	0.371	0.637	0.335	0.681	
	1.098	0.090	0.289	0.664	0.329	0.489	
Biofilm	1.102	0.081	0.322	1.002	0.346	0.767	
	1.655	0.102	0.384	0.884	0.381	3.762	
	1.396	0.089	0.332	0.680	0.452	0.808	

Growth	**Control**	**Melittin**	**OFNK**	**PNK**	**OII**	**OFNK**	**PNK**	**OII**
		117 µM	**10 µM**	**10 µM**	**10 µM**	**25 µM**	**25 µM**	**25 µM**
	1.514	0.301	1.500	1.487	1.566	1.461	1.370	1.547
	1.503	0.288	1.492	1.491	1.558	1.465	1.343	1.544
Total	1.497	0.293	1.508	1.466	1.537	1.453	1.417	1.546
	1.500	0.286	1.494	1.492	1.549	1.466	1.470	1.590
	1.515	0.303	1.486	1.352	1.544	1.472	1.502	1.559
	0.867	0.089	0.152	0.138	0.235	0.157	0.118	0.356
	0.780	0.107	0.155	0.154	0.328	0.186	0.119	0.503
Biofilm	0.313	0.190	0.160	0.186	0.347	0.145	0.127	0.358
	1.120	0.084	0.240	0.124	0.521	0.210	0.132	0.389
	1.060	0.086	0.239	0.169	0.357	0.210	0.101	0.523

[a]The AMPs NK= natural killer lysin, NKL= natural killer-like lysin, OII= Onchorhyncin II, OFNK= olive flounder (*Paralichthys olivaceus*) natural killer lysin and PNK= porcine natural killer lysin.

Appendix II. Raw data on the effect of striped bass (*Morone saxatilis*), white bass (*Morone chrysops*) and hybrid striped bass (*Morone saxatilis* x *Morone chrysops*) antimicrobial peptides (AMPs) on the total and biofilm growth of the *Escherichia coli* DH5α strain.

		AMPs[a]						
Growth	**Control**	**Melittin**	**SB P1**	**WB P1**	**SBWB P3**	**SB P1**	**WB P1**	**SBWB P3**
		117 µM	**10 µM**	**10 µM**	**10 µM**	**25 µM**	**25 µM**	**25 µM**
	1.386	0.308	0.219	1.514	1.343	0.867	1.454	0.217
	1.386	0.298	1.394	1.502	1.318	0.955	1.450	0.179
Total	1.385	0.304	1.412	1.516	1.402	0.264	1.448	0.183
	1.385	0.332	1.426	1.509	1.463	0.287	1.421	0.190
	1.388	0.290	1.405	1.503	1.303	0.242	1.449	0.193
	0.401	0.078	0.075	0.119	0.147	0.084	0.107	0.114
	0.515	0.078	0.089	0.133	0.120	0.084	0.115	0.085
Biofilm	0.481	0.075	0.090	0.123	0.127	0.093	0.118	0.098
	0.470	0.085	0.136	0.129	0.123	0.106	0.136	0.103
	0.477	0.085	0.162	0.146	0.137	0.103	0.103	0.111

Growth	**Control**	**Melittin**	**SB P4**	**WB P5**	**SB P4**	**WB P5**
		117 µM	**10 µM**	**10 µM**	**25 µM**	**25 µM**
	1.504	0.299	1.311	0.999	0.509	0.358
	1.508	0.311	1.218	1.144	0.564	0.360
Total	1.516	0.328	1.103	1.038	0.484	0.343
	1.567	0.304	1.412	1.343	0.533	0.334
	1.519	0.312	1.256	1.293	0.504	0.345
	1.271	0.088	0.090	0.152	0.085	0.211
	1.226	0.082	0.087	0.160	0.098	0.187
Biofilm	0.911	0.111	0.095	0.176	0.077	0.196
	0.965	0.094	0.131	0.181	0.096	0.192
	0.678	0.090	0.110	0.170	0.109	0.167

[a]The AMPs SB= striped bass piscidin, WB= white bass piscidin, SB/WB= hybrid striped bass.

Appendix III. Raw data on the effect of rainbow trout (*Oncorhynchus mykiss*) antimicrobial peptides (AMPs) on the total and biofilm growth of an *Aeromonas veronii* isolate.

		AMPs[a]				
Growth	**Control**	**Melittin**	**NK-1**	**NK-2**	**NK-3**	**NK-4**
		117 µM	**10 µM**	**10 µM**	**10 µM**	**10 µM**
	1.805	0.297	1.809	1.799	1.772	1.801
	1.800	0.290	1.801	1.786	1.767	1.802
Total	1.800	0.282	1.799	1.770	1.740	1.800
	1.804	0.281	1.802	1.778	1.754	1.779
	1.801	0.294	1.803	1.792	1.736	1.783
	1.023	0.072	0.834	0.749	0.776	0.738
	1.297	0.082	0.764	1.006	0.851	0.979
Biofilm	1.235	0.075	0.689	0.721	0.847	1.120
	1.751	0.076	0.945	1.187	0.800	0.881
	1.242	0.086	1.325	1.046	0.813	1.017
Growth	**Control**	**Melittin**	**NK-1**	**NK-2**	**NK-3**	**NK-4**
		117 µM	**25 µM**	**25 µM**	**25 µM**	**25 µM**
	1.805	0.297	1.802	1.710	1.731	1.755
	1.800	0.290	1.794	1.725	1.668	1.760
Total	1.800	0.282	1.789	1.734	1.724	1.746
	1.804	0.281	1.792	1.732	1.619	1.718
	1.801	0.294	1.791	1.769	1.720	1.738
	1.023	0.072	1.059	0.872	0.966	0.973
	1.297	0.082	1.111	1.169	0.873	1.030
Biofilm	1.235	0.075	1.107	0.901	1.204	1.562
	1.751	0.076	0.958	0.846	0.851	1.307
	1.242	0.086	1.107	0.841	0.991	1.260
Growth	**Control**	**Melittin**	**NK-1**	**NK-2**	**NK-3**	**NK-4**
		117 µM	**50 µM**	**50 µM**	**50 µM**	**50 µM**
	1.495	0.294	1.420	1.351	1.342	1.344
	1.487	0.280	1.393	1.345	1.260	1.298
Total	1.478	0.285	1.406	1.336	1.254	1.305
	1.473	0.279	1.418	1.348	1.325	1.295
	1.470	0.261	1.403	1.326	1.158	1.286
	0.906	0.072	0.439	0.168	0.232	0.167
	1.098	0.089	0.613	0.157	0.187	0.189
Biofilm	1.102	0.074	0.610	0.223	0.233	0.176
	1.655	0.092	0.775	0.152	0.233	0.218
	1.396	0.086	0.774	0.187	0.237	0.314
Growth	**Control**	**Melittin**	**NKLA**	**NKLB**	**NKLA**	**NKLB**
		117 µM	**10 µM**	**10 µM**	**25 µM**	**25 µM**
	1.708	0.255	1.714	1.707	1.667	1.654

	1.690	0.330	1.668	1.672	1.663	1.663
Total	1.681	0.277	1.668	1.679	1.678	1.679
	1.690	0.282	1.661	1.665	1.698	1.691
	1.689	0.288	1.663	1.653	1.722	1.720
	1.226	0.076	1.117	1.021	1.391	0.944
	1.402	0.072	1.112	0.836	1.453	0.990
Biofilm	1.326	0.088	0.943	0.847	1.473	0.984
	1.347	0.091	1.164	0.832	1.288	1.127
	1.540	0.090	1.225	0.857	1.526	1.292

Growth	**Control**	**Melittin**	**NKLA**	**NKLB**	**NKLA**	**NKLB**
		117 µM	**50 µM**	**50 µM**	**100 µM**	**100 µM**
	1.708	0.255	1.752	1.724	1.752	1.703
	1.690	0.303	1.737	1.704	1.737	1.676
Total	1.681	0.277	1.761	1.695	1.761	1.694
	1.690	0.282	1.732	1.670	1.732	1.716
	1.689	0.288	1.738	1.664	1.738	1.742
	1.226	0.076	1.037	1.014	1.404	1.353
	1.402	0.072	0.898	0.945	1.446	1.279
Biofilm	1.326	0.088	1.412	1.181	1.545	1.424
	1.347	0.091	0.872	1.205	1.192	1.354
	1.540	0.090	1.040	1.066	1.534	1.517

Growth	**Control**	**Melittin**	**OFNK**	**PNK1**	**OII**	**OFNK**	**PNK**	**OII**
		117 µM	**10 µM**	**10 µM**	**10 µM**	**25 µM**	**25 µM**	**25 µM**
	1.738	0.295	1.754	1.721	1.741	1.709	1.675	1.714
	1.712	0.277	1.714	1.692	1.716	1.712	1.669	1.711
Total	1.716	0.301	1.702	1.682	1.706	1.724	1.673	1.719
	1.710	0.288	1.708	1.680	1.708	1.727	1.685	1.726
	1.709	0.264	1.704	1.685	1.718	1.761	1.718	1.742
	1.077	0.088	0.713	0.825	0.741	0.840	1.039	1.410
	0.991	0.073	0.719	0.819	0.661	0.887	0.920	1.356
Biofilm	1.050	0.075	0.897	0.982	0.725	0.867	0.882	1.330
	1.566	0.083	1.019	1.102	0.926	0.750	0.912	1.272
	1.595	0.087	0.969	1.174	1.063	0.762	0.995	0.862

[a]The AMPs NK= natural killer lysin, NKL= natural killer-like lysin, OII= Onchorhyncin II, OFNK= olive flounder natural killer lysin and PNK= porcine natural killer lysin.

Appendix IV. Raw data on the effect of striped bass (*Morone saxatilis*), white bass (*Morone chrysops*) and hybrid striped bass (*Morone saxatilis* x *Morone chrysops*) antimicrobial peptides (AMPs) on the total and biofilm growth of an *Aeromonas veronii* isolate.

		AMPs[a]						
Growth	**Control**	**Melittin**	**SB P1**	**WB P1**	**SBWB P3**	**SB P1**	**WB P1**	**SBWB P3**
		117 µM	**10 µM**	**10 µM**	**10 µM**	**25 µM**	**25 µM**	**25 µM**
	1.823	0.243	1.820	1.811	1.817	1.798	1.786	1.795
	1.823	0.255	1.807	1.800	1.800	1.788	1.788	1.809
Total	1.813	0.307	1.798	1.793	1.807	1.800	1.790	1.798
	1.808	0.278	1.798	1.785	1.790	1.806	1.807	1.806
	1.812	0.284	1.794	1.785	1.791	1.809	1.811	1.825
	0.725	0.084	0.709	0.589	0.989	0.578	0.971	0.917
	0.794	0.086	0.692	0.952	0.841	0.795	0.661	0.824
Biofilm	0.804	0.077	0.614	0.891	0.815	0.916	0.940	0.838
	0.976	0.071	0.822	0.910	0.793	0.858	1.007	0.920
	0.659	0.099	0.691	0.609	1.054	0.722	0.945	1.009
Growth	**Control**	**Melittin**	**SB P1**	**WB P1**	**SBWB P3**	**SB P1**	**WB P1**	**SBWB P3**
		117 µM	**50 µM**	**50 µM**	**50 µM**	**100 µM**	**100 µM**	**100 µM**
	1.778	0.303	1.819	1.819	1.770	1.627	1.812	1.473
	1.767	0.289	1.800	1.810	1.756	1.787	1.674	1.732
Total	1.761	0.244	1.805	1.796	1.679	1.795	1.707	1.673
	1.760	0.283	1.800	1.804	1.781	1.758	1.808	0.168
	1.754	0.274	1.798	1.812	1.773	1.796	1.595	1.494
	1.101	0.078	0.845	1.080	1.593	0.814	0.729	1.778
	1.293	0.082	0.767	0.703	0.919	0.790	0.849	1.718
Biofilm	1.200	0.086	0.713	0.711	1.299	0.619	0.960	1.668
	1.111	0.088	0.851	0.769	1.037	0.797	1.017	0.170
	1.218	0.073	0.796	0.760	0.995	0.938	1.089	1.348
Growth	**Control**	**Melittin**	**SB P4**	**WB P5**	**SB P4**	**WB P5**		
		117 µM	**10 µM**	**10 µM**	**25 µM**	**25 µM**		
	1.778	0.297	1.789	1.746	1.723	1.685		
	1.765	0.290	1.767	1.718	1.700	1.681		
Total	1.766	0.282	1.755	1.708	1.742	1.677		
	1.773	0.281	1.754	1.703	1.758	1.696		
	1.772	0.294	1.746	1.717	1.744	1.701		
	1.138	0.072	0.821	0.989	0.994	1.251		
	1.299	0.082	0.797	0.924	1.038	1.319		
Biofilm	1.123	0.075	1.203	1.014	0.975	1.162		
	1.119	0.076	0.905	1.014	0.615	1.054		
	1.089	0.086	0.832	1.667	0.848	1.424		

[a]The AMPs SB P= striped bass piscidin, WB P= white bass piscidin, SBWB P= striped bass white bass piscidin.

Appendix V. Raw data on the effect of rainbow trout (*Oncorhynchus mykiss*) antimicrobial peptides (AMPs) on the total and biofilm growth of the *Aeromonas hydrophila* ALG-15-097.

		AMPs[a]				
Growth	**Control**	**Melittin**	**NK-1**	**NK-2**	**NK-3**	**NK-4**
		117 µM	**10 µM**	**10 µM**	**10 µM**	**10 µM**
	1.754	0.306	1.759	1.721	1.743	1.760
	1.742	0.364	1.745	1.713	1.740	1.747
Total	1.744	0.351	1.742	1.727	1.733	1.748
	1.732	0.294	1.750	1.707	1.723	1.747
	1.751	0.282	1.754	1.737	1.760	1.744
	0.833	0.084	0.396	0.384	0.420	0.336
	1.013	0.270	0.481	1.091	0.583	0.807
Biofilm	0.764	0.083	0.742	0.853	1.059	0.990
	1.020	0.074	0.619	0.948	1.123	0.997
	1.150	0.094	1.214	0.771	0.871	0.554
Growth	**Control**	**Melittin**	**NK-1**	**NK-2**	**NK-3**	**NK-4**
		117 µM	**25 µM**	**25 µM**	**25 µM**	**25 µM**
	1.754	0.306	1.761	1.728	1.743	1.775
	1.742	0.364	1.745	1.715	1.734	1.767
Total	1.744	0.351	1.748	1.725	1.731	1.751
	1.732	0.294	1.763	1.717	1.737	1.763
	1.751	0.282	1.762	1.732	1.766	1.748
	0.833	0.084	1.156	1.305	1.034	0.906
	1.013	0.270	1.122	1.187	1.059	1.001
Biofilm	0.764	0.083	1.137	1.010	1.045	1.001
	1.020	0.074	0.451	0.641	0.470	0.549
	1.150	0.094	0.637	0.680	0.624	0.430
Growth	**Control**	**Melittin**	**NK-1**	**NK-2**	**NK-3**	**NK-4**
		117 µM	**50 µM**	**50 µM**	**50 µM**	**50 µM**
	1.790	0.306	1.781	1.748	1.745	1.775
	1.778	0.364	1.773	1.731	1.786	1.729
Total	1.777	0.351	1.760	1.733	1.755	1.758
	1.776	0.294	1.771	1.729	1.748	1.777
	1.766	0.282	1.770	1.749	1.738	1.784
	0.629	0.107	0.594	0.524	0.704	1.117
	1.254	0.085	1.288	1.218	1.391	1.173
Biofilm	1.036	0.156	1.252	1.115	1.045	0.973
	0.650	0.071	0.710	0.630	0.771	1.327
	0.933	0.189	0.889	0.995	0.666	1.096
Growth	**Control**	**Melittin**	**NKLA**	**NKLB**	**NKLA**	**NKLB**
		117 µM	**10 µM**	**10 µM**	**25 µM**	**25 µM**
	1.710	0.613	1.776	1.792	1.754	1.777

	1.744	0.591	1.764	1.781	1.763	1.787
Total	1.740	0.589	1.764	1.777	1.759	1.787
	1.733	0.585	1.758	1.774	1.783	1.801
	1.732	0.589	1.756	1.766	1.799	1.805
	1.139	0.046	0.729	0.689	1.241	0.995
	1.358	0.047	1.053	0.879	1.153	1.301
Biofilm	1.369	0.054	1.100	1.518	1.100	1.001
	1.332	1.081	0.735	0.806	1.204	1.447
	1.435	0.565	1.044	1.412	1.364	0.880

Growth	**Control**	**Melittin**	**NKLA**	**NKLB**	**NKLA**	**NKLB**
		117 µM	**50 µM**	**50 µM**	**100 µM**	**100 µM**
	1.710	0.613	1.728	1.720	1.702	1.740
	1.744	0.591	1.708	1.743	1.676	1.743
Total	1.740	0.589	1.701	1.741	1.699	1.739
	1.733	0.585	1.716	1.740	1.676	1.752
	1.732	0.589	1.708	1.741	1.740	1.715
	1.139	0.046	0.992	1.061	1.505	1.121
	1.358	0.047	1.062	0.874	1.352	1.506
Biofilm	1.369	0.054	1.540	1.204	1.312	1.311
	1.332	1.081	1.181	1.099	1.486	1.244
	1.435	0.565	0.996	0.990	1.618	1.337

Growth	**Control**	**Melittin**	**OFNK**	**PNK**	**OII**	**OFNK**	**PNK**	**OII**
		117 µM	**10 µM**	**10 µM**	**10 µM**	**25 µM**	**25 µM**	**25 µM**
	1.808	0.463	1.800	1.796	1.815	1.800	1.796	1.795
	1.793	0.422	1.785	1.790	1.800	1.798	1.784	1.792
Total	1.794	0.375	1.797	1.796	1.800	1.798	1.775	1.794
	1.791	0.384	1.811	1.804	1.796	1.784	1.778	1.802
	1.780	0.399	1.812	1.803	1.798	1.797	1.789	1.812
	1.075	0.052	0.744	0.530	0.736	0.712	0.791	1.156
	1.097	0.088	0.891	0.665	1.228	0.850	0.803	1.342
Biofilm	1.373	0.061	0.676	0.690	1.315	0.793	0.748	1.264
	1.472	0.097	1.204	0.810	1.241	0.719	0.773	1.291
	1.596	0.099	1.041	0.917	1.446	0.644	0.714	1.247

Growth	**Control**	**Melittin**	**OFNK**	**PNK**	**OII**	**OFNK**	**PNK**	**OII**
		117 µM	**50 µM**	**50 µM**	**50 µM**	**100 µM**	**100 µM**	**100 µM**
	1.710	0.439	1.701	1.652	1.748	1.718	1.365	1.767
	1.744	0.455	1.692	1.643	1.732	1.689	1.493	1.748
Total	1.740	0.418	1.706	1.637	1.730	1.680	1.486	1.749
	1.733	0.393	1.698	1.635	1.730	1.684	1.479	1.746
	1.732	0.433	1.708	1.635	1.746	1.720	1.615	1.759
	1.139	0.064	0.589	0.824	1.361	0.972	1.800	1.690
	1.358	0.068	0.990	1.169	1.508	1.323	1.207	1.702

Biofilm	1.369	0.082	0.802	1.552	1.425	1.203	1.461	1.570
	1.332	0.098	1.410	1.317	1.721	1.151	1.547	1.459
	1.435	0.099	0.777	1.305	1.774	1.294	1.654	1.679

[a]The AMPs NK= natural killer lysin, NKL= natural killer-like lysin, OII= Onchorhyncin II, OFNK= olive flounder natural killer lysin and PNK= porcine natural killer lysin.

Appendix VI. Raw data on the effect of striped bass (*Morone saxatilis*), white bass (*Morone chrysops*) and hybrid striped bass (*Morone saxatilis* x *Morone chrysops*) antimicrobial peptides (AMPs) on the total and biofilm growth off the *Aeromonas hydrophila* ALG-15-097.

		AMPs[a]						
Growth	**Control**	**Melittin**	**SB P1**	**WB P1**	**SBWB P3**	**SB P1**	**WB P1**	**SBWB P3**
		117 µM	**10 µM**	**10 µM**	**10 µM**	**25 µM**	**25 µM**	**25 µM**
	1.787	0.613	1.762	1.760	1.772	1.764	1.746	1.757
	1.775	0.591	1.744	1.745	1.751	1.722	1.753	1.743
Total	1.765	0.589	1.742	1.733	1.744	1.766	1.740	1.737
	1.759	0.585	1.745	1.747	1.745	1.750	1.750	1.714
	1.757	0.589	1.761	1.753	1.773	1.766	1.752	1.762
	0.872	0.077	0.657	0.684	0.935	0.897	0.934	1.132
	1.081	0.056	0.839	1.050	0.820	1.253	0.803	1.213
Biofilm	0.896	0.081	1.048	0.835	0.958	0.669	1.003	1.170
	1.379	0.090	1.048	1.172	1.082	0.877	0.685	1.054
	1.353	0.098	1.200	1.133	1.207	0.772	0.644	0.939
Growth	**Control**	**Melittin**	**SB P1**	**WB P1**	**SBWB P1**	**SB P1**	**WB P**	**SBWB P1**
		117 µM	**50 µM**	**50 µM**	**50 µM**	**100 µM**	**100 µM**	**100 µM**
	1.784	0.311	1.781	1.794	1.786	1.740	1.721	1.509
	1.766	0.289	1.774	1.781	1.671	1.662	1.713	1.666
Total	1.766	0.357	1.804	1.778	1.783	1.706	1.714	1.552
	1.762	0.290	1.784	1.780	1.788	1.644	1.731	1.570
	1.76	0.344	1.769	1.795	1.806	1.721	1.492	1.575
	1.150	0.087	0.829	1.016	0.857	2.064	1.699	1.105
	1.434	0.093	1.149	1.116	1.280	2.083	1.809	1.446
Biofilm	1.965	0.095	1.134	1.294	1.935	1.842	2.190	1.131
	1.850	0.084	1.197	1.185	1.844	1.210	1.825	1.145
	1.942	0.108	1.486	1.709	1.428	1.546	1.138	1.097
Growth	**Control**	**Melittin**	**SB P4**	**WB P5**	**SB P4**	**WB P5**	**SB P4**	**WB P5**
		117 µM	**10 µM**	**10 µM**	**25 µM**	**25 µM**	**50 µM**	**50 µM**
	1.778	0.421	1.771	1.771	1.760	1.717	1.683	1.730
	1.768	0.459	1.754	1.750	1.725	1.714	1.676	1.729
Total	1.751	0.396	1.748	1.746	1.749	1.714	1.668	1.719
	1.770	0.399	1.746	1.757	1.740	1.703	1.672	1.703
	1.755	0.438	1.752	1.767	1.771	1.732	1.699	1.698
	1.414	0.099	0.849	0.814	1.720	1.698	1.153	0.913
	1.545	0.078	1.151	1.032	1.618	1.661	1.416	1.289
Biofilm	1.615	0.092	1.173	1.212	1.658	1.775	1.795	1.525
	1.472	0.087	1.113	1.099	1.566	1.623	2.148	1.558
	1.444	0.091	1.048	1.354	1.554	1.791	2.036	1.719

[a]The AMPs SB P= striped bass piscidin, WB P= white bass piscidin, SBWB P= striped bass white bass piscidin.

Appendix VII. Raw data on the effect of rainbow trout (*Oncorhynchus mykiss*) antimicrobial peptides (AMPs) on the total and biofilm growth of the *Flavobacterium columnare* LV-359-01.

		AMPs[a]				
Growth	**Control**	**Melittin**	**NK-1**	**NK-2**	**NK-3**	**NK-4**
		117 µM	**10 µM**	**10 µM**	**10 µM**	**10 µM**
	0.559	0.472	0.568	0.641	0.613	0.524
	0.547	0.360	0.557	0.674	0.618	0.588
Total	0.560	0.468	0.554	0.645	0.606	0.479
	0.531	0.446	0.562	0.623	0.648	0.562
	0.561	0.341	0.550	0.621	0.612	0.584
	0.950	0.069	0.707	0.647	0.674	0.631
	1.054	0.083	1.020	0.724	0.727	0.766
Biofilm	1.033	0.075	0.724	0.695	0.749	0.710
	0.986	0.096	0.883	0.802	0.702	0.779
	0.876	0.069	0.862	0.766	0.899	0.788
Growth	**Control**	**Melittin**	**NK-1**	**NK-2**	**NK-3**	**NK-4**
		117 µM	**25 µM**	**25 µM**	**25 µM**	**25 µM**
	0.559	0.472	0.524	0.708	0.598	0.434
	0.547	0.360	0.566	0.686	0.676	0.513
Total	0.560	0.468	0.626	0.692	0.624	0.397
	0.531	0.446	0.554	0.653	0.686	0.424
	0.561	0.341	0.641	0.771	0.631	0.475
	0.950	0.069	0.956	0.786	0.857	0.969
	1.054	0.083	0.890	0.742	0.817	0.658
Biofilm	1.033	0.075	0.916	0.882	0.880	0.664
	0.986	0.096	1.014	0.763	0.751	0.664
	0.876	0.069	0.881	1.087	0.814	0.707
Growth	**Control**	**Melittin**	**NK-1**	**NK-2**	**NK-3**	**NK-4**
		117 µM	**50 µM**	**50 µM**	**50 µM**	**50 µM**
	0.995	0.488	0.971	1.078	0.908	0.957
	0.995	0.451	0.990	1.047	0.833	0.819
Total	0.986	0.470	0.989	1.010	0.866	0.809
	0.991	0.379	0.998	1.065	0.912	0.840
	0.988	0.399	0.998	1.056	0.792	0.813
	0.414	0.080	0.224	0.490	0.187	0.211
	0.423	0.083	0.367	0.501	0.214	0.190
Biofilm	0.412	0.068	0.408	0.542	0.272	0.220
	0.390	0.101	0.365	0.560	0.299	0.214
	0.413	0.093	0.389	0.848	0.347	0.248
Growth	**Control**	**Melittin**	**NK-1**	**NK-2**	**NK-3**	**NK-4**
		117 µM	**100 µM**	**100 µM**	**100 µM**	**100 µM**
	0.995	0.488	1.006	0.893	0.703	0.679

	0.995	0.451	0.997	0.930	0.679	0.651
Total	0.986	0.470	0.991	0.957	0.819	0.719
	0.991	0.379	0.987	1.172	0.751	0.661
	0.988	0.399	0.973	1.000	0.700	0.675
	0.414	0.080	0.410	0.498	0.201	0.207
	0.423	0.083	0.406	0.651	0.184	0.206
Biofilm	0.412	0.068	0.420	0.608	0.153	0.160
	0.390	0.101	0.385	0.784	0.180	0.243
	0.413	0.093	0.398	0.607	0.212	0.259

Growth	**Control**	**Melittin**	**NKLA**	**NKLB**	**OFNK**	**PNK**	**OII**
		117 µM	**10 µM**	**10 µM**	**10 µM**	**10 µM**	**10 µM**
	0.952	0.488	0.887	0.867	0.870	0.887	0.888
	0.932	0.449	0.871	0.880	0.878	0.895	0.877
Total	0.933	0.383	0.874	0.865	0.879	0.886	0.895
	0.927	0.428	0.879	0.901	0.894	0.871	0.886
	0.918	0.461	0.890	0.892	0.890	0.874	0.909
	1.200	0.115	1.079	0.836	0.739	0.599	0.693
	1.153	0.106	1.074	1.094	0.899	0.752	0.921
Biofilm	0.987	0.098	1.040	1.112	0.911	0.895	1.227
	0.949	0.113	0.987	1.015	0.914	0.854	0.789
	0.960	0.104	1.160	0.983	0.882	0.887	0.814

Growth	**Control**	**Melittin**	**NKLA**	**NKLB**	**OFNK**	**PNK**	**OII**
		117 µM	**25 µM**	**25 µM**	**25 µM**	**25 µM**	**25 µM**
	0.952	0.488	0.906	0.905	0.898	0.879	0.908
	0.932	0.449	0.910	0.909	0.894	0.890	0.899
Total	0.933	0.383	0.919	0.901	0.902	0.906	0.896
	0.927	0.428	0.895	0.887	0.882	0.903	0.888
	0.918	0.461	0.886	0.803	0.894	0.905	0.905
	1.200	0.115	1.449	1.242	0.993	1.165	0.930
	1.153	0.106	1.337	1.180	0.926	0.964	1.074
Biofilm	0.987	0.098	1.431	1.140	1.043	0.914	0.923
	0.949	0.113	1.394	1.053	0.948	0.774	0.991
	0.960	0.104	1.218	1.077	0.885	0.856	1.043

Growth	**Control**	**Melittin**	**NKLA**	**NKLB**	**OFNK**	**PNK**
		117 µM	**50 µM**	**50 µM**	**50 µM**	**50 µM**
	1.001	0.472	1.064	1.027	0.964	1.167
	1.029	0.360	1.043	1.030	0.961	1.024
Total	1.004	0.468	1.029	1.020	0.961	1.002
	0.997	0.446	1.004	1.009	0.983	1.005
	1.018	0.341	1.026	1.020	1.001	1.040
	0.297	0.069	0.533	0.240	0.326	0.180
	0.291	0.083	0.425	0.242	0.351	0.283

Biofilm	0.250	0.075	0.506	0.260	0.404	0.274
	0.285	0.096	0.501	0.266	0.379	0.218
	0.276	0.069	0.571	0.332	0.453	0.168
Growth	**Control**	**Melittin**	**NKLA**	**NKLB**	**OFNK**	**PNK**
		117 µM	**100 µM**	**100 µM**	**100 µM**	**100 µM**
	1.001	0.472	0.689	0.960	0.942	1.078
	1.029	0.360	0.968	0.965	1.019	1.137
Total	1.004	0.468	1.061	0.969	0.973	1.032
	0.997	0.446	1.070	0.962	0.977	1.121
	1.018	0.341	1.051	0.983	0.984	1.063
	0.297	0.069	0.973	0.322	0.564	0.262
	0.291	0.083	1.285	0.287	0.299	0.257
Biofilm	0.250	0.075	0.312	0.267	0.365	0.248
	0.285	0.096	0.383	0.308	0.379	0.249
	0.276	0.069	0.302	0.295	0.326	0.214

[a]The AMPs NK= natural killer lysin, NKL= natural killer-like lysin, OII= Onchorhyncin II, OFNK= olive flounder natural killer lysin and PNK= porcine natural killer lysin.

Appendix VIII. Raw data on the effect of striped bass (*Morone saxatilis*), white bass (*Morone chrysops*) and hybrid striped bass (*Morone saxatilis* x *Morone chrysops*) antimicrobial peptides (AMPs) on the total and biofilm growth of the *Flavobacterium columnare* LV-359-01.

		AMPs[a]					
Growth	**Control**	**Melittin**	**SB P1**	**WB P1**	**SBWB P3**	**SB P4**	**WB P5**
		117 µM	**10 µM**	**10 µM**	**10 µM**	**10 µM**	**10 µM**
	0.490	0.399	0.473	0.531	0.613	0.827	0.755
	0.474	0.383	0.469	0.464	0.526	0.727	0.677
Total	0.494	0.387	0.473	0.438	0.546	0.778	0.692
	0.492	0.415	0.438	0.439	0.558	0.694	0.638
	0.481	0.427	0.438	0.436	0.582	0.708	0.640
	0.950	0.074	0.695	0.561	0.530	0.226	0.317
	1.054	0.091	0.690	0.686	0.562	0.326	0.340
Biofilm	1.033	0.094	0.851	0.892	0.641	0.282	0.408
	0.986	0.104	0.891	0.845	0.705	0.327	0.449
	0.876	0.106	0.878	0.859	0.800	0.371	0.583
Growth	**Control**	**Melittin**	**SB P1**	**WB P1**	**SBWB P3**	**SB P4**	**WB P5**
		117 µM	**25 µM**	**25 µM**	**25 µM**	**25 µM**	**25 µM**
	0.490	0.399	0.443	0.395	0.613	0.719	0.973
	0.474	0.383	0.447	0.447	0.526	0.747	1.009
Total	0.494	0.387	0.499	0.405	0.546	0.728	1.022
	0.492	0.415	0.446	0.398	0.558	0.754	1.020
	0.481	0.427	0.408	0.419	0.582	0.803	1.052
	0.950	0.074	0.936	0.861	0.475	0.086	0.202
	1.054	0.091	0.735	0.608	0.508	0.078	0.318
Biofilm	1.033	0.094	0.846	0.780	0.460	0.074	0.240
	0.986	0.104	0.810	1.059	0.467	0.084	0.250
	0.876	0.106	0.839	0.804	0.403	0.150	0.229
Growth	**Control**	**Melittin**	**SB P1**	**WB P1**	**SBWB P1**	**SB P1**	**WB P1**	**SBWB P1**
		117 µM	**50 µM**	**50 µM**	**50 µM**	**100 µM**	**100 µM**	**100 µM**
	1.057	0.384	0.862	0.740	0.619	0.464	0.660	0.436
	1.072	0.444	0.774	0.774	0.672	0.463	0.610	0.464
Total	1.064	0.438	0.776	0.759	0.642	0.474	0.612	0.429
	1.023	0.387	0.765	0.811	0.652	0.506	0.614	0.417
	1.060	0.399	0.812	0.775	0.627	0.462	0.579	0.451
	0.952	0.087	0.199	0.198	0.762	0.311	0.350	0.289
	0.872	0.099	0.522	0.391	0.935	0.688	0.565	0.479
Biofilm	0.976	0.124	0.359	0.273	0.874	0.344	0.394	0.439
	1.287	0.107	0.316	0.388	0.884	0.731	0.761	0.275
	1.028	0.970	0.688	0.641	0.845	0.750	0.762	0.256

[a]The AMPs SB P= striped bass piscidin, WB P= white bass piscidin, SBWB P= striped bass white bass piscidin.