THE STUDY OF BACTERIAL COMMUNITIES IN CATFISH (*ICTALURUS PUNCTATUS*) DURING STORAGE

by

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DEDICATION

This thesis is dedicated to my parents Mario and Loretta Accumanno, who instilled in me the importance of pursuing an education. Without their continual prayers, encouragement and support, my current academic goals would not have been accomplished.

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The Study of Bacterial Communities on Catfish (*Ictalurus punctatus*) During Storage

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ABSTRACT

Fish is a lean source of protein and abundant in vitamins and minerals. Unfortunately, fresh fish deteriorates rapidly mainly due to microbial spoilage. With consumers' health concerns about using harsh chemical preservatives, natural antimicrobials would be a safer, alternative solution to prevent microbial spoilage. Metagenomics refers to investigating microbial communities from foods, environmental samples etc. and understanding how they interact with one another. Bacterial taxonomy, as a whole can be classified using high throughput sequencing platforms. In this study a commercial store-bought catfish and a pond-raised catfish were treated individually with three natural preservatives. From the catfish samples under refrigerated storage, DNA libraries were sequenced on the Illumina MiSeq platform.

Of the 3 treatments, N#1 showed the most significant bacteria reduction at the midpoint (99%) in the store-bought fish and in the pond-raised fish in the late phase (>99%). *Pseudomonas* dominated the other bacteria in early phase for all sample treatments in the store-bought fish but was suppressed by the N#1 in middle phase which allowed for more diversity. In the pond-raised fish, *Pseudomonas* also dominated other bacteria in the sample treatments for the middle and late phases but never became the dominant bacterial species for the pond-raised N#1 samples which allowed for greater diversity overall. This knowledge can suggest effective natural preservative treatments to extend the shelf-life of fishery products.

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LIST OF ABBREVIATIONS

bp	Base Pair
GFS	Grape Fruit Seed Extract
LAB	Lactic Acid Bacteria
Ng	Nano Gram
NGS	Next Generation Sequencing
OTUs	Operational Taxonomic Units
PacBio	Pacific BioScience
PCoA	Principal Coordinate Analysis
spp	Species
SSOs	Specific Spoilage Organisms

CHAPTER I: INTRODUCTION

Fish is considered a good source of dietary protein, relatively low in calories and saturated fat, and abundant in beneficial nutrients, such as vitamins (A, B12, and sometimes D), iron, zinc, magnesium, phosphorous, potassium, and omega-3 fatty acids (EPA and DHA) (Kantor, 2016). The 2015-20 Dietary Guidelines for Americans and supporting USDA "my plate" recommends that Americans should consume at least 8 ounces of fish weekly as a part of a healthy diet (Kantor, 2016).

Consequently, fresh fish spoils very rapidly even at low temperatures (Ghaly, Dave, Budge, & Brooks, 2010). Although fish spoilage is caused by a combination of enzymatic autolysis, oxidation, and microbial growth, the latter is considered the main cause (Hickey, Accumanno, McIntosh, Blank, & Lee, 2015; Rodríguez-Vaquero, Aredes-Fernández, & Manca de Nadra, 2013). These microbes, classified as specific spoilage organisms (SSOs) (Wang et al., 2017), produce metabolites and can cause changes in the sensory properties such as off-odors and off-flavors, rendering it unsuitable for human consumption (Rodríguez-Vaquero, Aredes-Fernández, & Manca de Nadra, 2013; Wang et al., 2017). On newly processed seafood, SSOs are usually low in number, contribute to a small part of the total microflora and are unique to the type of seafood (Gram & Dalgaard, 2002). The most frequently cultivated bacteria from freshwater fish include: *Acinetobacter, Pseudomonas, Aeromonas, Flavobacterium, Shewanella, Micrococcus, Carnobacterium* and *Moraxella* spp. (Wang et al., 2017).

In order to suppress the growth of microbes and extend the shelf life of foods, various approaches (low temperature storage, modified-atmosphere packaging, curing, antimicrobial

additives, smoking, freezing, brining, fermenting and canning, etc.) have been used (Batt, 2016; Chaillou, 2015; Ghaly, Dave, Budge, & Brooks, 2010).

In recent years, there has been interest in more natural alternatives to traditional preservation methods. Consumers want foods that are not only free from foodborne pathogens but also are less processed and contain fewer added "chemical ingredients" (Batt, 2016; Lingham, Besong, Ozbay, & Lee, 2012; Rodríguez-Vaquero, Aredes-Fernández, & Manca de Nadra, 2013). Consumers' avoidance of foods treated with chemical preservatives they deem "artificial" have stimulated interest in developing alternative approaches (Batt, 2016; Rodríguez-Vaquero, Aredes-Fernández, & Manca de Nadra, 2013).

Grapefruit seed extract (GSF) has been shown to have antimicrobial properties. When combined with thymol, lemon extract and modified atmospheric pressure (MAP), grapefruit seed extract extended the shelf life of fresh blue fish burger to 18 days when stored at 4ºC (Del Nobile et al., 2009). Additionally, researchers have found that lemon juice inhibited the growth of inoculated *S. Typhimurium* in mussels. The lemon juice's inhibitory effects also increased with time (Kisla, 2007).

Weak acids have been known for their antimicrobial properties (Budak, Aykin, Seydim, Greene, & Guzel-Seydim, 2014; Halstead et al., 2015; Lingham, Besong, Ozbay, & Lee, 2012). In its undissociated form, acetic acid passes through the cell wall and dissociates into anions and protons due to the internal neutral pH. As a result of the freed protons, the pH decreases which leads to eventual cell death (Halstead et al., 2015; Lingham, Besong, Ozbay, & Lee, 2012; Ricke, 2003). Acetic acid reduces food-borne pathogens, *L. monocytogenes, Salmonella* Enteritidis, *S. sonnei,* and *Yersinia spp.,* to levels below the detection limit (Medina, Romero, Brenes, & De

Castro, 2007). Acetic acid has been used on many different food items as an antimicrobial but to a lesser extent on fish products. According to Lingham et al (2012), malt vinegar exhibited antimicrobial activity against spoilage bacteria when applied to catfish.

During the storage of fish products, the microbial community changes due to the microbes' tolerance to preservatives (Gómez‐Sala et al., 2015). These changes can be monitored by a metagenomics approach, an approach providing "the direct genetic analysis of genomes contained within an environmental sample (Thomas, Gilbert, & Meyer, 2012)." This method is preferable to traditional culture-dependent methods as more than 90% of microbes in nature are nonculturable (NRCCM, 2007) nor do isolated and identified microbes alone explain microbial communities and interactions. When analyzing the composition of bacterial communities, sequencing of 16S rRNA gene, which covers variable regions of the gene, is the method of choice (Panek et al., 2018). This method, known as reference gene metagenomics (Oulas et al., 2015), is desirable because it is quick, cost-effective, and provides an in-depth community/taxonomic distribution profile (Oulas et al., 2015; Panek et al., 2018). The 16S rRNA gene is composed of nine variable regions disconnected by conserved regions and averages 1500 bp in length (Panek et al., 2018). In metagenomics investigations, the V3-V4 region is commonly studied (Akinsanya, Goh, Lim, & Ting, 2015; Hong et al., 2016; Paul, Cortez, Vera, Villena, & Gutiérrez-Correa, 2016; Rintala et al., 2017). While metagenomics has been thoroughly used to observe microbial communities in the air, soil, water, plants, and in humans (digestive tract, lungs, skin) it has been studied less so in foods, perhaps due to the assumption of low richness in regards to diversity (Kergourlay, Taminiau, Daube, & Vergès, 2015). Nevertheless, a metagenomics approach would be useful and informative in tracking changes that occur in the microbial community during food spoilage (Ercolini, 2013).

Objectives

- To observe the differences in total bacteria counts between the natural preservative-treated and control samples of catfish.
- To analyze the relationships between different bacterial communities in single samples.
- To observe differences and similarities between the pond-raised and store-bought catfish.

Hypothesis

$H_0$1: The natural preservatives will effect SSOs and therefore there will be no change in the microbial profile of pond-raised and store-bought catfish (*Ictalurus punctatus*).

H_A1: The natural preservatives will effect SSOs and therefore there will be a change in the microbial profile of pond-raised and store-bought catfish (*Ictalurus punctatus*).

$H_0$2: Non-SSOs and SSOs will contribute to the chao1.

H_A2: SSOs will mainly contribute to the chao1.

CHAPTER II: LITERATURE REVIEW

Food preservation has been necessary for the survival of the human race (Gram et al., 2002). Microbes that cause food spoilage must be put in an environment that suppresses their growth, shortens their survival or causes their death (Leistner, 2000). This can be accomplished by manipulating intrinsic factors (pH, water activity, and substrate type and availability) and extrinsic factors (temperature, atmosphere, and relative humidity) (Batt, 2016). Common methods used through the centuries include drying, salting, heating or fermentation but can have a negative effect on the food or consumer health (Gram et al., 2002). Salt is used as a food preservative by reducing the water activity but is unhealthy in large quantities and affects sensory properties of the food (Batt, 2016). Thermal and non-thermal processes, including infrared heating, high pressure, pulsed electric field, and high-intensity light, have been developed that suppress microbes but are less harsh on food products (Batt, 2016). A combination of effective methods is known as hurdle technology (Leistner, 2000). Common hurdles used include: temperature (low or high), water activity, acidity, preservatives (additives) and competitive organisms (Leistner, 2000). While food processing and additives can effectively suppress microbial growth, today's consumers desire "fresher" foods with fewer additives and interventions (Batt, 2016).

Lactic acid bacteria (LAB) are a controversial topic in regard to food spoilage. Certain LAB are known to prevent spoilage and pathogenic bacteria by producing organic acids and proteins known as bacteriocins while others contribute to spoilage and the production of unpleasant metabolites (Batt, 2016; Kannappan, Sivakumar, & Sivagnanam, 2017; Pothakos, Devlieghere, Villani, Björkroth, & Ercolini, 2015). Probiotic LAB have been used to control

aquatic bacterial pathogens in aquaculture but strict safety precautions must be applied (Cruz, Ibanez, Hermosillo, & Saad, 2012; Kannappan, Sivakumar, & Sivagnanam, 2017).

The investigation of plant-derived antimicrobials is of interest because they often lack undesirable side effects associated with synthetic antimicrobials (Oikeh, Omoregie, Oviasogie, & Oriakhi, 2016). Studies on the antimicrobial properties of essential oils (EOs) are especially popular (Kačániová et al., 2017). Oregano, rosemary, thyme, laurel, sage, cinnamon, clove, and basil-extracted essential oils have shown the most antimicrobial activity when applied to fish and seafood products (Hassoun & Çoban, 2017) but reports of the effectiveness of different EOs have been inconclusive (Hassoun & Çoban, 2017; Karoui & Hassoun, 2017; Makri, 2013). According to Hassoun and Coban (2017), the effectiveness of EOs applied to fish and seafood products is dependent on the plant source, the harvesting season, the spice variety, the part of the plant used for EOs extraction, geographical origin of the plant, and the type of fish/seafood product used. More research is needed in this area to make conclusive recommendations regarding the efficacy of essential plant-derived oils as antimicrobials.

Grapefruit seed extract (GSF) ruptures the bacterial membrane and the cytoplasmic contents exposed within 15 min (Heggers et al., 2002). Some commercially available products contain artificial agents, such as benzethonium chloride, benzalkonium chloride, cetrimonium bromide, triclosan, etc. Avula et al., (2016) suggested that GSF's antimicrobial activity is mostly due to the artificial preservatives included in the product, while Cvetnic & Vladimir- Knezevic (2004) reported potent antimicrobial activity of pure ethanolic extract of grapefruit seed/pulp. According to Kanmani & Rhim (2014) GSF incorporated into agar films inhibited the growth of pathogenic and non-pathogenic microorganisms. In another study, GSF (0.5%) treatment was

shown to significantly reduce the growth of *E. coli* O157:H7, *L. monocytogenes*, and *S.* Typhimurium individually inoculated in lettuce (Kim, Kwon, & Oh, 2016).

Juices from citrus plants are consumed because of their pleasant flavor and high Vitamin C (ascorbic acid) content. They are also believed to have antioxidant, anti-inflammatory, and antimicrobial properties due to bioactive compounds such as phenolic compounds, flavonoids, vitamins, and essential oils (Oikeh, Omoregie, Oviasogie, & Oriakhi, 2016). Since lemons are relatively inexpensive and are easy to acquire (de Castillo et al., 2000), their juice has potential to be used as a "natural antimicrobial". Other researchers compared the inhibitory effect of lactic, hydrochloric, and citric acids on the growth of *S.* Typhimurium in skim milk and found that out of the 3 acids, citric acid showed the most inhibitory effect (Subramanian & Marth, 1968). Lemon juice has also been found to inhibit the growth of several *Vibrio* spp. in vitro including *V. anguillarum, V. alginolyticus* (Tomotake, Koga, Yamato, Kassu, & Ota, 2006) and *V. cholera* (de Castillo et al., 2000).

Organic acids are commonly used as antimicrobials because they are inexpensive and effective (Lucera, Costa, Conte, & Del Nobile, 2012). Acetic, lactic, propionic, sorbic, and benzoic acids are considered the most potent (Lucera, Costa, Conte, & Del Nobile, 2012). Various weak acids display different cytotoxicities at the same pH (Halstead et al., 2015). The antimicrobial activity of organic acids is not only dependent upon a reduction in pH, but also the ratio of un-dissociated species of the acid and cell physiology and metabolism (Kim, Kwon, & Oh, 2016). Acetic acid has been used to suppress the growth of *E.coli* O157:H7, and *S.* Typhimurium inoculated in tabbouleh salad (Al-Rousan et al., 2018). When treated with 0.4% acetic acid, *S.* Typhimurium and *E. coli* O157:H7 were not detected in tabbouleh salad after 5 and 7 days of incubation, respectively (Al-Rousan et al., 2018). When combined with citric acid,

S. Typhimurium, and *E. coli* O157:H7 were not detectable after 2 and 3 days of incubation, respectively (Al-Rousan et al., 2018). In a study (Sengun & Karapinar, 2004), grape vinegar (4% acetic acid) treatments significantly reduced *S.* Typhimurium inoculated on carrots after 60 min incubation time. In the same study lemon juice also reduced the pathogen but to a lesser extent. The greatest reduction was observed, however, when researchers combined both antimicrobials (Sengun & Karapinar, 2004). Additionally, *Bacillus* spp*., Clostridium* spp., *Listeria* spp*., Salmonella* spp., *S. aureus, Pseudomonas* spp., *E. coli, Campylobacter* spp., *Aspergillus* spp., *Rhizopus* spp., *Penicillium* spp. and *Saccharomyces* spp. have been reported to be sensitive to acetic acid (Bordignon et al., 2016).

The North American catfish, also known as Channel catfish (*Ictalurus punctatus*) are in the top 10 consumed fish products in the US (Painter, 2016). In 2016, 386 million dollars of catfish were sold in the US (USDA, 2017). Over half of the seafood and/or fish products produced annually in the US are pond-raised catfish (Anonymous, 2018). Although channel catfish is considered a moderately fatty fish (Casallas, Casallas, & Mahecha, 2012), it does offer nearly 21 g of high quality protein per 4 oz serving (dry heat cooked) (USDA, 2016).

Unfortunately, Americans do not meet the recommendations for fish consumption. In 2015, it was estimated that Americans consumed only 15.5 lbs of seafood (fish) yearly (NOAA, 2016), which is roughly equivalent to 4 oz weekly or half the suggested amount in the Dietary Guidelines for Americans (Painter, 2016). It has been hypothesized that Americans eat less fish due to cost, taste, lack of exposure to seafood at an early age and fear of mercury poisoning (Painter, 2016). Another reason may be the rapid rate at which fresh fish spoils. This occurs because fish products contain high levels of soluble nitrogen compounds in the muscle

(Venugopal, 1990). Also, fish contain high water activity and high nutrient content that can support the growth of microbes (Abbas, Saleh, Mohamed, & Lasekan, 2009; Batt, 2016).

According to Ghaly et al. (2010), fish spoilage involves three mechanisms: enzymatic autolysis, oxidation, and microbial growth. Soon after fish are caught and killed, enzymatic and biological transformation occurs as a result of enzymatic disintegration of fish tissue. Autolysis of muscle proteins result in peptides and free amino acids which can contribute to microbial growth and production of biogenic amines (Ghaly, Dave, Budge, & Brooks, 2010).

Fish is especially vulnerable to enzyme degradation as well as lipid oxidation . Enzymatic degradation occurs on fish held at low temperatures because of the production of extracellular enzymes by psychrotrophic microbes. Previous research indicated that extracellular DNAse and protease production by bacteria contributed to the spoilage of catfish during refrigerated storage. (Hickey, Accumanno, McIntosh, Blank & Lee, 2015). Lipid oxidation can occur (A) enzymatically or (B) non-enzymatically. (A) Lipolysis (fat deterioration) occurs when enzymes, known as lipases, break down triglycerides and form free fatty acids. This results in "off flavors" or rancidity. (B) Non-enzymatic oxidation is caused by "hematin compounds" (hemoglobin, myoglobin and cytochrome) catalysis producing hydroperoxides. Denaturation of proteins occurs as a result of interactions between fatty acids produced by lipolysis and sarcoplasmic and myofibrillar proteins (Ghaly, Dave, Budge, & Brooks, 2010).

The major cause of fish spoilage is considered to be microbial growth (Hickey, Accumanno, McIntosh, Blank, & Lee, 2015; Rodríguez-Vaquero, Aredes-Fernández, & Manca de Nadra, 2013) during which amines, biogenic amines (putrescine, histamine and cadaverine), sulphides, alcohols, aldehydes and ketones are produced and unpleasant off-flavors develop

(Ghaly, Dave, Budge, & Brooks, 2010). Trimethylamine oxide (TMAO) is used to avoid dehydration and tissue waterlogging in marine and fresh water fish, respectively (Ghaly, Dave, Budge, & Brooks, 2010). Ammonia-like off-flavors are created by bacteria acquiring energy by the reduction of TMAO to trimethylamine (TMA) which serves as an indicator of microbial decay of fish (Ghaly, Dave, Budge, & Brooks, 2010). Such bacteria include *Shewanella putrifaciens*, *Aeromonas* spp., *Enterobacteriacceae*, *P. phosphoreum* and *Vibrio* spp. (Ghaly, Dave, Budge, & Brooks, 2010; Gram & Dalgaard, 2002).

Gram and Dalgaard (2002) describe the spoilage domain as the set of conditions (pH, temperature, water activity and atmosphere) that SSOs can thrive under to generate spoilage metabolites. The spoilage domain of SSOs can be influenced by fish feeding habits, geographical location, season, water temperature, location of fish harvesting, and storage conditions, including temperature and composition of the packaging atmosphere (Tryfinopoulou, Tsakalidou, & Nychas, 2002). The microflora of food products is specific and characteristic for the foods (Gram et al., 2002). Initially, the microflora of newly caught fish is composed of microbes on the skin, in the intestines and from the water in which the fish lived (Chaillou et al., 2015; Ghaly, Dave, Budge, & Brooks, 2010). The most frequently isolated bacteria from freshwater fish include: *Acinetobacter*, *Pseudomonas*, *Aeromonas*, *Flavobacterium*, *Shewanella*, *Micrococcus*, *Carnobacterium* and *Moraxella* spp. (Wang et al., 2017).

SSOs constitute a very small percentage of the total microflora on newly processed fish. Typically, SSOs are unique to the seafood host and can be dominated by a single species (Gram & Dalgaard, 2002). This changes as a result of processing, preservation, storage conditions and contamination during processing (Chaillou et al., 2015; Gram et al., 2002). At the point of microbial spoilage, the microflora consists of the dominant microbes that contributed to the

spoilage (SSOs) and the microbes that grew but did not participate in unpleasant changes (Gram et al., 2002; Pothakos, Devlieghere, Villani, Björkroth, & Ercolini, 2015). Interactions in the complex population results in spoilage activity that dictates the changes in the final product (Adreani & Fasolato, 2016).

Although microbes associated with food spoilage are dependent on the type of food (Batt, 2016), under the same conditions, similar microbes can surface in different food products (Gram et al., 2002). Meats and other animal products spoil due to Gram-negative bacteria (*Pseudomonas*, *Enterobacteriaceae*, *Shewanella*, *Moraxella*, *Acinetobacter*) and Gram-positive bacteria (lactic acid bacteria (LAB), *Brochothrix thermosphacta*, *Clostridium*, *Bacillus*) that became dominant under various conditions (Batt, 2016; Pothakos, Devlieghere, Villani, Björkroth, & Ercolini, 2015). Fish and other protein rich foods stored aerobically at low temperatures will be dominated by *Pseudomonas* spp. and other Gram-negative psychrotrophic bacteria (Gram et al., 2002). Specifically, different microbes can tolerate different preservation techniques (Gram & Dalgaard, 2002). *Shewanella* has been identified as an SSO of fish, especially marine fish, notably due to its altering of fish odor by trimethylamine (TMA) and H_2S production (Adreani & Fasolato, 2016). Previously, *S. putrefaciens* has been reported to dominate spoilage organisms in marine fish (Adreani & Fasolato, 2016; Gram et al., 2002; Wang et al., 2017) and other seafood, but recent studies (Adreani & Fasolato, 2016; Beaz-Hidalgo, Agüeria, Latif-Eugenín, Yeannes, & Figueras, 2015; Vogel, Venkateswaran, Satomi, & Gram, 2005) have identified *S. baltica* was the major H_2S-producing strain in iced marine fish. Both *S. baltica* and *S. putrefaciens* are among the most common spoilage bacteria found in refrigerated foods, including seafood and freshwater fish (Adreani & Fasolato, 2016; Beaz-Hidalgo, Agüeria, Latif-Eugenín, Yeannes, & Figueras, 2015).

Whereas microbial growth is understood to be a causative agent of seafood spoilage, the diversity, competition, and interactions within the spoilage communities are less understood (Chaillou et al., 2015). Bacterial interactions occur within and between spp while also reacting to extraneous stimulants from their surroundings (Stubbendieck, Vargas-Bautista, & Straight, 2016). Competing microbes can exist in the same environment with specific nutrient concentrations, however, under nutrient limiting conditions, particular microbes can be "outcompeted" for nutrients (Hibbing, Fuqua, Parsek, & Peterson, 2010). This exploitation can occur by consuming or sequestering nutrients, building up toxic waste products and producing secondary metabolites such as enzymes, antibiotics and toxins (Stubbendieck & Straight, 2016; Stubbendieck, Vargas-Bautista, & Straight, 2016). The latter is also to make used territory uninhabitable to trespassing competitors (Stubbendiecka & Straight, 2016). This approach, known as interference competition (Stubbendiecka & Straight, 2016), occurs when species directly antagonize one another (Kergourlay, Taminiau, Daube, & Verges, 2015). Microbes can also undertake intraspecies competition which in turn influences interspecies interactions including competitive approaches necessitating collaboration among individuals (Hibbing, Fuqua, Parsek, & Peterson, 2010). 'Social cheating' however can occur from this when individuals in populations benefit from cooperative traits that they themselves do not express (Hibbing, Fuqua, Parsek, & Peterson, 2010). Theoretically, this can be prevented if cheaters and cooperators can be differentiated or if groups that harbor cheaters are disadvantaged because they detract from the overall competitiveness (Hibbing, Fuqua, Parsek, & Peterson, 2010).

In short, investigating microbial diversity in foods in regards to food quality and safety would be beneficial for human health (Flores et al., 2013; Wang et al., 2017). Although this was traditionally implemented through culturing methods, it proved unreliable for the complete

microbial characterization of foods (Mayo et al., 2014). The environment of the foods and their storage conditions may make microbes physiologically viable but not cultivable. Additionally, low populations of microbes can be outcompeted by more abundant microbes, which might prevent their detection in culture. These limitations can result in underestimated microbial diversity, as well as possible failure to identify the major microbial groups (Mayo et al., 2014). Several culture independent methods were developed such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP), temporal temperature gradient gel electrophoresis (TTGE), single stranded conformation polymorphism (SSCP), real-time quantitative PCR (qPCR), the construction and analysis of 16S rRNA gene libraries, terminal restriction fragment length polymorphism (TRFLP) and others (Kergourlay, Taminiau, Daube, &Verges, 2015; Mayo et al., 2014,). These methods allow for accurate characterization of part of the microbial communities (Kergourlay, Taminiau, Daube, & Verges, 2015).

Next Generation Sequencing (NGS) technology or high-through-put sequencing have revolutionized microbial and environmental studies (Oulas et al., 2015). Next Generation Sequencing technology is able to analyze a higher number of nucleic acid sequences and therefore allows for a more comprehensive identification of microbial populations in a system (Mayo et al., 2015). This can be accomplished by detecting DNA fragments from the total DNA isolate (Panek et al., 2018).

In 2006, the Illumina sequencing platform was commercialized (van Dijk, Auger, Jaszczyszyn, & Thermes, 2014). DNA libraries are denatured and bound to the surface of a flow cell coated with adapter oligos. Bridge amplification occurs as the other end of the DNA fragment ‘bends over’ and hybridizes to a complementary adapter on the surface, and the

complementary strand synthesis begins. Clusters of 1000s of copies of single stranded DNA are synthesized after multiple cycles of amplification and denaturation. This form of chemistry is dubbed “sequence by synthesis” or SBS (Heather & Chain, 2016; van Dijk, Auger, Jaszczyszyn, & Thermes, 2014). This technology is less “error prone” in 16S metagenomics data analysis as there is no need for the time-consuming “noise” removal algorithms usually required for pyrosequencing platforms (Oulas et al., 2015). The Illumina platform typically produces 300 bp that can be expanded to 600 bp by paired end sequencing (sequencing from both ends of the DNA cluster) (Oulas et al., 2015; Reuter, Spacek, & Snyder, 2015). This “greater coverage” results in less errors (Oulas et al., 2015). Because of the technological advances made by the Illumina platform, the cost per sequencing run has greatly been reduced. As a result, Illumina dominates the HTS market and is the preferred platform for metagenomics studies (Mayo et al., 2014; Oulas et al., 2015; Reuter, Spacek, & Snyder, 2015).

CHAPTER III: MATERIALS & METHODS

A "fresh" commercial North American (*Ictalurus punctatus)* catfish fillet was acquired from a local retail source, cut up, and divided into bags for the control and three separate bags for catfish treated with natural preservatives (two weak acids and seed extract). The natural preservatives were drained prior to applying sterile saline solution to all "Day 1 bags" including the control. The other bags were stored at 4ºC for the remainder of the trial. The bags were stomached at medium speed. All the "juice" was pipetted out and centrifuged to collect any fish muscle at the bottom. Avoiding any fish muscle, the supernatant was removed and centrifuged to collect a bacteria pellet at the bottom. The supernatant was discarded and the pellet was washed in sterile saline solution twice and then stored at -80ºC until DNA was to be extracted. This process was repeated every other day until the bacterial growth curve reached the stationary phase which was Day 15.

The stomached "juice" was serially diluted with tryptic soy broth (TSB). Select dilutions were applied to Tryptic Soy Agar (TSA) (Carolina Biological Supply Company, Burlington, NC) plates in duplicate and incubated at 27ºC for 48 hours. Bacterial colonies were enumerated and Log CFU/g were calculated. Each day was plotted using Excel, and a bacteria growth curve was generated for the control and the three natural preservative treatments. This process was repeated every other day until the stationary phase was reached (Day 15).

Bacterial genomic DNA were extracted using a Microbial DNA Isolation kit before a Qubit fluorimeter 3.0 (Thermo Fisher Scientific, Waltham, MA) quantified them. PCR was also performed to confirm the DNA had been amplified (~550bp region).

Twelve ng of each DNA sample were thoroughly mixed with Kappa HiFi master-mix (Kappa Biosystems Ltd., Cape Town, South Africa), primers , and PCR water (MoBio, Carlsbad, CA). Using a thermal cycler (Bio-Rad Laboratories, Hercules, CA), a formulated PCR reaction was performed. The resulting PCR product was cleaned and confirmed on a bioanalyzer (Agilent Technologies, Santa Clara, CA). Then Nextera XT indices (Illumina Inc., San Diego, CA) were tagged to the DNA samples through second round PCR. The products were cleaned and confirmed using a bioanalyzer. The products were quantified by the Qubit fluorimeter, normalized and pooled to one micro centrifuge tube. Denatured samples and a PhIX was added to ensure quality.

Samples were loaded onto a v3 kit (Illumina Inc., San Diego, CA) and sequenced using an Illumina MiSeq (Illumina Inc., San Diego, CA) system. The sequencing run successfully finished after three days.

After the sequencing was complete, FASTQ files (raw sequencing data) were generated in the Cloud database and analyzed using two pipelines: BaseSpace and QIIME. The former is a web-based database and the latter is a Linux-based system. In both pipelines, the alpha and beta diversities were observed.

Standard deviation was calculated for the growth curves using Excel. α diversity was used to measure evenness and richness within individual samples. This includes Shannon diversity index, rarefaction curves and OTU number (Operational Taxonomical Unit). Weighted

and unweighted distance matrices were used for β diversity analysis among samples. These includes hierarchal clustering dendrograms and Principal Coordinate Analysis (Mayo et al., 2016).

The entire process was repeated with North American catfish from the Delaware State University Aquaculture facility. The catfish were humanely killed by the percussive "stunning and spiking" method before filleting. This method involves rendering the fish instantaneously insensible by a blunt instrument before spiking the fish in the brain with a sharp thin object. The catfish were then aseptically filleted using sterilized knives and cutting board. Fillets were transferred on ice to the food microbiology lab and were aseptically sampled as previously stated above. Pellets were acquired every other day and the bacterial growth curve was prepared until all the fish was used (Day 27).

CHAPTER IV: RESULTS

BACTERIAL GROWTH ON STORE-BOUGHT FISH

In store-bought catfish (Figure 1) on day 1, the control showed 6.5 Log CFU/g. After 30 min of marinating, the treatments showed bacterial reduction to 6.3, 5.8 and 5.7 Log CFU/g for N#2, N#3 and N#1 respectively. On subsequent days, an increasing gap developed between the treatment groups and the control. By the endpoint (Day 15), the control and N#3 treated samples entered the death phase while N#2 and N#1 treated samples were just entering the stationary phase.

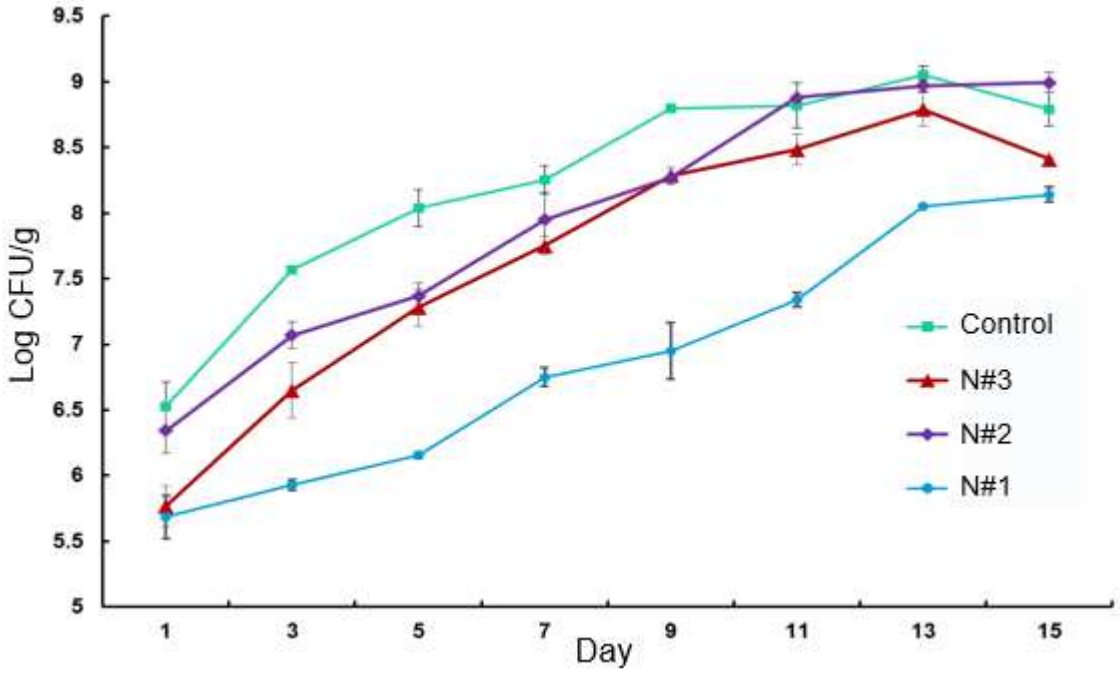

Figure 1. Total bacterial population of treated and untreated store-bought fish. Samples were applied to TSA plates in duplicate.

In pond-raised catfish (Figure 2) on day 1, the control contained 3.7 Log CFU/g. After marinating, the treatment samples showed bacterial reduction to 2.8, 2.5, and 2.8 Log CFU/g for N#2, N#3 and N#1 respectively. Unlike the commercial catfish, the control entered the stationary phase by day 11 and remained constant until the endpoint at day 27. As in the commercial fish trial, N#2 and N#3 samples had similar bacterial growth patterns. At the midpoint, both treatment samples showed the most antimicrobial activity by suppressing the bacterial growth to 5.1 Log CFU/g in comparison to the control at 6.7 Log CFU/g. The bacterial population on the N#1 treatment samples grew very slowly throughout the entire trial. At the endpoint (Day 27) the N#1 treatment had suppressed the growth of the bacterial population by almost 4 Log CFU/g compared to the control which contained 9.1 Log CFU/g. From the results, N#1 proved to be the most effective antimicrobial treatment for both the commercial and aquaculture catfish.

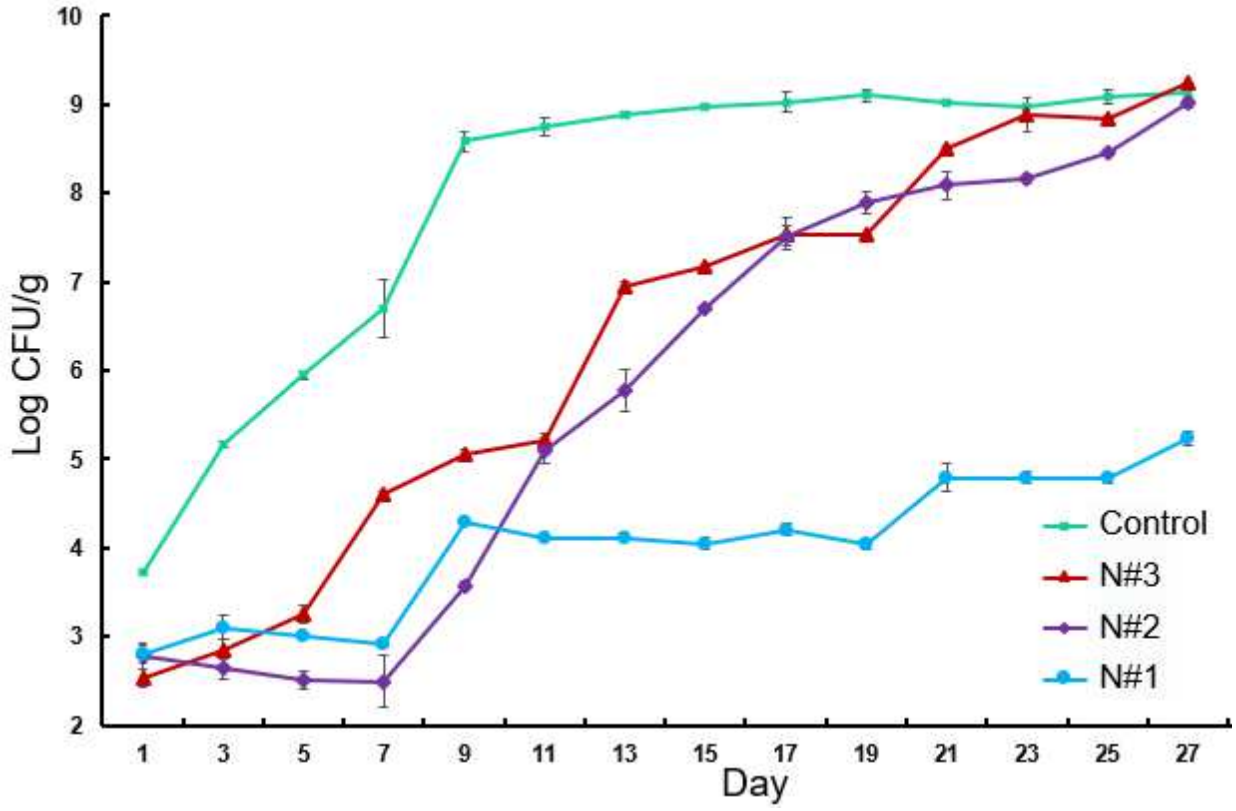

Figure 2. Total bacterial population of treated and untreated pond-raised fish. Samples were applied to TSA plates in duplicate.

OPERATIONAL TAXONOMIC UNITS OBSERVED

The rarefaction curves for the store-bought samples (Figure 3) showed high OTUs detected for all early phase samples and the middle phase N#1 sample. The OTUs detected decreased with the N#1 late phase sample being the lowest. The control middle and late phase samples were lower than the early phase samples but were still relatively high compared to the treatment samples. In contrast, the rarefaction curves for the pond-raised samples (Figure 4) showed the early phase samples and all N#1 samples detecting high OTUs.

The control, N#3, and N#2 middle and late phase samples decreased with the former being the highest. The control also showed less OTUs detected in the middle and late phase samples but were higher than the N#3 and N#2 samples in the same phases.

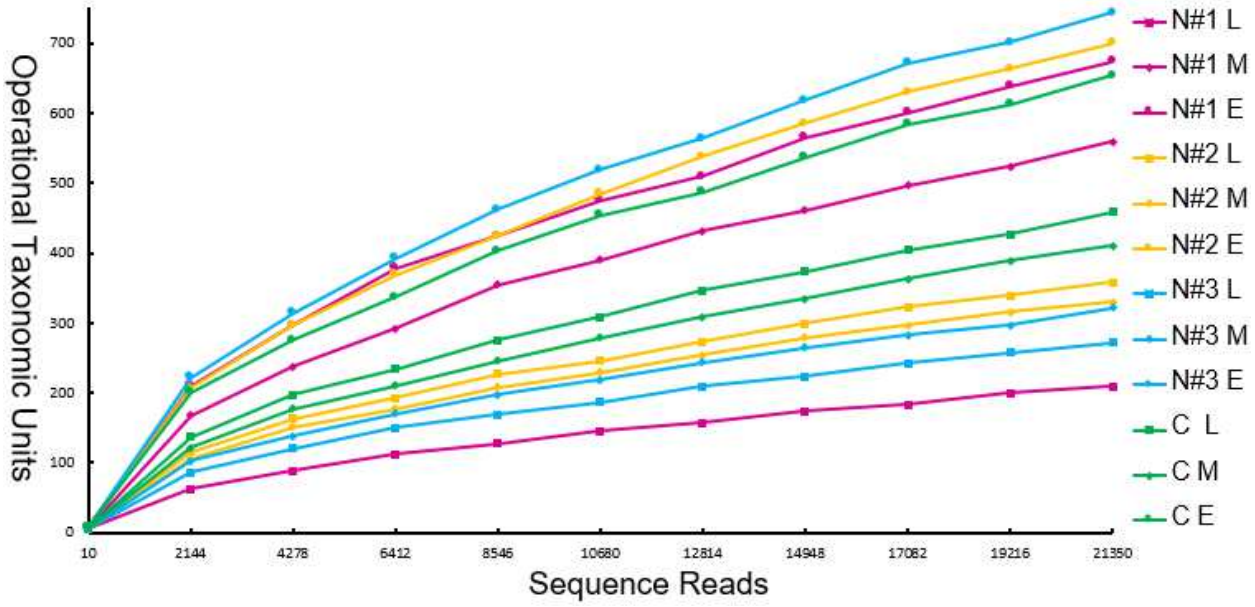

Figure 3. Rarefaction curve for store-bought samples. Samples ending in E correspond to the early phase; M correspond to the middle phase; L correspond to the late phase.

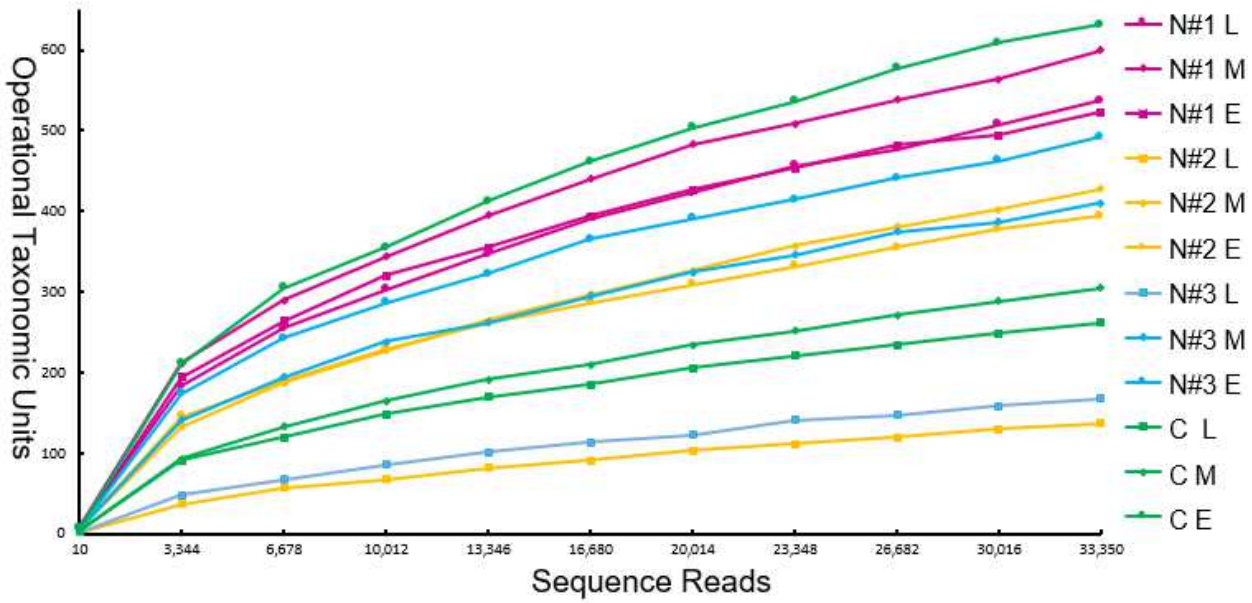

Figure 4. Rarefaction curve for pond-raised samples. Samples ending in E correspond to the early phase; M correspond to the middle phase; L correspond to the late phase.

SEQUENCING DATA

In the store-bought fish samples, the QIIME and BaseSpace were able to consistently classify the aggregate counts from the Phylum to the Genus level. The top 10 bacteria genera classified were mainly discussed (Figure 5 and Figure 6). On day 1, *Pseudomonas* accounted for over 60% of microbes in the control and ~50% of microbes in all treatment groups. By the midpoint, *Pseudomonas* spp. became the dominant spp. in the N#3 and N#2 treatments. The N#1 samples showed no increase in *Pseudomonas* spp. but increased in other microbes such as *Lactobacillus*, *Leuconostoc*, *Brochothrix* and *Flavobacterium*. By the endpoint, however, the *Psueodomonas* spp. dominated all treatment groups (>79%). In the control, *Pseudomonas* was abundant to a lesser extent and *Shewanella*, *Janthinobacterium* and *Flavobacterium* still present.

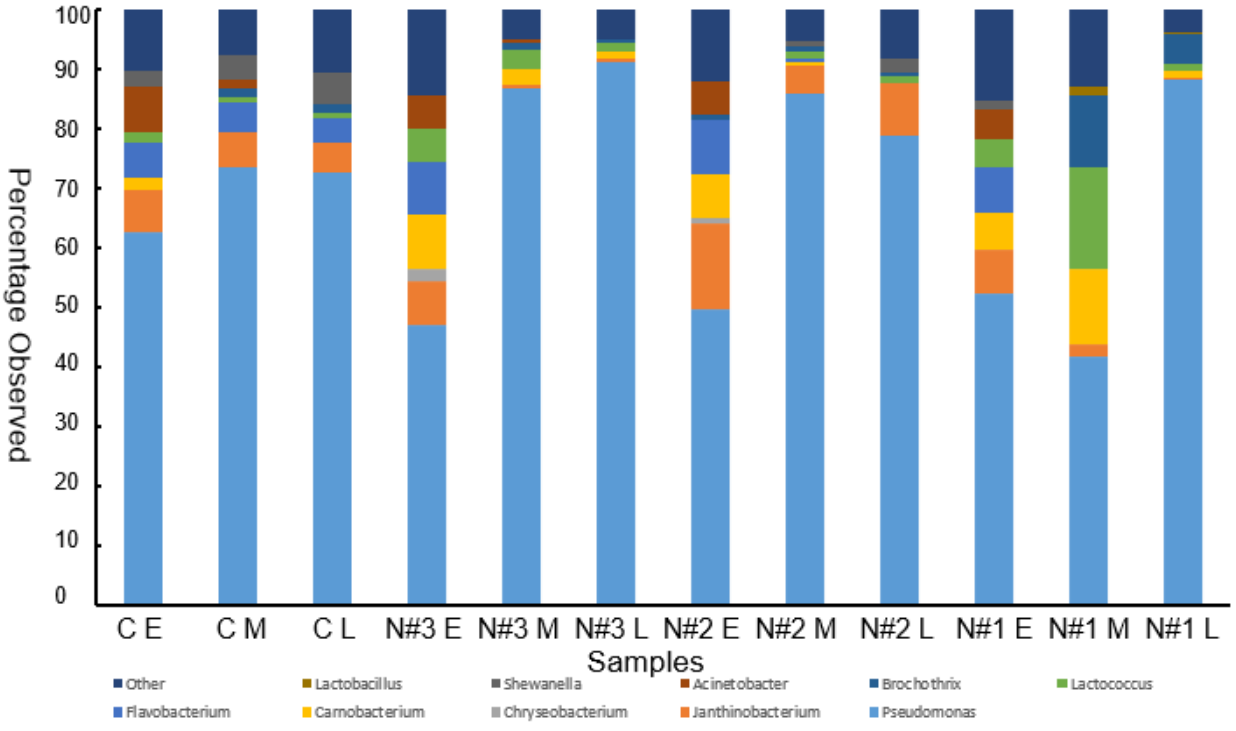

Figure 5. Metagenomics application illustrates the Genus classification of store-bought fish samples for the early, middle, and late phases for the control and treatments.

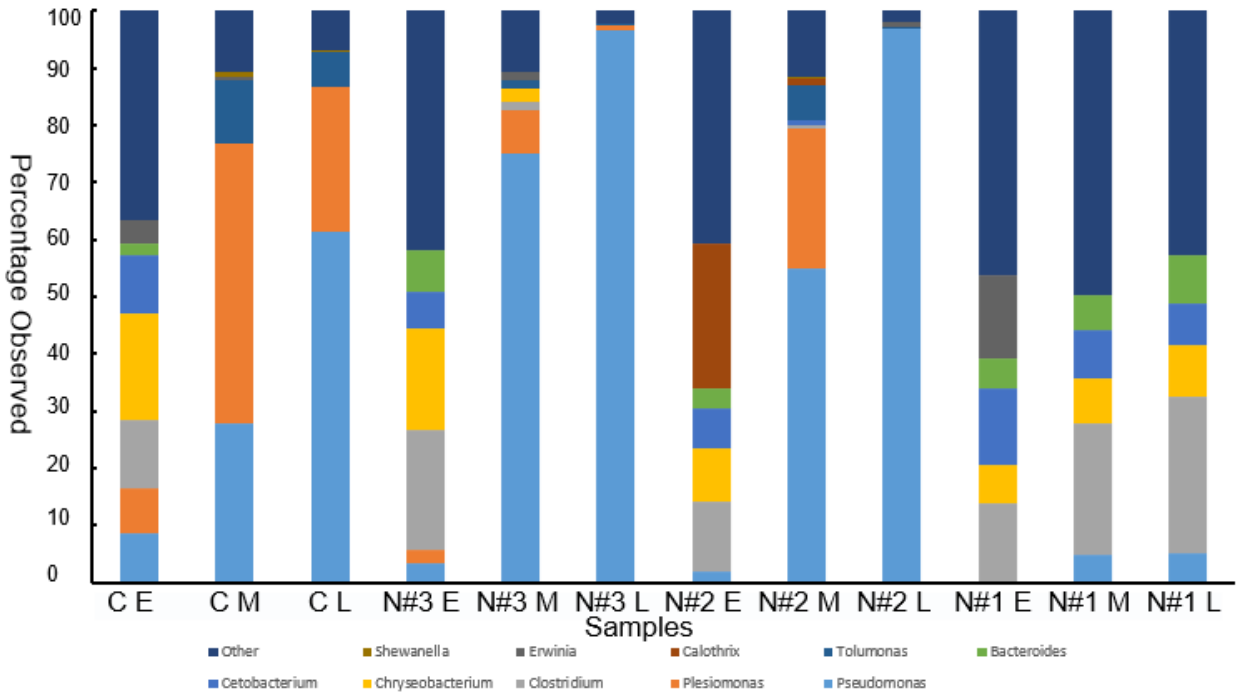

Figure 6. Metagenomics application illustrates the Genus classification of pond-raised fish samples for the early, middle, and late phases for the control and treatments.

In store-bought samples, the 16S metagenomics application in QIIME (Figure 7) was able to classify the aggregate counts from Phylum to the Genus level but was unable to classify to the Genus level for many of the pond-raised samples (Figure 8). The BaseSpace data will be the focal point for the discussion and the QIIME data will act as complementary data.

In comparison to the store-bought catfish, the control initially showed *Chryseobacterium* spp. as its dominant spp. but the population shifted over time, ending with *Pseudomonas* spp as the most abundant microbe followed by *Plesiomonas* and *Tolumnas* spp.

The N#3 treatment group showed *Clostridium* and *Chryseobacterium* spp. as the most abundant classification on day 1. In the N#2 treated samples, *Clostridium* and *Chryseobacterium* spp. were also abundant but *Calothrix* was highest on day 1. As in the commercial samples,

Pseudomonas became the dominant bacteria by the midpoint followed by *Plesiomonas* and *Tolumnus*. By the endpoint, *Pseudomonas* was still the dominant spp . The N#1 treatment samples had a very diverse bacterial profile with *Erwinia* as the most abundant bacteria in the early phase. As time progressed in the N#1 sample, the bacterial population did not change with the exception of *Clostridium* becoming the most abundant genus present. Interestingly, *Pseudomonas* never became the dominant genus in the N#1 samples and only reached ~5% in the total bacterial population. At the endpoint, the bacterial profile for the N#1 samples remained fairly similar to the early phase.

The QIIME data differed from BaseSpace in the control, in that with the exception of the *Pseudomons* classification, the majority of bacteria were classified as belonging to the *Aeromonadaceae* family. Also, across all samples *Clostridium* counts were very low. This differed from BaseSpace where *Clostridium* was a major bacterium in the N#1 samples. Instead, the N#3, N#2 and N#1 samples showed a bacteria belonging to the Class CK-1C4-19. Also, the N#2 samples showed no detection of *Calothrix*. Instead, *Streptophyta* was detected. Other samples were classified as belonging to the *Bacteroidaceae* and *Pseudomonadaceae* families.

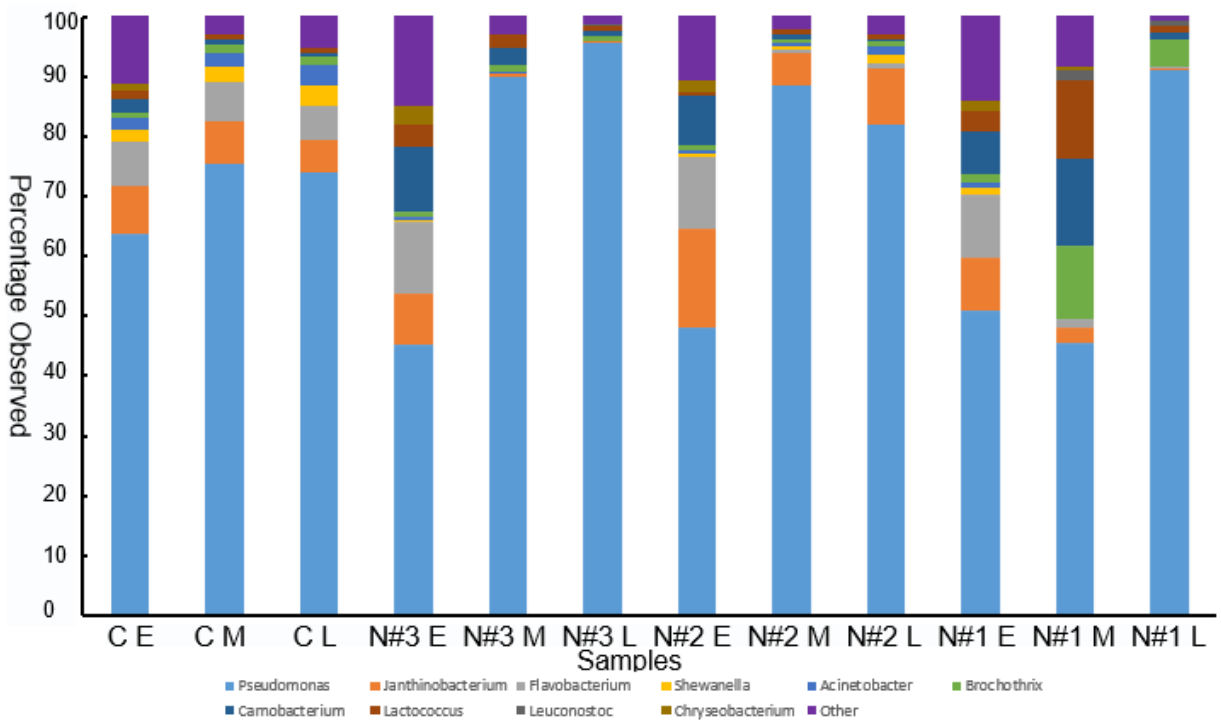

Figure 7. Metagenomics application in QIIME illustrates the Genus classification of store-bought fish samples for the early, middle, and late phases for the control and treatments.

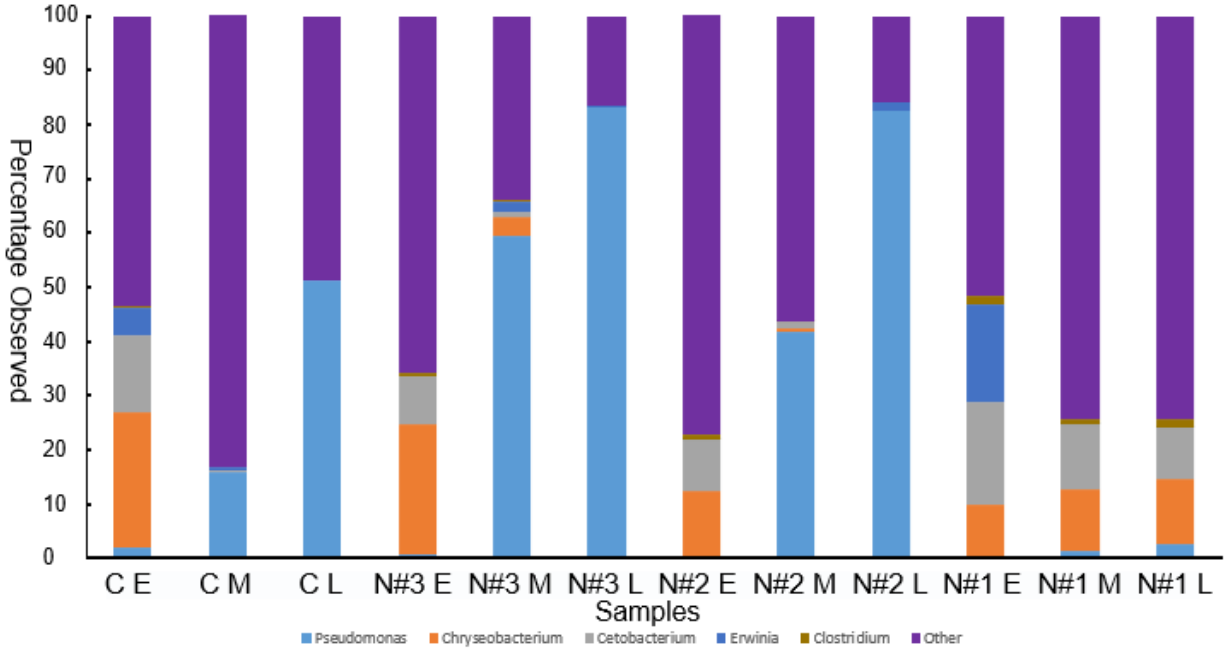

Figure 8. Metagenomics application in QIIME illustrates the Genus classification of pond-raised fish samples for the early, middle, and late phases for the control and treatments.

DIVERSITY INDEX

In the store-bought control samples (Table 1), the diversity scores remained relatively high for all phases. In the store-bought treatment samples, lower scores were seen as times passed. In the pond-raised (Table 2) control samples, the diversity score was high in the early phase and decreased slightly during the middle and late phases. In the pond-raised N#3 and N#2 treatment samples, the results were comparable to the store-bought treatment samples. The diversity was high on the initial day but decreased drastically as time passed. The pond-raised N#1 treatment samples showed only a slight decrease from day 1 to the middle and late phase days.

Table 1: Shannon Diversity Index for store-bought fish samples for the early, middle, and late phases.

Sample ID	Shannon Species Diversity
N#1 Early	2.867
N#1 Middle	2.276
N#1 Late	1.044
N#2 Early	2.838
N#2 Middle	1.707
N#2 Late	1.901
N#3 Early	2.964
N#3 Middle	1.604
N#3 Late	1.405
C Early	2.599
C Middle	2.112
C Late	2.305

Table 2: Shannon Diversity Index for pond-raised fish samples for the early, middle, and late phases.

Sample ID	Shannon Species Diversity
N#1 Early	2.747
N#1 Middle	2.433
N#1 Late	2.490
N#2 Early	2.294
N#2 Middle	1.732
N#2 Late	0.897
N#3 Early	2.592
N#3 Middle	1.723
N#3 Late	0.919
C Early	2.674
C Middle	1.438
C Late	1.880

HEIRARCHAL CLUSTERING DENDROGRAM

The clustering dendrogram (Figure 9) split the samples into 2 main branches with subgroups beneath. Beneath the left branch, the day 1 pond-raised samples for the control, N#3, and N#2 treatment were grouped with all the N#1 samples. On the other branch, the rest of the pond-raised samples are clustered together separate from the store-bought samples. The store-bought samples were divided into 3 clusters. The first contained the initial days for the treatment samples (N#2, N#3 and N#1) with the middle phase for N#1. The next cluster contains the middle/late phases for N#3 and N#2 treated samples with the late phase for N#1. The last cluster contained all the phases for the control.

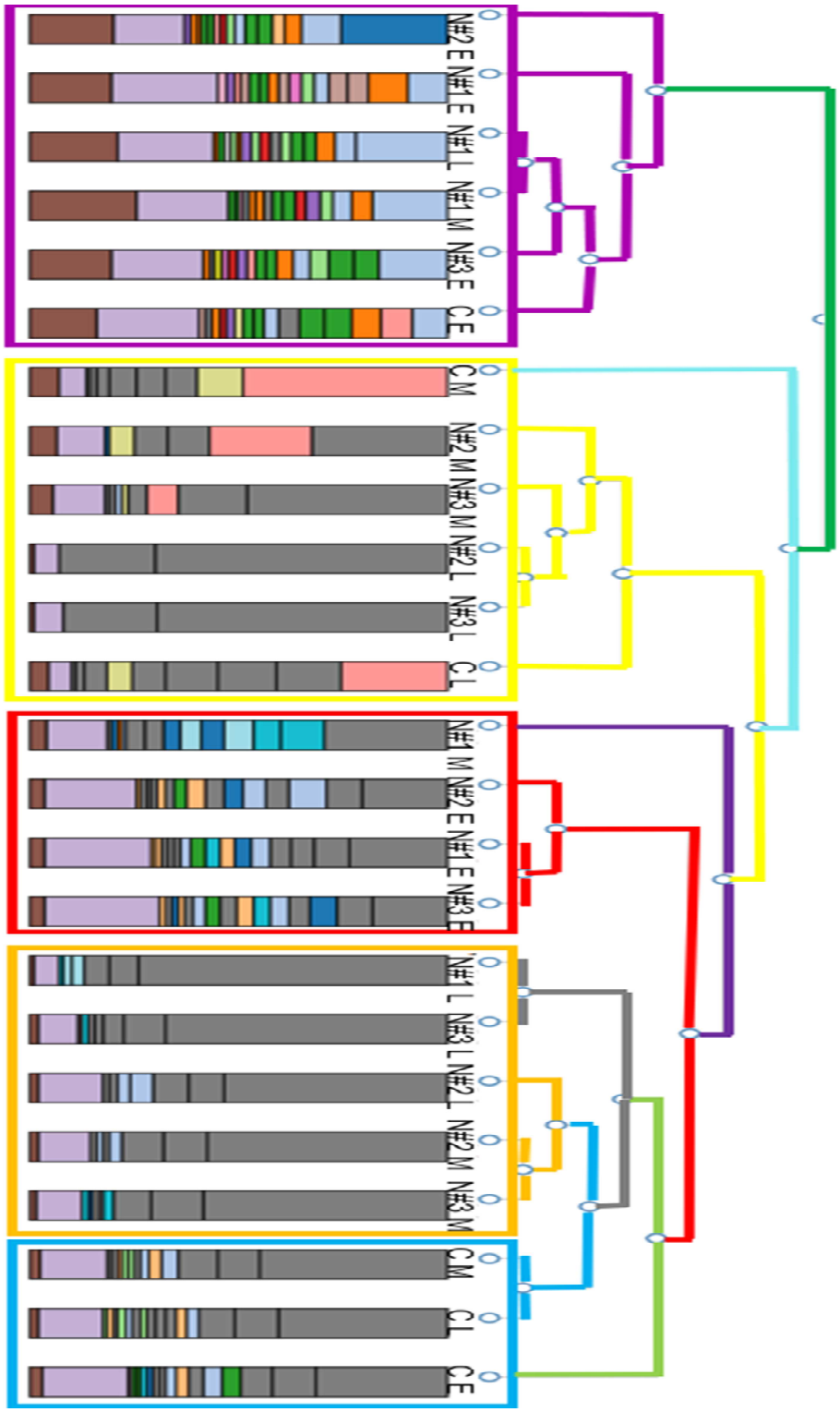

Figure 9. Hierarchal clustering dendrogram of treated and untreated store-bought and pond-raised catfish.

DISTANCE MATRIX OF MICROBIOME BETWEEN CATFISH SAMPLES

In the weighted Principal Coordinate Analysis (PCoA) (Figure 10), Beta diversity was observed between samples. All the pond-raised N#1 treated samples were grouped together with the early day samples for the control, N#2, and N#3 samples. The middle and late phase samples for the control, N#3, and N#2 samples remained close together as well. In the store-bought samples, all the treatment samples for day 1 were grouped together with the middle phase N#1 samples. All the control samples were grouped together. Finally, the middle and late phase samples for N#3 and N#2 were grouped together with the late phase sample for N#1. In the unweighted analysis (Figure 11), similar grouping was seen.

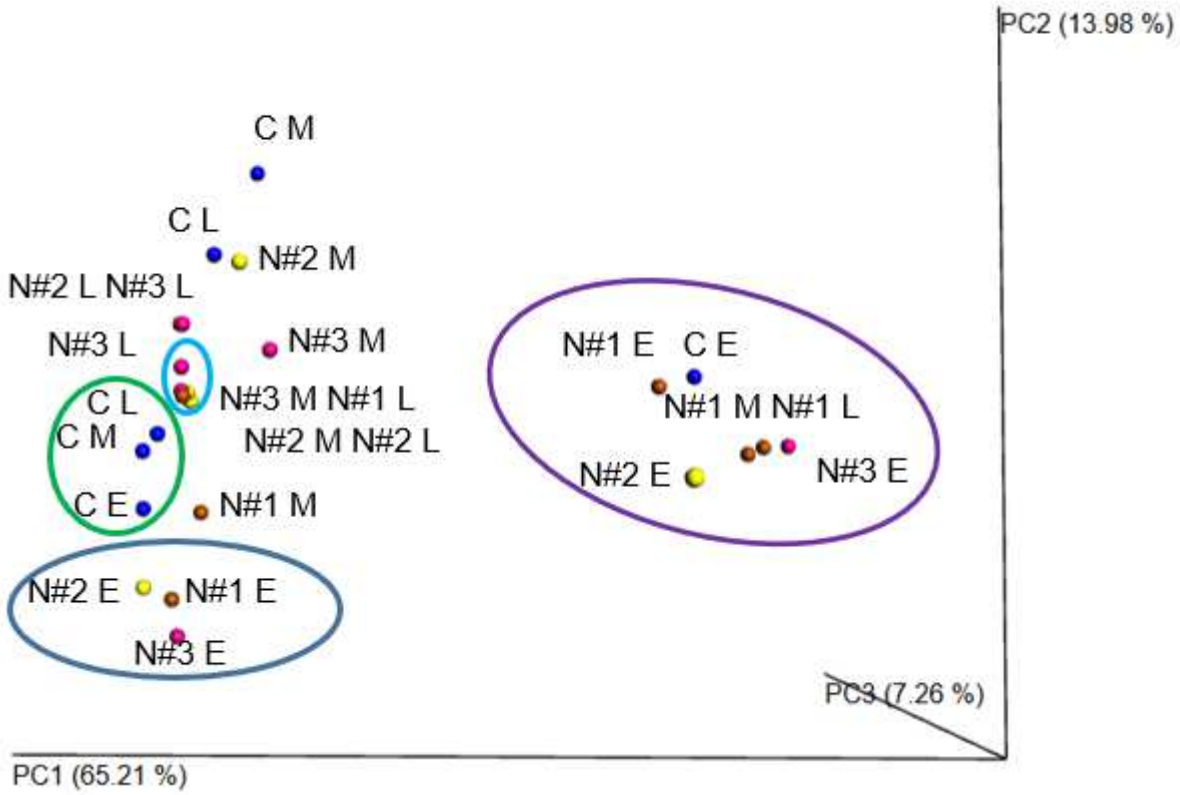

Figure 10. The Weighted PCoA for all samples shows distances based on similarities. The control (C) samples are in blue. The N#1 samples are in brown. The N#2 samples are in yellow. The N#3 samples are in pink.

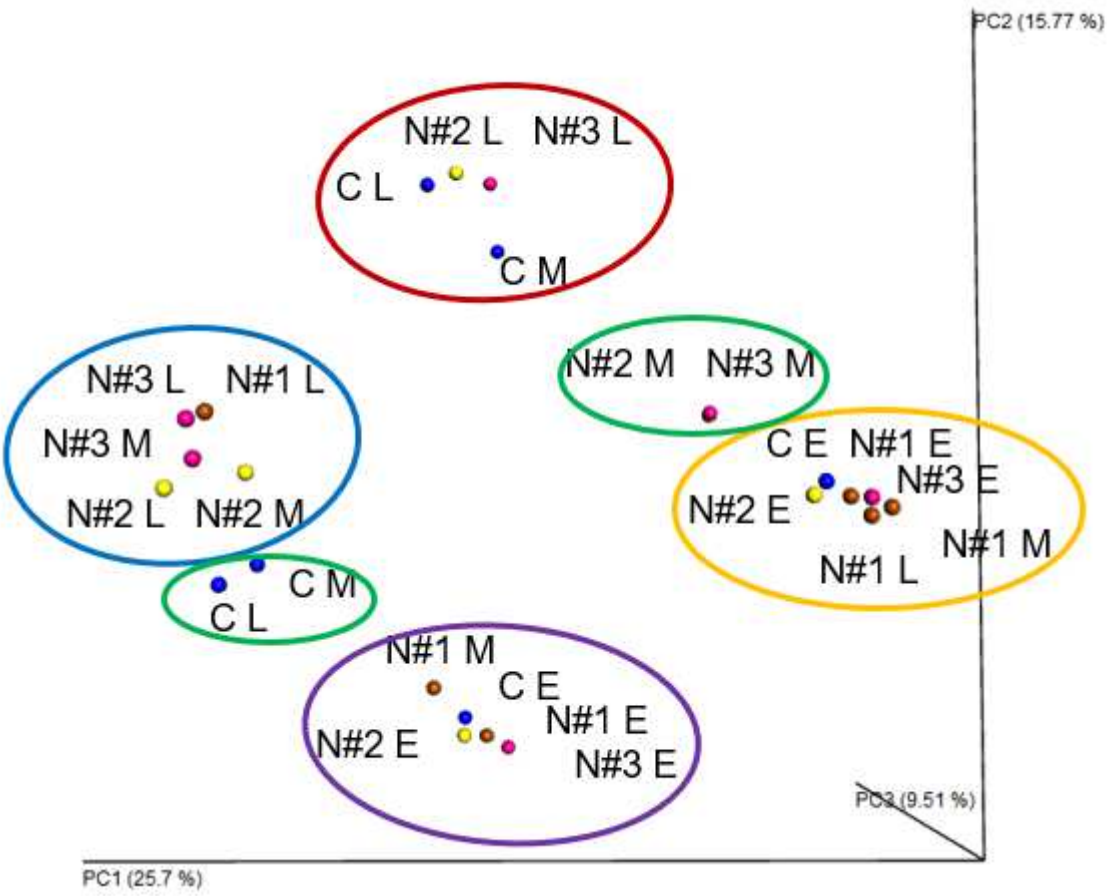

Figure 11. The Unweighted PCoA for all fish samples shows distances based on similarities. The control (C) samples are in blue. The N#1 samples are in brown. The N#2 samples are in yellow. The N#3 samples are in pink.

CHAPTER V: DISCUSSION

Although N#2 and N#3 treatments showed antimicrobial activity, N#1 treated samples of catfish showed the most significant bacterial reduction of 2 Log CFU/g for store-bought fish and 5 Log CFU/g for the pond-raised fish. It is suspected that the enhanced effectiveness of the N#1 treatment on the pond-raised catfish would be due to less bacterial load initially present on the pond-raised catfish compared to the store-bought catfish. In addition, the different bacteria present on the pond-raised catfish may be more susceptible to N#1 treatment.

Past research (Lingham, Besong, Ozbay, & Lee, 2012) reported that retail catfish treated with malt vinegar showed little bacterial proliferation remaining constant at approximately 6 Log CFU/g. When the acetic acid concentration was reduced to 0.3%, the bacterial pattern was very similar to the control with little bacterial reduction (Lingham, Besong, Ozbay, & Lee, 2012).

In the rarefaction curves, all early phase samples had very high OTUs (bacterial spp.). Initially, many observed genera could be identified, but, as the spoilage bacteria, including *Pseudomonas* increased during storage, other genera are observed less and the rarefaction curves diminish. This is reflected in the store-bought and pond-raised N#3 and N#2 treatment samples but only the N#1 samples from the store-bought fish. In the pond-raised N#1 samples, the high OTU's reflected the many bacterial spp. still present in the middle and late phases.

In the store-bought fish, N#3 and N#1 were able to suppress the growth of *Shewanella* and *Acientobacter* spp. In the pond-raised fish, N#3 and N#2 were able to suppress the growth of *Erwinia* spp. The treatments however were not as effective on suppressing the growth of *Pseudomonas* spp. *Pseudomonas*, ubiquitous (Adreani & Fasolato, 2016) as they are found in a variety of environments such as soil, water, plants, animal tissues, foods, etc. (Caldera et al.,

2016), are also able to adapt to various conditions (Adreani & Fasolato, 2016) due to their complex enzymatic systems (Caldera et al., 2016). Although they are aerobic, some are able to thrive with nitrate, fumarate or other electron acceptors in oxygen depleted environments (Adreani & Fasolato, 2016). Also, they are able to compete with other bacteria for iron due to their production of iron-binding siderophores. This is especially useful in iron limited foods such as fish (Adreani & Fasolato, 2016; Gram & Dalgaard, 2002). *Pseudomonas* spp. have been classified as SSOs in chilled meat (Gu, Sun, Tu, Dong, & Pan, 2016) and other protein-rich food products such as dairy products, eggs and freshwater fish (Adreani & Fasolato, 2016). In refrigerated meats, *Pseudomonas* have become the dominant bacteria and contributed significantly to spoilage due to their ability to break down glucose and amino acids (Ercolini, Russo, Torrieri, Masi, & Villani, 2006). According to Stanborough et al. (2018), *P. fragi* contribute to the spoilage of fish, meat products and ultra-pasteurized dairy products. *P. lundensis, P. fluorescens*, and *P. gessardii* also contribute to the spoilage of milk products (Adreani & Fasolato, 2016).

In our study we also identified several lactic acid bacteria (LAB) that contributed to catfish spoilage. LAB and *Carnobacterium*, can dominate the microflora of chilled fish products in vacuum packs when treated with salt (NaCl) and acids (Gram & Dalgaard, 2002). *Lactobacillus* are able to grow in seafood treated with acids and other chemical preservatives (Gram & Dalgaard, 2002). *Leuconostoc* also contributes to refrigerated raw meat spoilage (Ercolini, Russo, Torrieri, Masi, & Villani, 2006). Under specific conditions, LAB are also competitors of other spoilage-related microbial groups (Ercolini, Russo, Torrieri, Masi, & Villani, 2006).

Other notable spoilage bacteria such as *Brochothrix* and *Shewanella* spp were also identified in this study. *Brochothrix thermosphacta* has commonly been isolated aerobically and anaerobically from meats but also from spoiled fish and seafood (Ercolini, Russo, Torrieri, Masi, & Villani, 2006; Remenant, Jaffrès, Dousset, Pilet, & Zagorec, 2015). At the expense of other bacteria such as *Carnobacterium*, *Lactobacillus* or *Leuconostoc*, *B*. *thermosphacta* has been reported to become the dominating organism in modified atmosphere and vacuum packaged meat products. *Shewanella* has been found in freshwater and marine environments. It also causes spoilage of food products such as poultry, fish and other meat products (Adreani & Fasolato, 2016).

The major taxonomy that were identified through QIIME in the pond-raised samples that were not in agreement with the data from BaseSpace included the class CK-1C4-19 and *Streptophyta* genus. The class CK-1C4-19 has been identified in samples from the gut of fathead minnow fish (Narrowe et al., 2015) and zebrafish (Roeselers et al., 2011). *Streptophyta* is an alga found in freshwater and marine environments (Becker & Marin, 2009).

Hickey et al. (2015) suggested in a previous study that rapid deterioration of store-bought fish in comparison to pond-raised fish at refrigerated storage is due to mishandling of tissue during processing. The different sources of the catfish samples can also influence the taxonomy of bacterial composition detected from the samples in addition to the change due to the application of natural preservatives.

The Shannon index (H') is based on uncertainty. Higher values are assigned to unknown individuals that could belong to any species in a highly diverse, evenly distributed system. The control samples in both the store-bought and pond-raised fish showed high diversity that

decreased slightly after day 1, reflected by the control bacterial profile staying relatively stable throughout storage. Interestingly, the pond-raised N#1 treated samples showed very little decrease in diversity values and this complements the taxonomic data where many of the same species identified on day 1 were seen on the final day in similar proportions.

Unifrac was used for studying weighted and unweighted Principal Coordinate Analysis (PCoA). It provides relative abundance of different bacterial species and is essential for tracking changes in the bacterial community and determines the presence or absence of bacteria in β-diversity (Lozupone, Hamady, Kelley, & Knight, 2007). In both the weighted and unweighted PCoA, groupings were seen that reflected similarity between samples.

In the hierarchal clustering dendrogram, the first cluster contained the pond-raised early phase samples and the N#1 middle and late phase samples. This shows that the N#1 samples were all very similar while the other pond-raised treated and control samples became dissimilar over time. The store-bought samples also grouped with early phase treatment samples together with the N#1 middle phase sample. This showed that they were similar. The middle and late phases for store-bought N#3 and N#2 samples grouped with the late phase store-bought N#1 sample. The store-bought control samples were very similar through all phases and this was reflected in their being grouped by themselves.

In conclusion, of the 3 treatments, N#1 showed the most significant bacteria reduction at the midpoint in the store-bought fish but also in the pond-raised fish in the late phase. The control had the highest diversity in the store-bought fish and the pond-raised fish but the N#1 samples also had high diversity scores in the pond-raised fish samples. This knowledge can suggest effective natural preservative treatments to extend the shelf-life of fishery products.

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APPENDIX

N#3 Store-bought

Top Kingdom Classification Results

Classification	Number of Reads	%Total Reads
Bacteria	186,028	99.93%
Unclassified at Kingdom Level	118	0.06%
Viruses	4	00.0%

Top Phylum Classification Results

Classification	Number of Reads	% Total Reads
Proteobacteria	121,323	65.17%
Firmicutes	33,416	17.95%
Bacteroidetes	26,139	14.04%
Actinobacteria	2,609	1.4%
Fusobacteria	1,742	0.94%
Unclassified at Phylum Level	715	0.38%
Cyanobacteria	59	0.03%
Verrucomicrobia	42	0.02%

Top Class Classification Results

Classification	Number of Reads	% Total Reads
Gammaprotobacterium	104,216	55.98%
Bacilli	32,536	17.48%
Flavobacteriia	21,270	11.43%
Betaproteobacteria	16,004	8.60%
Bacteroidia	3,022	1.62%
Actinobacteria	2,603	1.40%
Fusobacteria	1,742	0.94%
Unclassified at Class level	1,599	0.86%

N#3Store-bought

Top Kingdom Classification Results

Classification	Number of Reads	%Total Reads
Bacteria	189,573	99.91%
Unclassified at Kingdom Level	173	0.09%
Viruses	2	0.00%

Top Phylum Classification Results

Classification	Number of Reads	%Total Reads
Proteobacteria	173,949	91.67%
Firmicutes	14,921	7.86%
Unclassified at Phylum Level	439	0.23%
Bacteroidetes	210	0.11%
Actinobacteria	93	0.05%
Fusobacteria	42	0.02%
Nitrospirae	42	0.02%
Tenericutes	24	0.01%

Top Class Classification Results

Classification	Number of Reads	% Total Reads
Gammaprotobacterium	172,141	90.72%
Bacilli	14,773	7.79%
Betaproteobacteria	1,272	0.67%
Unclassified at Class level	761	0.4%
Alphaproteobacteria	244	0.13%
Flavobacteriia	167	0.09%
Actinobacteria	92	0.05%
Clostridia	86	0.05%

N#3 Store-bought

Top Kingdom Classification Results

Classification	Number of Reads	%Total Reads
Bacteria	125,006	99.84%
Unclassified at Kingdom Level	126	0.10%
Viruses	72	0.06%

Top Phylum Classification Results

Classification	Number of Reads	%Total Reads
Proteobacteria	119,565	95.5%
Firmicutes	4,990	3.99%
Unclassified at Phylum Level	247	0.2%
DNA	126	0.1%
Bacteroidetes	118	0.09%
Actinobacteria	91	0.07%
Nitrospirae	41	0.03%
Tenericutes	6	0.00%

Top Class Classification Results

Classification	Number of Reads	% Total Reads
Gammaprotobacterium	118,129	94.35%
Bacilli	4,913	3.92%
Betaproteobacteria	893	0.71%
Unclassified at Class Level	539	0.43%
Alphaproteobacteria	250	0.2%
Group II	126	0.1%
Flavobacteriia	126108	0.09%
Actinobacteria	88	0.07%

N#2 Store-bought

Top Kingdom Classification Results

Classification	Number of Reads	%Total Reads
Bacteria	236,435	99.94%
Unclassified at Kingdom Level	128	0.05%
Viruses	6	0.0%

Top Phylum Classification Results

Classification	Number of Reads	% Total Reads
Proteobacteria	181,280	76.63%
Bacteroidetes	27,152	11.48%
Firmicutes	24,574	10.39%
Actinobacteria	1,893	0.8%
Unclassified at Phylum Level	724	0.31%
Cyanobacteria	484	0.20%
Fusobacteria	334	0.14%
Tenericutes	51	0.02%

Top Class Classification Results

Classification	Number of Reads	% Total Reads
Gammaprotobacterium	139,977	59.17%
Betaproteobacteria	39,770	16.81%
Flavobacteria	25,496	10.78%
Bacilli	24,149	10.21%
Actinobacteria	1,891	0.8%
Unclassified at Class Level	1,643	0.69%
Sphingobacteria	1,280	0.54%
Alphaproteobacteria	719	0.3%

N#2 Store-bought

Top Kingdom Classification Results

Classification	Number of Reads	%Total Reads
Bacteria	190,667	99.91%
Unclassified at Kingdom Level	173	0.09%
Viruses	2	0.00%

Top Phylum Classification Results

Classification	Number of Reads	%Total Reads
Proteobacteria	182,923	95.85%
Firmicutes	5,919	3.1%
Bacteroidetes	1,267	0.66%
Unclassified at phylum level	417	0.22%
Actinobacteria	144	0.08%
Fusobacteria	54	0.03%
Nitrospirae	46	0.02%
Cyanobacteria	38	0.02%

Top Class Classification Results

Classification	Number of Reads	% Total Reads
Gammaprotobacterium	171,673	89.96%
Betaproteobacteria	10,542	5.52%
Bacilli	5,758	3.02%
Flavobacteriia	1,155	0.61%
Unclassified at Class Level	843	0.44%
Alphaproteobacteria	320	0.17%
Actinobacteria	144	0.08%
Clostridia	112	0.06%

N#2 Store-bought

Top Kingdom Classification Results

Classification	Number of Reads	%Total Reads
Bacteria	214,754	99.89%
Unclassified at Kingdom Level	134	0.06%
Viruses	106	0.05%

Top Phylum Classification Results

Classification	Number of Reads	%Total Reads
Proteobacteria	207,361	96.45%
Firmicutes	5,4110	2.52%
Bacteroidetes	1,659	0.77%
Unclassified at Phylum Level	306	0.14%
DNA	134	0.06%
Actinobacteria	61	0.03%
Nitrospirae	23	0.01%
Tenericutes	10	0.00%

Top Class Classification Results

Classification	Number of Reads	% Total Reads
Gammaprotobacterium	185,014	86.06%
Betaproteobacteria	21,658	10.07%
Bacilli	5,325	2.48%
Flavobacteriia	1,582	0.74%
Unclassified at Class level	730	0.34%
Alphaproteobacteria	264	0.12%
Group II	134	0.06%
Sphingobacteriia	72	0.03%

N#1 Store-bought

Top Kingdom Classification Results

Classification	Number of Reads	%Total Reads
Bacteria	189,179	99.9%
Unclassified at Kingdom Level	184	0.1%
Viruses	3	0.%

Top Phylum Classification Results

Classification	Number of Reads	%Total Reads
Proteobacteria	135,391	71.5%
Firmicutes	26,907	14.21%
Bacteroidetes	22,128	11.69%
Fusobacteria	2,198	1.16%
Actinobacteria	1,759	0.93%
Unclassified at Phylum Level	810	0.43%
Tenericutes	45	0.02%
Cyanobacteria	36	0.02%

Top Class Classification Results

Classification	Number of Reads	% Total Reads
Gammaprotobacterium	117,477	62.04%
Bacilli	25,675	13.56%
Flacobacteriia	17,449	9.21%
Betaproteobacteria	16,693	8.82%
Bacteroidia	2,880	1.52%
Fusobacteria	2,198	1.16%
Unclassified at Class level	1,776	0.94%
Actinobacteria	1,758	0.93%

N#1 Store-bought

Top Kingdom Classification Results

Classification	Number of Reads	%Total Reads
Bacteria	187,636	99.98%
Unclassified at Kingdom Level	41	0.02%
Viruses	4	0%

Top Phylum Classification Results

Classification	Number of Reads	%Total Reads
Proteobacteria	86,856	46.28%
Firmicutes	95,496	50.88%
Bacteroidetes	3,528	1.88%
Fusobacteria	419	0.22%
Actinobacteria	467	0.25%
Unclassified at Phylum Level	818	0.44%
Tenericutes	31	0.02%
Nitrospirae	28	0.01%

Top Class Classification Results

Classification	Number of Reads	% Total Reads
Gammaprotobacterium	81,532	43.44%
Bacilli	94,901	50.57%
Flavobacteriia	2,497	1.33%
Betaproteobacteria	4,730	2.52%
Bacteroidia	688	0.37%
Fusobacteria	419	0.22%
Unclassified at Class level	1,319	0.7%
Actinobacteria	463	0.25%

N#1 Store-bought

Top Kingdom Classification Results

Classification	Number of Reads	%Total Reads
Bacteria	137,652	99.91%
Unclassified at Kingdom Level	78	0.06%
Viruses	51	0.04%

Top Phylum Classification Results

Classification	Number of Reads	%Total Reads
Proteobacteria	124,141	90.10%
Firmicutes	12,963	9.41%
Bacteroidetes	323	0.23%
DNA	78	0.06%
Actinobacteria	13	0.01%
Unclassified at Phylum Level	202	0.15%
Tenericutes	19	0.01%
Nitrospirae	24	0.02%

Top Class Classification Results

Classification	Number of Reads	% Total Reads
Gammaprotobacterium	123,321	89.51%
Bacilli	12,852	9.33%
Flavobacteriia	308	0.22%
Betaproteobacteria	444	0.32%
Clostridia	87	0.06%
Alphaproteobacteria	168	0.12%
Unclassified at Class level	411	0.3%
Group II	78	0.06%

N#3 Pond-Raised

Top Kingdom Classification Results

Classification	Number of Reads	%Total Reads
Bacteria	178,888	92.8%
Unclassified at Kingdom Level	13,886	7.2%
Viruses	1	0%

Top Phylum Classification Results

Classification	Number of Reads	%Total Reads
Proteobacteria	34,893	18.1%
Firmicutes	64,000	33.2%
Bacteroidetes	57,939	30.06%
Unclassified at Phylum Level	16,747	8.69%
Fusobacteria	14,406	7.47%
Actinobacteria	1,439	0.75%
Cyanobacteria	1,229	0.64%
Verrucomicrobia	1,136	0.59%

Top Class Classification Results

Classification	Number of Reads	% Total Reads
Gammaprotobacterium	18,901	9.8%
Clostridia	44,083	22.87%
Flavobacteriia	35,528	18.43%
Unclassified at Class Level	26,323	13.65%
Bacteroidia	19,543	10.14%
Fusobacteria	14,406	7.47%
Betaproteobacteria	10,329	5.36%
Bacilli	7,016	3.64%

N#3 Pond-Raised

Top Kingdom Classification Results

Classification	Number of Reads	%Total Reads
Bacteria	191,492	97.71%
Unclassified at Kingdom Level	4,492	2.29%
Viruses	4	0%

Top Phylum Classification Results

Classification	Number of Reads	%Total Reads
Proteobacteria	177,784	90.71%
Firmicutes	5,565	2.84%
Bacteroidetes	5,736	2.93%
Unclassified at Phylum Level	4,990	2.55%
Fusobacteria	1,250	0.64%
Actinobacteria	93	0.05%
Cyanobacteria	262	0.13%
Tenericutes	122	0.06%

Top Class Classification Results

Classification	Number of Reads	% Total Reads
Gammaprotobacterium	176,300	89.95%
Clostridia	4,323	2.21%
Flavobacteriia	4,288	2.19%
Unclassified at Class Level	5,944	3.03%
Bacteroidia	1,277	0.65%
Fusobacteria	1,250	0.64%
Alphaproteobacteria	577	0.29%
Bacilli	571	0.29%

N#3 Pond-Raised

Top Kingdom Classification Results

Classification	Number of Reads	%Total Reads
Bacteria	203,344	99.97%
Unclassified at Kingdom Level	58	0.03%
Viruses	1	0%

Top Phylum Classification Results

Classification	Number of Reads	%Total Reads
Proteobacteria	202,619	99.61%
Firmicutes	210	0.1%
Bacteroidetes	201	0.1%
Unclassified at Phylum Level	229	0.11%
Fusobacteria	35	0.02%
Actinobacteria	16	0.01%
Nitrospirae	49	0.02%
Tenericutes	22	0.01%

Top Class Classification Results

Classification	Number of Reads	% Total Reads
Gammaprotobacterium	201,953	99.29%
Clostridia	87	0.04%
Flavobacteriia	158	0.08%
Unclassified at Class Level	553	0.27%
Bacilli	102	0.05%
Alphaproteobacteria	266	0.13%
Betaproteobacteria	78	0.04%
Nitrospira	49	0.02%

N#2 Pond-Raised

Top Kingdom Classification Results

Classification	Number of Reads	%Total Reads
Bacteria	250,210	95.53%
Unclassified at Kingdom Level	11,700	4.47%
Viruses	2	0%
Archaea	2	0%

Top Phylum Classification Results

Classification	Number of Reads	%Total Reads
Proteobacteria	44,245	16.89%
Cyanobacteria	77,186	29.47%
Bacteroidetes	39,515	15.09%
Firmicutes	57,986	22.14%
Fusobacteria	21,833	8.34%
Unclassified at Phylum leve	16,733	6.39%
Actinobacteria	1,060	0.4%
Verrucomicrobia	1,030	0.39%

Top Class Classification Results

Classification	Number of Reads	% Total Reads
Gammaprotobacterium	17,854	6.82%
Nostocophycideae	72,196	27.56%
Clostridia	37,856	14.45%
Unclassified at Class Level	35,107	13.4%
Flavobacteriia	25,177	9.61%
Fusobacteria	21,833	8.34%
Bacteroidia	12,314	4.7%
Alphaproteobacteria	11,607	4.43%

N#2 Pond-Raised

Top Kingdom Classification Results

Classification	Number of Reads	%Total Reads
Bacteria	264,715	99.09%
Unclassified at Kingdom Level	2,434	0.91%
Viruses	6	0%

Top Phylum Classification Results

Classification	Number of Reads	%Total Reads
Proteobacteria	250,339	93.71%
Cyanobacteria	3,613	1.35%
Bacteroidetes	2,985	1.12%
Firmicutes	3,974	1.49%
Fusobacteria	2,424	0.91%
Unclassified at Phylum level	3,308	1.24%
Actinobacteria	125	0.05%
Verrucomicrobia	106	0.04%

Top Class Classification Results

Classification	Number of Reads	% Total Reads
Gammaprotobacterium	248,034	92.84%
Nostocophycideae	3,299	1.23%
Clostridia	2,376	0.89%
Unclassified at Class Level	5,052	1.89%
Flavobacteriia	1,528	0.57%
Fusobacteria	2,424	0.91%
Bacteroidia	1,242	0.46%
Alphaproteobacteria	813	0.30%

N#2 Pond-Raised

Top Kingdom Classification Results

Classification	Number of Reads	%Total Reads
Bacteria	165,892	99.95%
Unclassified at Kingdom Level	71	0.04%
Viruses	4	0%

Top Phylum Classification Results

Classification	Number of Reads	%Total Reads
Proteobacteria	165,270	99.58%
Cyanobacteria	28	0.02%
Bacteroidetes	158	0.1%
Firmicutes	210	0.13%
Fusobacteria	29	0.02%
Unclassified at Phylum level	193	0.12%
Nitrospirae	41	0.02%
Tenericutes	15	0.01%

Top Class Classification Results

Classification	Number of Reads	% Total Reads
Gammaprotobacterium	164,785	99.29%
Bacilli	127	0.08%
Clostridia	69	0.04%
Unclassified at Class Level	455	0.27%
Flavobacteriia	132	0.08%
Nitrospira	41	0.02%
Betaproteobacteria	42	0.03%
Alphaproteobacteria	192	0.12%

N#1 Pond-Raised

Top Kingdom Classification Results

Classification	Number of Reads	%Total Reads
Bacteria	304,367	95.35%
Unclassified at Kingdom Level	14,831	4.65%
Viruses	4	0%

Top Phylum Classification Results

Classification	Number of Reads	%Total Reads
Proteobacteria	117,091	36.68%
Firmicutes	77,277	24.21%
Fusobacteria	51,453	16.12%
Bacteroidetes	49,467	15.5%
Unclassified at Phylum level	19,835	6.21%
Verrucomicrobia	1,283	0.4%
Actinobacteria	698	0.22%
Cyanobacteria	694	0.22%

Top Class Classification Results

Classification	Number of Reads	% Total Reads
Gammaprotobacterium	92,280	28.91%
Fusobacteria	51,453	16.12%
Clostridia	50,571	15.84%
Unclassified at Class Level	33,742	10.57%
Flavobacteria	23,034	7.22%
Bacteroidia	22,664	7.1%
Betaproteobacteria	18,325	5.74%
Bacilli	10,461	3.28%

N#1 Pond-Raised

Top Kingdom Classification Results

Classification	Number of Reads	%Total Reads
Bacteria	250,677	90.31%
Unclassified at Kingdom Level	26,904	9.69%
Viruses	6	0%

Top Phylum Classification Results

Classification	Number of Reads	%Total Reads
Proteobacteria	42,257	15.22%
Firmicutes	111,373	40.12%
Fusobacteria	28,948	10.43%
Bacteroidetes	52,190	18.8%
Unclassified at Phylum level	32,674	11.77%
Verrucomicrobia	3,236	1.17%
Actinobacteria	2,510	0.9%
Cyanobacteria	2,330	0.84%

Top Class Classification Results

Classification	Number of Reads	% Total Reads
Gammaprotobacteria	26,787	9.65%
Fusobacteria	28,948	10.43%
Clostridia	71,823	25.87%
Unclassified at Class Level	52,597	18.95%
Flavobacteriia	23,200	8.36%
Bacteroidia	24,160	8.7%
Erysipelotrichi	8,901	3.21%
Bacilli	14,794	5.33%

N#1 Pond-Raised

Top Kingdom Classification Results

Classification	Number of Reads	%Total Reads
Bacteria	226,876	93.07%
Unclassified at Kingdom Level	16,888	6.93%
Viruses	10	0%

Top Phylum Classification Results

Classification	Number of Reads	%Total Reads
Proteobacteria	34,443	14.13%
Firmicutes	100,309	41.15
Fusobacteria	21,751	8.92%
Bacteroidetes	56,208	23.06%
Unclassified at Phylum level	21,658	8.88%
Verrucomicrobia	3,658	1.5%
Actinobacteria	3,004	1.23%
Tenericutes	1,099	0.45%

Top Class Classification Results

Classification	Number of Reads	% Total Reads
Gammaprotobacteria	23,514	9.65%
Fusobacteria	21,751	8.92%
Clostridia	72,768	29.85%
Unclassified at Class Level	36,934	15.15%
Flavobacteriia	22,947	9.41%
Bacteroidia	28,344	11.63%
Erysipelotrichi	5,987	2.46%
Bacilli	10,521	4.32%