Effect of Aquaculture Practices on Fish Microbial Communities

by

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Abstract

Channel and hybrid catfish are the main aquaculture species in the USA. One of the most significant problems that catfish producers face is the presence of bacterial diseases throughout production. These diseases can cause high mortalities and consequently have an economic impact on the catfish industry. The microbiome is a complex group of microorganisms living in a symbiotic relationship with their host. A balanced microbiome is key to maintain fish health. If the fish microbial community structure is disrupted, it opens the door for opportunistic pathogens that directly affect the host's health. Standard practices in aquaculture affect the fish physiology, which in turn affects the host symbiosis. In this dissertation, I aimed to characterize the microbiome in different tissues (gut, gill, and skin) of channel catfish (*Ictalurus punctatus*) and the gut microbiome in the zebrafish (*Danio rerio*) using metagenomic approaches to study the effect of standard aquaculture practices on freshwater fish microbial communities. I have described the structure of the bacterial microbiome in both the healthy state and diseased state, dysbiosis. In Chapter 2 of this dissertation, I characterized the development of the gut microbiome in channel catfish reared under standard industry practices. I identified critical time points during their development in which bacterial communities significantly changed from one ontogenic state to the next. Besides characterizing how their microbiome shifted and shaped overtime, I pinpointed the time after which the communities became stable. In Chapter 3 and 4, I focused on investigating the effect of antibiotis on the gut microbiome in zebrafish and catfish,

respectively. As expected, antibiotic treatment induced a dysbiosis state in both hosts with an overall decreased in bacterial richness. Gut dysbiosis in zebrafish made the host more susceptible to opportunistic bacterial infections, an effect that was not observed in catfish. In Chapter 5, I assessed the effect of an external mechanical injury to the gut microbiome in channel catfish. I observed significant changes in the composition of the gut microbiome in injured fish, which suggest the presence of the 'brain-gut' axis in channel catfish previously described in mamals. Finally, in Chapter 6, I tested the effect of vaccines on the skin, gill, and gut microbiomes of channel catfish. No significant effect in those communities was found due to vaccination.

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List of Abbreviations

ACE	Abundance-based coverage estimation
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
Bp	Base pair
CFU	Colony forming unit
MDS	Multidimensional scaling
OUT	Operational taxonomic unit
SIMPER	Similarity percentages
EWSFC	E.W. Shell Fisheries Center
AML	Aquatic Microbiology Laboratory
OD	Optical density
RPS	Relative percent survival
PERMANOVA	Permutational multivariate analysis of variance
PCo	Principal Coordinate Analysis
PERMIDISP	Permutational Analysis of Multivariate Dispersions
SEI	Shannon-evenness index
ACE	Abundance-based coverage estimation

CHAPTER 1. LITERATURE REVIEW

Introduction

Fish are a vital source of food for humans, providing ~16% of the high quality of animal protein. Fish are the main animal protein consumed by the world's population, according to the Food and Agriculture Organization (FAO) of the United Nations [1]. Aquaculture is currently the fastest-growing food production system worldwide [2] and the modern aquaculture industry provides effective means for intensive fish production. However, this rapidly growing industry continues to face severe disease problems, many of which are caused by opportunistic bacterial pathogens. Despite all the information available regarding disease prevention on farmed fish, common farming practices place a lot of pressure on the animals and their symbionts that are part of their well-being.

Fish serve as host to a wide range of microorganisms, such as bacteria, yeast, virus, archaea, and protozoans [3]. These complex commensal communities are commonly referred to as the microbiota or microbiome [4]. As I will describe henceforth, microbes inhabiting many niches on and in the host including skin, gill, and the gastrointestinal tract and are well-adapted to live in intimate contact with the hosts' mucosas [5, 6]. Besides providing important health benefits to their host's, the microbiome also protects the host from incoming pathogens through various mechanisms, including competition for space or binding sites, competition for nutrients

with bacteria with similar nutrient requirements, and by direct inhibition through the release of inhibitory molecules [7].

Studies of bacterial abundance, diversity, host-symbionts interactions, are essential to recognize and understand the benefits that a healthy microbiome, brings to its host.

Objectives

This doctoral dissertation aimed at filling some of the gaps in our current knowledge on the factors affecting the microbiome of cultured fish. A better understanding on the bacterial microbiome in both the healthy and diseased state, dysbiosis as a result to the standard routine industry and laboratory practices is of critical importance. Thus, the overarching goal of my dissertation research was to answer three main questions:

1. The first one is how microbiome gets establish in the host?
2. The second one is how dysbiosis affects host health?
3. And finally, how routine practices in aquaculture affect the microbiome?

To answer those questions, I proposed the following objectives:

1. Characterize how the gut microbiome develops during the early life stage.
2. Determine if therapeutic doses of Aquaflor® medicated feed (containing florfenicol) induce dysbiosis in the gut microbiome of zebrafish and channel catfish.
3. Test if dysbiotic gut microbiome of zebrafish were more susceptible to infection by the opportunistic bacterial pathogen *Aeromonas hydrophila*.
4. Determine if the fin clipping protocol required to obtain consistent challenges in the lab altered the fish gut microbiome.

5. Evaluate the effect of vaccination on the microbiome composition of soft tissue (gut, gill, and skin) in a field trial.

Fish Species Used

All fish samples used in the following studies were reared under laboratory conditions or under farm-raised conditions. Both specied used, channel catfish (*Ictalurus punctatus*) and zebrafish (*Danio rerio*) are ray-finned, bony fishes of the class Actinopterygii.

Order: Siluriformes
Family: Ictaluridae
Ictalurus punctatus

Channel catfish (*Ictalurus punctatus*) is the primary farm-raised fish in the United States [8]. It belongs to the family Ictaluridae (order Siluriformes) that contains 51 species. Channel catfish is native to North America where it has a broad distribution. They prefer clear water streams, but they can also survive in the muddy water [9]. Catfish live in moderate to swiftly flowing streams, but they are also abundant in large reservoirs, lakes, ponds and some sluggish streams. This species can be found throughout the Lawrence, Great Lakes system and Mississippi Rivers basins from southern Quebec to Montana, spreading southward to the Gulf of Mexico [10, 11]. They are usually found in areas that have sand, gravel or rubble, instead of mud bottoms, as well as areas covered by dense aquatic weeds [9]. In the United States, there are 39 species of catfish but only six are commercially produced. These commercial species include blue catfish (*I. furcatus*), white catfish (*I. catus*), yellow bullhead (*I. natalis*), brown bullhead (*I.*

nebulosus), black bullhead (*I. melas*), flathead catfish (*Pylodictis olivaris*), channel catfish (*I. punctatus*) [9].

In the southeastern United States, channel catfish are generally cultured in earthen ponds, at relatively high densities and feed artificial diets. Commercial channel catfish feeds are a mixture of feedstuffs (animal or vegetal protein sources), vitamins, and mineral supplements that provide adequate amounts of essential nutrients and digestible energy. Dietary requirements are based on age, size, water temperature, natural food availability in the pond; daily feed allowance and fish stock density. Diet formula, components, and products vary based on the life stage of the fish, nutrient requirements, digestibility, price, and availability of feed ingredients. The recommended dietary levels of crude protein range based on protein requirement from 36%-40% during the fry and pre-fingerling stages to 25%-36% in fingerling. In the hatchery, fry are fed a finely ground feed powder but after they are moved into the nursery ponds, animals add natural foods to their diets (large zooplankton, insect larvae, and small insects) in addition to a regular catfish diet. Small fingerlings typically receive floating pellets of ~3 mm in diameter [12, 13]. In winter, due to the decrease in feeding, animals are fed with slow-sinking feeds (that are produced by extrusion) [14, 15].

Channel catfish can live up to forty years. Their life cycle begins with the fertilized eggs that hatch within 4 to 6 days post fertilization (temperature dependent process), followed by sac fry stage (they start to absorb the egg yolk sac and beging to swim), the swim-up stage (first feeding), and the subsequent fingerling and adult stages. Optimal water quality parameters for channel catfish rearing include temperature between 28-30 °C, dissolved oxygen >4.0 mg/l, pH 6.5-9.0, Total ammonia nitrogen (TAN) < 0.5-1 mg/l [15].

Spawning typically occurs between April to June, but it can vary depending on the climate [15]. In the wild, male catfish build hole nest and females lead the eggs to be fertilized, and the male cares the eggs until the fry leave the nest. Females spawn only once a year producing ~3,000 to 4,000 eggs per pound of body weight [9]. In commercial hatcheries, spawning can be triggered by hormones or by natural spawning is also allowed (by selecting mating pairs and provide them with the right environment). Once the eggs are fertilized, they are removed, and the matrix is dissolved to allow for egg disinfection. Cleaned eggs are moved to hatchery jars to continue with their regular life cycle until the swim-up stage at which they are later move directly into earth ponds or keep in recirculating aquaculture system (RAS) and growth up until commercial size [15].

Order Cypriniformes

Family: Cyprinidae

Zebrafish (*Danio rerio*)

The freshwater zebrafish *Danio rerio* is native to floodplains of the South Asia (India, Bangladesh, Nepal, Myanmar, and Pakistan). It is found in shallow, slow-flowing waters. In the wild, the water quality parameters include temperatures between 16.5 to 33 ℃ and a pH from 7.9 to 8.2. Zebrafish are group spawners and egg scatterers. Females are choosy to sites of oviposition and males defend territories around such sites [16]. Because of its small size, zebrafish require minimal facility space. Moreover, a pair of fish can produce hundreds of embryos each week that develop rapidly outside of the mother [17]. Under laboratory conditions, they are reared in re-circulating systems (with 5 to 10% water exchange per day). Fish spawn in the morning (till noon) with a ration of 2:1 females: male. After spawning, parents should be

removed from the breeding tanks as they eat their progeny. It takes approximately five days for the transition between embryos to larvae, after which they star to eat natural food (paramecia or rotifers) followed by artificial diets. Adults grow to ~2.5 cm in length and sexual maturity is reached within five weeks[18].

Zebrafish have become increasingly important to scientific research due to their small size, robustness in artificial environments, high fecundity, fast growth rate, and near transparent embryos that allows examining the developmental of internal organs. Zebrafish have been used extensively in neurobiology, developmental biology, drug research, virology, microbiology, inflammation process, immune response, microbial pathogenesis, pathology, and infection studies [19-21]. In veterinary medicine zebrafish have been used as infectious model for *Streptococcus iniae*, *Vibrio anguillarum*, *Listeria monocytogenes* [22, 23], *Aeromonas* sp., and with *Vibrio parahaemolyticus* [24].

Understading the fish microbiome

Fish harbor the greatest taxonomic and ecological diversity of all vertebrates [25]. As in other groups, the bi-directional relationship between the microbiome and its host has proved to be vital to host health. In comparison with terrestrial vertebrates, fish are in a more intimate contact with microbes present in the environment they occupy [26].

Initially, studies aimed at characterizing the microbial community of fish relied on culture-based methods [27]. Culture-dependent methods, besides being time consuming and complex, typically only cover less than 10% of the microbial diversity [28-30]. With the development of metagenomic and bioinformatics tools, the field changed. The expansion of the information characterizing the composition of microbial communities, their operations, and

dynamic relationship with their habitats increased dramatically, revealing a new picture of the real microbial diversity [25, 31-33]. Next-generation sequencing technology overcame the limitations of the first-generation sequencing (Sanger) due to their higher throughput and lower cost. Currently, next-generation methods are the most utilized platform to study microbiomes regardless of the host [34, 35]. Sequencing of 16S rRNA gene is a popular method for profiling and comparing microbial communities, and is broadly used for bacterial classification [36-38]. The small subunit 16s rRNA contains ~1500 bp, and it is composed of variable and conserved regions (named V1 to V9) and small fragments targeting areas of high variability can be used to revealed the microbial diversity of a sample at least at the genus level (in many cases even down to the species level) [39-42].

Practical considerations when studing the microbiome

Even though the 16S rRNA gene is considered the best marker to characterize the bacterial diversity, there are some factors to consider before starting a microbiome study including amplification primers, sequencing primers, sequencing technologies, as well as quality filtering and clustering. Results could be affected by these factors, and data produced with different protocols are hard to compare [38]. One problem known when using the Illumina platform involves derived artifact related to amplicon sequencing [43]. It is well known that many bacterial species contain more than one copy of 16S rRNA [44] within each cell and those overestimate bacterial diversity indexes. On the contrary, different bacterial species could have the same (in the fragment sequenced) 16S rRNA gene, which leads to an underestimation of the bacterial diversity [45].

In addition to intrinsic limitations to the use of 16S rRNA gene as marker and the Illumina platform, there are many other factors to consider. For example, it is essential to carefully chose the sample type (e.g., gastrointestinal tract (GI)) and what specific are of the organ or the tissue will be analyzed and maintain consistency throughout the study [27]. When analyzing the gut microbiome, even the time of sampling is critical as the digestion process produces dynamic changes in the gut that reflect on the bacterial composition [46].

The core microbiome concept

The bacteria consortium (microbiota) comprises bacterial genes, proteins, and metabolites and collectively are known as the microbiome [47]. The core microbiome is defined as the group of microbes that are present in all individuals of a host species [48]. Identifying the core microbiome is the first step in defining the 'healthy' community and predicting community response to perturbation [49]. The term 'core' not only describes host-associated microbes, but it can also be used to describe members shared across non-biological systems (i.e., soil, water, feed).

The healthy microbiome

Recent studies have attempted to describe what constitutes a 'healthy' microbiome [4, 17, 50]. To be considered as normal microbiota, the community of microbes has to: 1) be present in most individuals of a population or a species and cause no harm to the host [51], 2) possess the ability to resist change under unfavorable conditions (resistance) as well as its' ability to return to the equilibrium state following stress (resilience)[52], and 3) present high microbial diversity

[52, 53]. In the absence of identified factors that produce significant changes in the microbiome composition and structure, the normal individual's microbiome may be quite stable long-term [54].

Gut microbiome

The vertebrate gut harbors a co-evolved consortium of microbes composed of aerobic, facultative anaerobic, and obligate anaerobic bacteria [55]. These bacteria can be classified as autochthonous or indigenous, once they are capable of colonizing the host's epithelial surface or are associated with the microvilli, or as allochthonous or transient when they are associated with digesta or are present in the lumen [5, 56-59].

The human gut microbiome contains more than 1,000 bacterial species including more than 5 million genes, performing many of the functions required for overall host health [60]. The role of microorganisms resident in the intestinal tract of fish include the ability to degrade complex molecules and extract nutrients, transform residuals components into a useful material (i.e.: vitamin K, B12, biotin, folic acid, pantothenate) to the host, modify the host immune response, shape gut development, metabolize drugs, produce energy, and maintain homeostasis [27, 32, 53, 59, 61-64]. The gut microbiome is considered as the 'other' genome and it has been proposed that host + its microbiome constitutes the holobiome unit and that should be considered the unit for evolution [31, 32, 65]. Sequencing different regions of the 16S rRNA has been widely used to explore the fish gut microbial community composition in both wild and captivity [66, 67]. This technique has also been used to characterize the effect of a variety of biotic and abiotic factors that affect the healthy balance and symbiotic relationship with their host [32]. The contribution of resident bacteria in the intestine goes further than metabolic benefits to the host.

The intestinal epithelium provides a physical barrier between the intestinal lumen and sterile tissues, as well as an immunological barrier capable of sensing and responding to microbial incursion [21, 68-71]. Alterations in one species or group of bacteria may affect the entire microbial community [72] causing profound effects within microbiome functions and metabolic responses [72-74] including higher susceptibility to bacterial infections [72, 75]. The mucosal immune system is responsible for clearing pathogens, a process that requires competitive metabolic interactions, localization to intestinal niches and induction of host immune responses that activate the pro-inflammatory signaling cascade maintained by the microbiome [76, 77].

The phyla Proteobacteria, Firmicutes, Fusobacteria, Bacteroidetes, Actinobacteria, and Verrucomicrobia, have been reported as typical residents of the gut in a variety of fishes [58]. But their relative abundance varies among fish species the part of the GI analyzed [78, 79]. Proteobacteria, Firmicutes and Bacteriodetes could represent up to 90% of the fish intestinal microbiome depending on the fish species [80-83] and comprised bacterial species that contribute to the digestive process by providing a variety of enzymes [84]. Proteobacteria is a major phylum of gram-negative bacteria. This phylum contains 1,534 species or 32.3% of all known bacteria. They include a wide variety of pathogens mostly belonging to γ-proteobacteria. Represent a diverse range of organism responsible for nitrogen fixation, reduce ammonia and ammonium to nitrate, fermentation of carbohydrates, oxidation of methane and sulfur [85, 86]. Members of the phylum Fusobacteria, specifically the species *Cetobacterium somerae* are found in the gut of different freshwater species in high abundance [87-94]. *C. somerae* can secrete butyrate [95] and synthesize vitamin B12 (cobalamin) [87, 91, 96], which may exert a positive effect on fish health. The phylum Actinobacteria represents one of the largest taxonomic units among the 18 major lineages currently recognized within the domain Bacteria. Members of this

phylum exhibit diverse physiological and metabolic properties, such as the production of extracellular enzymes and the formation of a wide variety of secondary metabolites [97]. Verrucomicrobia belongs to the superphylum Planctomycetes/ Verrucomicrobial/ Chlamydiae (PVC) [98]. The phylum Verrucomicrobia comprises up to 10% of the total bacteria in soil and different aquatic environments including drinking water [99-101]. Member of this phylum has been detected from the gut of sea cucumber *Stichopus japonicus*, termite, and human intestine and contribute with mucine degradation, however, most members of this phylum remain uncultivated [102-104].

Common bacterial genera detected in the gut of different species of marine and freshwater fish include: *Cetobacterium, Psychrobacter, Clostridium, Vibrio, Aeromonas, Flavobacterium, Plesiomonas, Pseudomonas, Micrococcus, Acinetobacter, Fusarium, Bacteroides, Mycoplasma, Streptococcus, Pasteurella, Photobacterium, Lactococcus, Edwardsiella, Yersinia, Renibacterium, Arthrobacter, Brochothrix, Bacillus,* and *Mycobacterium* [59, 105, 106]. A few species within some of these genera can be classified as opportunistic bacterial pathogens that are commensals in healthy fish but can become pathogenic if circumstances change [58, 107, 108].

Skin microbiome

The skin of fish harbors a complex and diverse microbiome that is continuously in close, intimate contact with the surrounding aquatic environment [109]. The presence of indigenous bacteria inhabiting the outer-surface mucus in fish has been widely described [27, 58, 109-111]. In the aquatic environment, free-living aquatic microorganisms move actively, looking towards nutrients that are more abundant on water or surface interfaces. One of the most nutrient-rich

surfaces available to aquatic microorganisms is the mucosal surface of fish [112]. The role of the mucus layer and its associated microbiome is particularly relevant to cultivated fish species. Especially given that fish are often stocked at higher densities than found in the wild, which elevates stress and vulnerability to infectious agents and thus hinders the full success of an aquaculture enterprise [113].

The skin microbiome, in conjunction with the skin and mucosal epithelia, is thought to act as the first line of defense providing both physical and molecular barriers against invading pathogens. Also, the outer surface microbial communities facilitate the movement and homeostasis of cellular immunity mechanism of protection [114, 115] providing resistance to pathogen colonization [115, 116]. Latest studies on the changes in outer-surface bacteria have shown that the undisturbed mucosal surface, along with its commensal bacterial communities, could be a reflection of the health status of the fish [117-119].

The healthy balance between commensal and opportunistic bacteria can be easily altered by disturbance factors such as physical or chemical stress [120-122] including netting, sorting, and transporting [123], which could eliminate the outer surface mucus, providing a portal of entry to bacteria. These practices could potentially affect the balance of fish-skin microbe, reducing the bacterial biodiversity, and promoting proliferation of opportunistic bacteria, a process that has been previously documented [124-126].

The phylum Proteobacteria and Bacteroidetes are the most commonly identified in fish mucus and skin [116-118], however other phyla, such as, Actinobacteria, Acidobacteria, Chlorobi, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Firmicutes, Planctomycetes, and Verrucomicrobia have been detected in the skin and mucus of many fish species [118]. Freshwater fishes commonly contain members of the genera *Acinetobacter*, *Aeromonas*,

Alcaligenes, Enterobacter, Moraxella, and *Pseudomonas* [27, 127, 128]. Less commonly identified genera include *Flavobacterium*, *Methylobacterium*, Lysobacter, *Rheinheimera, Undibacterium, Duganella, Lactobacillales* [113, 116]

Gill microbiome

Endogenous, symbiotic bacteria have been documented in the cutaneous mucus and on the surface of gills in teleost fish [58, 116]. Aerobic counts indicate bacterial loads $\sim 10^{-5}$ CFU/g [129]. Interestingly, the mucosal surface that covers the soft tissues, such as, gill, gut, and skin has the same ontological origin and therefore the microbial communities associated with those tissues are similar. However, gills tend to harbor lesser diversity compared with that in skin [128]. Like skin, the gills are dominated by bacterial class such as Bacteroidetes and Gammaproteobacteria [58, 117, 118, 129]. Other phyla, like Acidobacteria, Actinobacteria, Cyanobacteria, Firmicutes, Planctomycetes, Verrucomicrobia, Ascomycota, Basidiomycota, and Fusobacteria has been detected [118, 130]. Gills also harbor a wide diversity of bacterial genera including the genera *Photobacterium, Aeromonas, Enterobacter, Flavobacterium, Allivibrio, Acinetobacter, Vibrio, Paracoccus, Shewanella and Tenacibaculum* [129-131]

Bacteria associated with the gills recycle and remove waste products [132], which clearly benefits the host. Their relationship with host health has been studied in yellowtail kingfish (*Seriola lalandi*) that were infected with enteritis. The bacterial shift observed in their gut microbiome translated into the gill microbial composition by reducing the diversity and increasing the abundance of the phyla Proteobacteria and Actinobacteria. This evidence shows that the gut microbiota can affect the microbiomes associated to other tissues [117].

Factors influencing microbiome

The stable bacteria-host symbiosis can be disrupted when successional changes occur as the fish respond to different factors. Abiotic factors such as water temperature, salinity, nutrients, pH, diet, fish management practices, medical interventions, disease, and stocking densities have the potential to impact bacterial groups, OTU abundance, and might even result in the disappearance (or appearance) of some bacteria groups [133]. Additionally, the previous factors mentionated also can influence activation and expression of genes related to virulence and secondary metabolites in the bacteria metagenome.

In this study, I explored a few factors associated with conventional practices applied in population management on fish reared under laboratory condition and in production facilities, and how these factors affect the microbiome in soft tissues such as gut, gill, and skin in the fish.

Age

The age-related changes in the gut microbiome have been very well characterized in human studies. Studies showed that as soon as humans are born, bacteria begin to colonize the gut. Development of the intestinal microbiome in infants is characterized by rapid and substantial changes in microbial abundance, diversity, and composition until it becames stable during the first year of life [134]. The bacterial shifts observed are influenced by different factors including mode of delivery, diet, family, environment, culture, travel, illness and therapies used [60, 135].

The microbial communities in newborns differ from those adults and as the host ages; there is a decrease in individual members of the dominant phyla while another members' increase. Interestingly, the elderly individuals have more variation in the evenness or relative proportion of different bacterial species [62, 136]. There is a prominent bacterial signature associated with changes in the gut bacterial composition due to aging. In infants, the Firmicutes: Bacteroidetes ratio is 0.4, and shifts to 10.9 in adults, and then down into 0.6 in the elderly. Infants have lower levels of total bacteria, with a higher abundance of the genus *Clostridium*, while the elderly exhibit a high abundance of *Escherichia coli* and *Bacteroides* and a decrease in a vital organism, *Bifidobacteria* [137, 138].

As in humans, the pattern of the fish gut colonization is quite similar concerning factors influencing their bacterial composition, the process of aging, stage-specific bacteria signatures in the intestinal microbiome and extensive interindividual variation. Most ontogenesis studies in fish have focused on the development of the microbial structure in the gastrointestinal tract (GI) of the fish. These studies have covered most of the early stages of the fish development (egg, sac-fry, swim-up, fingerlings) and have analyzed contributing factors such as water and diet. The differences found in the gut of the microbial communities between the early stages and adult fish suggest changes in the functional contribution of microbiome over time [83, 94, 139, 140]

Several studies in fish egg surface have shown the presence of bacteria as soon as they are expelled and fertilized, suggesting vertical transmission by bacteria living in the female oviduct [141]. In aquaculture, eggs are kept in jars system in hatcheries, which is an entirely different habitat compared with the natural environment that allows a heavily overgrown with bacteria and other microbes within just a few hours after fertilization [142]. Right after the eggs are expelled from the female oviduct, the microorganisms living in rearing environment start the

colonization process [27, 143]. Factors such as host genetics and the microbial diversity found in the parents rearing environment (ponds, tanks, etc.) will shape the diversity and composition of the egg-larvae associated bacterial communities that will have a definite effect on the life history of their hosts [144, 145]. This fish first microbiome, however, rapidly evolves to the one associated with the sac fry state, in which the larvae absorb their yolk-sac and bacteria are coming from the surrounding water to control osmoregulation [142, 146]. At this point, the incipient gut microbiome is characterized by relatively low diversity and instability [5, 53]. Once the fry starts eating natural and commercial food, a new shift, lead by diet type, is characterized by an increase in bacterial diversity [83, 139, 140, 147]. Once the fish reach juvenile stage, the microbial community stabilizes and the gut microbiome composition is drastically different from that of the surrounding environment (and feed) demonstrating a very strong host-selection [94, 139, 148]. Interestingly, bacterial diversity seems to decrease as fish ages [149, 150]. The host-selection microbial selection has been described in different fish species living in the same habitat [81, 93] and by diet preferences [66, 151] that include specialized bacteria to aid in the digestion and absorption of nutrients from a variety of food sources within the fish intestine [152-154].

Bacterial communities in eggs and larvae are commonly dominated by the phylum Proteobacteria, Bacteroidetes, Verrucomicrobia, Planctomycetes and Firmicutes, with changes among the different developmental stages that persisted to juvenile stage, suggesting their participation as autochthonous microbiome in the fish [139, 143, 155]. Despite the natural shift in the microbiome, the bacterial communities share a core microbiome between them along with all the ontogeny stages, revealing their importance across host' development [54]. The control of colonization using beneficial microorganisms (probiotics) or improving the conditions for a

settlement of commensal bacteria at early fish life stages is an opportunity to enhance the future immune health of the animal.

Diet

Several studies on fish gut microbiome has revealed that fish harbor a diverse microbiota, dependent on host species [57, 93, 156], life cycle stage [83, 140, 157, 158], and diet [159]. However, some studies support the hypothesis that diet is the primary driver that shapes fish microbiome composition. Diet creates metabolic niches in the immature gut that could shape microbial communities [160]. It is undoubtedly that feeding; trophic habitat and diet play essential roles in the gut microbiome composition and dynamics. For example, the diet of Amazonian catfish *Panaque nicrolineatus* is rich in plant components and, their microbial community harbors microorganisms capable of cellulose degradation and nitrogen fixation characterized by the presence of phylum Cytophaga, Flavobacterium, and Bacteroides [161, 162]. Animals with more generalized diet had less diverse gut microbiome than a dietary specialist [163] as seen in carnivorous with higher microbial diversity compared to omnivores [57, 93, 151]

A similar scenario is observed in animals under culture, where their diets are composed of natural food supplemented with artificial diets. The components in commercial diets are under constant modifications lead by the costly fishmeal-based diets [46]. The replacement of fishmeal by vegetable-based feed has been studied in different commercial fish and has shown their impact in the gut microbial composition [46, 159, 164, 165]. Most of these studies concluded that the host-selection is mainly guided by the metabolism required based on the type of diet. In plant-derived food sources, the presence of bacterial groups with fermentative metabolism and

degradation of carbohydrates are widespread, and most of the members belong to phyla Fusobacteria, Firmicutes, Proteobacteria and Bacteroidetes [152, 166]. However, something particularly interesting is that not all fish species show a shift in their microbial communities due to changes in their diets, which has been observed in adult rainbow trout [165]. The bacterial genera that change with diet alterations are often similar between fish species with a particular response on the bacterial shift based on plant-based components replacement [164]. Bacterial shifts due to differing diets primarily affect the abundance of specific genera such *Pseudomonas, Carnobacterium, Aeromonas, Bacillus, Flavobacterium, Vibrio, Weissella, Streptococcus, Cetobacterium, Shewanella* [92, 159, 167]

The shifts in the gut microbial communities and abundance are not only reported as a change in diets but also to a response to periods of prolonged fasting or starvation, as has been observed in Asian seabass [168] and tilapia [169]. Other fish tissues also can be affected by diet, as has been observed with the increase of bacterial load associated with skin in Atlantic salmon [170], puffer fish *Takifugu [171]*, and yellow grouper [172].

Probiotic, prebiotic and functional diets

The most common additives used in aquaculture diets are probiotics, prebiotics, immunostimulants, vitamins, and nucleotides [173]. The use of probiotics in farmed animals has increased considerably as an environmentally friendly alternative to the use of antibiotics. Probiotics are living organisms that confer health benefits to the host when they are administered in appropriate and regular quantities [115, 174-176]. A growing number of studies have asserted the positive influence of probiotic in fish feed. Probiotic bacteria must colonize the gut where it either: a) produce antimicrobials [177], b) compete for space and nutrients with opportunistic pathogens [174, 177], c) promote a stable healthy microbiota resistant to pathogen invasion

[178], d) survive to the gastrointestinal conditions [179], e) enhance host growth [180], f) improved lipid metabolism, g) stimulate immune responses [181, 182], and/or f) result in a better physiological status for the gut [115].

The probiotics used in fish farmed and hatcheries are members of the microbiota of the species of interest [115, 175, 177, 183, 184]. Thus, the characterization of the healthy microbiome of a particular fish species is key for choosing the right probiotic strain. There are many commercial probiotics (either as a single strain or as a combination of several strains) that can be administered to fish, along with feed, during the entire culture cycle [185]. *Lactobacillus*, *Bacillus*, *Enterococcus*, and *Pediococcus* are among the most common bacteria used as probiotics in fish. Several studies have characterized the effect of commercial probiotics in the fish gut microbiome. For example, the use of Aqualase (yeast mix) in rainbow trout (*Oncorhynchus mykiss*) increased bacterial abundance as well as some biomolecules related to the immune responses in skin [180]. The administration of a multi-species probiotic such as AquaStar ® Growout that contains *Lactobacillus reuteri*, *Bacillus subtilis*, *Enterococcus faecium* and *Pediococcus acidilactici* in tilapia resulted in higher levels of lactic acid bacteria (LAB), *Enterococci* and *Bacillus spp*. in the intestinal mucosa and digesta of the fish while significantly reduced diversity and species richness in the host [181]. A study in when a mixed probiotic containing four different strains of *Lactobacillus*, one strain of *Enterococcus*, *Bacillus*, and *Saccharomyces* was used, revealed a significant reduction in mortalities due to *Aeromonas hydrophila* in seabass [183]. However, other studies monitoring gut microbiome composition alongside probiotic administration reported mixed results with no observable effect on host health [185-187]

Similarly, the incorporation of prebiotics (non-digestible ingredients) into feed diets has become widely used in aquculture. The reasoning for using prebiotics is that they will selectively favor the growth of beneficial bacteria and/or that the by-products produced during prebiotic digestion by microbes has a beneficial effect on the host. For example, short-chain fatty acids (SCFAs) produced during the fermentation of complex carbohydrates have been recognized as beneficial to the host, especially during high-stress occurrences [188, 189]. It has been suggested that plant- based diet with high content of complex carbohydrates could favor the presence of beneficial bacterial taxa that provides a protective effect to the host [159]. The used of arabinoxylan oligosaccharides (AXOS) in Siberian sturgeon (*Acipenser baerii*) showed that gut-associated bacterial communities shifted towards to butyrate-producing bacteria, including lactic acid bacteria and *Clostridium*, increasing the concentration of short chain fatty acids in the gut [190].

The combination of a prebiotic and a probiotic is another alternative used as a resource to improve the general health of the fish. The combined administration of short chain fucto-oligosaccharides (scFOS) and *Lactobacilli* in hybrid tilapia revealed that the composition of the gut adherent microbiota was altered and resulted in siginificant protection against *A. hydrophila* infections. However, there was no significant difference in survival rate, weight gain, or feed conversion in the fish [191]. The incorporation of immunostimulants like β- 1,3/1,6-glucans and plant essential oils as functional diets in European sea bass (*Dicentrarchus labrax*) showed significant shifts in the dominant gut microbial genera *Dysgonomonas* (Bacteroidetes) and *Ralstonia* (Beta-proteobacteria)[80].

Despite the growing number of publications describing the benefits of administering pro- and/or prebiotics to fish, few studies thoroughly characterized the microbial shifts due to the

administration of these supplements. In addition, many of them only induced transitory changes in the gut microbiome that reverted back to its previous status after treatment ceased. One of the reasons for this lack of consistency across studies could be the fact that they used adult animals that had a well-established gut microbiome. Breaking the equilibrium between the members of well established a microbial complex is likely to be more difficult than when the same pressure is applied while the communities are still developing.

Disease

Disease is any condition, which results in the disorder of a structure or function in a living organism. The appearance and development of a fish disease is the result of the interaction among host-pathogen-environment [192]. As aquaculture developed, an increased number of diseases appeared and severely hampered production of many fish species. Infectious diseases are caused by parasites, bacteria or viruses, which can spread quickly within a system and between susceptible hosts. In some cases, high mortalities occur rapidly and entire productions can be lost in a matter of days. Standard practices to treat infectious diseases heavely rely on the use of chemotherapeutics, including antibiotics.

Infectious diseases and dysbiosis

Infectious diseases occur in fish just like in any other animal. There are different therapies to treat them. Detergents alone may kill some viruses quickly, while others are difficult to eradicate. A few parasites can be destroyed with disinfectants. Some parasites such Myxozoa (spore producer) are difficult to inactivate and some monogeneans and crustaceans produce eggs that can be resistant to many disinfectants [193]. Infectious agents can be found on fish, in the

water, in tanks, floors, filter beds, on other husbandry equipment such as nets and siphons. Some can be transmitted by water, while others require fish to fish contact. Vectors, including humans and carrier fish, and fomites, including feeds, are responsible for introduction of pathogens in aquaculture facilities [194, 195].

Among all the possible infectious agents that affect fish, from hereon, I will focus on bacterial pathogens. Pathogenic bacteria can be divided into primary pathogens and secondary or opportunistic pathogens. Primary pathogens are those capable of causing disease in a healthy host while opportunistic patahogens need the host to be 'weakened' or compromised before they cause clinical signs. These terms should not be confused with obligate and facultative pathogens; while the first need a host to survive, facultative pathogens can multiply and thrive in the absence of a host. Bacteria identified as fish pathogens have been isolated in the surrounding water, biofilm, and in the tissue of the fish. Mostly are gram-negative, including *Aeromonas hydrophila, A. salmonicida, Flavobacterium columnare*, *Vibrio* species, *Pseudomonas* species, *Edwardsiella ictaluri*, and *Yersinia ruckeri* [196, 197], and the primary group of gram-positive bacteria that cause disease in fish is *Streptococcus, Renibacterium salmonarium* [198].

There are only four antibiotic approved by the U.S. Food and Drug Administration (FDA) to treat bacterial infections in fish [195, 196]. Among those only three antibiotics are commercially available: Terramycin ® (Terramycin 200), Romet ® (Romet 30 and Romet TC) and Florfenicol (Aquaflor ®). Sulfamerazine ® is also approved, but it is no longer in the market [196]. Once diagnostic fish lab confirms the bacterial disease, antibiotics should be administered at the maximum recommended dose for that species and for the total number of days prescribed. The percent active ingredient, mechanism of action, dosages, and treatment regimens are

different for each antibiotic. Adminsitration routes are orally for food fish but could be by immersion fish for some ornamental fish species [198]. Terramycin ® (oxytetracycline dihydrate) is a broad spectrum antibiotic interfering with the ability of bacteria to produce essential proteins and has been used in salmonids, freshwater, catfish to treat bacterial disease such as ulcerative disease, furunculosis, bacterial hemorrhagic septicemia, pseudomonas disease [199-202]. Romet ® (sulfadimethoxine and ormetoprim) acts in different parts of the folic acid metabolic pathway and has been used to treat furunculosis in salmonids and enteric septicemia in catfish [203, 204]. Aquaflor ® (florfenicol) is a broad-spectrum antibiotic that is banned Veterinary Feed Directive (VFD) and acts by inhibiting bacterial protein synthesis. Florfenicol has been used in catfish, freshwater salmonids, freshwater raised finfish and warm water finfish to treat mortalities due to *E. ictaluri*, furunculosis, coldwater and columnaris diseases, and mortality due to streptococcal septicemia [205, 206].

Antibiotics are not selective in their bacteriostatic or bactericidal effect thus as a side effect of the use of antibiotics is the destruction of commensal bacterial populations. The original microbial community may or may not recover after the cessation of antibiotic treatment [207]. The resilience of the healthy microbiome protects the host from dysbiosis-related diseases, such as inflammatory or metabolic disorders. By contrast, a resilient dysbiotic microbiome is likely to result in illness [208].

Dysbiosis represents a microbial imbalance that is characterized by an increase of the gut permeability, a change in the microbial diversity [47, 54, 209], that results in an increase of the abundance of gamma-proteobacteria [76], along with the reduction of key bacterial members [210] of the core microbiota. More and more evidence supports the hypothesis of dysbiosis beign responsible for an increased susceptibility to disease [210-212]; probably due to a decrease in

colonization resistance [213] and lack of essential metabolites. Dysbiosis could be caused by several factors such as diet [72], chemical [214], stress and chemotherapeutics [53, 211]. Between all the factors mentioned, antibiotics have been very well documented in several vertebrates, including humans, as one of the main factor that cause dysbiosis. The bacterial perturbation (unbalanced microbiome) can be short or long term. Changes induced by antibiotic treatment include the loss of bacterial ligands that are recognized by the host, alterations in the metabolites, produced by the microbiome and the loss of specific bacterial signals [215]. Changes in the community dynamics of gastrointestinal microbiome following antibiotic perturbation has been well documented in humans and animal models [17, 135, 209, 216, 217].

Contrary to surmounting evidence of disease linked to antibiotic-bacterial disruption in humans, the effect of medicated (antibiotic treated) feed in fish and its possible effect to induce dysbiosis in cultured fish is for the most part lacking. Most of the medicated feed studies are focused on exploring their efficacy or their safety [218, 219]. However, Carlson et al. [121] and He et al. [220] started to explore this phenomenon in Western mosquitofish (*Gambusia affinis*) treated with rifampicin and adult zebrafish (*Danio rerio*) treated with olaquindox. In both cases, the mucosal gut microbiomes lost diversity, and there was a shift in the composition with an increase of susceptibility to specific pathogens in treated fish.

Rearing system and lifestyle

Fish reared under aquaculture practices typically are housed at different facilities during their life cycle. From the hatchery phase to the grow-out phase, fish are maintained in facilities that are appropriate (or should be) to each life stage and should take into consideration the requierements of each fish species. Biosecurity should be maintained in order to minimize the

introduction of unwanted pathogens into the system. However, it is inevitable that microbes that inhabit water and sediments are not in close contact with fish. It has been shown that captivity induces changes in the microbiome composition on wild fish and that domesticated fish exhibit different microbial communities to their wild counterparts [125, 221, 222]. Studies on how lifestyle can affect the gut microbiome are thoroughly investigated in animals with different trophic level but sharing the same habitat [87, 223-226]. Liu et al. [66] conducted a study comparing herbivorous *Megalobrama amblycephala* (blunt snout bream,) and *Ctenopharyngodon idellus* (grass carp), carnivorous *Siniperca chuatsi* (mandarin fish) and *Culter alburnus* (topmouth culter), omnivorous *Cyprinus carpio* (common carp) and *Carassius auratus* (crucian carp), and filter-feeding *Hypophthalmichthys molitrix* (silver carp) and *Hypophthalmichthys nobilis* (bighead carp). Despite having different diets and lifestyle, fish shared a large number of gut OTUs (core microbiome) that influence the structure and composition of gut microbiota, metabolic capacity, and gut content enzyme activity.

Complementary to the location, lifestyle can affect the gut microbial composition. In addition, it has been reported that the stability of the gut microbiota may be influenced by seasonal changes [227, 228]. Depending on the fish species, habitats studied, the presence of specific bacteria in higher abundance as a response to the seasonal changes has been documented. For example, *Lactococcus lactis* was founded in higher abundance in summer, while *Lactococcus raffinolactis* was in winter in the intestine of common carp [228]. Al-Harbi et al., [227] described the higher abundance of *Aeromonas*, *Shewanella*, and *Corynebacterium* in summer with a decrease of them by winter with the increase of *Pseudomonas* in the same season in the intestine microbiome of hybrid tilapia. In the intestine sample of Atlantic salmon, *Lactobacillus* strains seem very stable to season changes while Gram-negative bacteria such as

Vibrio and *Photobacterium* are intermittent [229]. But, bacteria not only change in the gut of the fish; the surrounding bacteria where the fish live also changes as it has been observed by Resende [230] in water samples analyzed in an aquaculture system in dry and rainy season with the particularly negative correlation between seasonality in bacterial and flagellates abundance.

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CHAPTER 2. BACTERIAL DIVERSITY AND COMMUNITY STRUCTURE OF THE INTESTINAL MICROBIOME OF CHANNEL CATFISH *(ICTALURUS PUNCTATUS)* DURING ONTOGENESIS

Abstract

The acquisition of gut microbes does not occur randomly and is highly dependent on host factors, environmental cues, and self-assembly rules exerted by the microbes themselves. The main objective of this project was to characterize how the gut microbiome develops during the early life stages of channel catfish and to identify i) which bacteria are the main constituents of the gut microbiome at different ontogenesis stages, and ii) at which time point (s) the gut microbiome stabilizes. High-throughput Illumina MiSeq DNA sequencing of the V4 domain of the 16S rRNA gene was used to assess the microbial community composition during the life stages of Channel Catfish along with water and feed samples. Microbiomes from fertilized eggs, sac fry, swim up fry, pre-fingerlings, and fingerlings were all significantly distinct. OTUs analyses showed that the phylum Proteobacteria, Firmicutes, Fusobacteria and Cyanobacteria dominated the Channel catfish gut microbiome. During the early stages of ontogenesis, the fish microbiome was dynamic and highly diverse, with significant shifts occurring between fertilized eggs to sac fry (6 dph), and from sac fry to swim up fry (15 dph). The gut microbiome stabilized between the pre-fingerlings and fingerlings stage (≤ 90 dph) with an observed reduction in species richness. Feed had a more significant contribution to the microbial colonization of the

gut than water. We have identified the period in which the gut microbiome changes rapidly from 15 dph until 21 dph before stabilizing after 90 dph.

Introduction

The gut microbiome has received a lot of attention in recent years due to its effect on host nutrition, immune status, disease susceptibility, growth, and reproduction [1, 2]. Understanding how microbes interact with their host and characterizing the symbiotic relationships that occur in the gut of vertebrates is key to inducing specific microbiome modifications that will improve host health [3-9]. An obvious prerequisite to this approach is to characterize the gut microbiome of the species in question and to identify its' core microbiome (i.e. members of the microbial community present in all individuals of a species) [10]. The 'healthy core' confers protection against pathogens by providing essential developmental factors, activating the immune system and optimizing nutrient conversion. All vertebrates develop in close association with a specific and complex assembly of microbes that can affect host ontogenesis [11]. The gut microbiome is shaped by intrinsic and extrinsic factors such as host age [12], genetics [13, 14], nutritional status [15], environmental conditions [16, 17], diets [18-20] and location in the gastrointestinal tract [21, 22]. Although many studies have characterized the gut microbiome of fishes, few [23-27] have attempted to describe how these microbial communities get established in the host and at which point(s) these communities become stable.

The relationship between the composition of the gut microbiome and the development stage of the host has been documented in some vertebrates including humans [28-31]. Those studies revealed that significant shifts occurred during the early stages of development and that diversity diminishes over time [32, 33]. It has also been reported that diet is the determining

factor leading to the observed sequence of microbial colonization during ontogenesis [19, 26, 34].

Channel catfish (*Ictalurus punctatus*), is the top farmed-raised fish in United States with a production of more than 750 million pounds per year [35, 36]. The demand for farmed catfish is strong and growing. However, the industry faces losses during the entire commercial cycle due primarily to infectious diseases. Producers have been looking for alternative strategies to improve feed efficiency, growth rate and, disease resistance while minimizing the use of chemicals and antibiotics [37, 38]. Probiotics (live microbes) or prebiotics (feed additives that promote the growth of naturally occurring probiotics) are considered environmentally friendly alternatives to prevent and control infectious diseases due to the positive effect they have on the host [24, 38-41]. Several studies have investigated the efficacy of different probiotics and prebiotics in channel catfish as alternative treatments to control bacterial infections and improve host health [42-45]. Although some have shown positive results when *Bacillus* sp. was administered with the feed [43] others have shown no significant effect [44]. In other fish species [4, 6, 8, 9, 46, 47] the use of pre- or probiotics requires continuous supplementation to exert a positive effect on the host. We hypothesize that influencing the gut microbiome composition during the early stages of development could result in a more permanent change if the modifier (pre- or probiotics) is established before the gut microbiome becomes stable. If our hypothesis is correct, the best time to administer pre- or probiotics will be between the first feeding and the point of stabilization. This target-specific approach should exert a stronger effect on the core microbial community. From that point, pre- or probiotics might only be required on occasional basis as boosters or administered at much lower doses with the consequent cost benefit to producers.

The objective of this study was to characterize the development of the gut microbiome in channel catfish using different fish cohorts taking into account the contribution of feed and water as source of microbes. Specifically, we used high-throughput sequencing to assess the taxonomic composition and microbial diversity of the gut microbiome during channel catfish ontogenesis to identify i) which bacteria were the main constituents of the gut microbiome throughout development, and ii) at which point the gut microbiome became stable.

Material and Methods

Ethics statement.

The Auburn University Institutional Animal Care and Use Committee, IACUC number 2012-2094, approved the experiment. During the entire experiment the animals were kept at the E. W. Shell Fisheries station following standard protocols.

Experimental design and fish husbandry.

Our experimental design followed the life cycle of channel catfish from fertilized egg until they reached the juvenile stage (fingerlings) as described by Wellborn [48], Tucker and Hargreaves [36], Wyatt, Barkoh, Martinez and Sparrow [49] and Chapman [50]. Based on their morphological and physiological characteristics, the following life stages were considered (see Figure 2-1 for a detailed description of the experimental plan): i) fertilized eggs, ii) sac fry, iii) swim up fry, iv) pre-fingerlings, and v) fingerlings. Except for the fertilized egg stage, all other life stages were represented by more than one sampling event.

Three sexually mature couples of channel catfish (Marion strain) from broodstock were maintained in outdoor ponds at the E. W. Shell Fisheries Station (Auburn University, Auburn, AL) and were randomly selected for this project into the hatchery in late June 2014. Each mated

pair was maintained in independent recirculating system tanks provided with pond water. The water chemistry was as follow: temperature (27°C), pH (7.4), ammonia (0-0.02 ppm), hardness (50 ppm) and alkalinity (80 ppm). Females were injected intramuscularly with a single dose of synthetic hormone, the luteinizing hormone-releasing hormone analog (LHRHa), at a dose of 100 µg/kg body weight. Pairs were allowed to mate in individual tanks and eggs masses were promptly removed after fertilization and washed with saline solution (0.9%). Fertilized egg masses were separated into individual eggs using sodium sulfite solution (150 mg/L) to dissolve the matrix tissue. Eggs were rinsed 2 or 3 times with de-chlorinated city water and transferred into 6 L McDonald jars placed inside 1 m^3 recirculating aquaculture system (Hatchery system). The system was supplied with de-chlorinated city water and the water quality was maintained as follows: temperature (27-28°C), pH (7-7.6), hardness (< 75 ppm), alkalinity (40-80 ppm). Hatching was temperature dependent. Eggs hatched between 4 to 6 days post-fertilization at temperatures ranging from 21 to 27°C. Eggs from each mated pair were maintained in separate tanks throughout the study. After hatching, the fry absorbed the yolk sac (2-5 dph), became darker and reached the swim up stage at which point they were offered the first feed.

After 9-days post hatch (9 dph), fry were transferred into 300 L tanks connected to a recirculating system (G-System) provided with biofilter and UV filters. The system was supplied with de-chlorinated city water. Water quality during the rearing period was as follow: temperature at 27 ± 1.3°C, pH value maintained at 7.12 ± 0.14, total ammonia was 0, nitrate was less than 20 ppm, nitrite was 0 ppm, hardness was maintained between 25-75 ppm, and alkalinity between 40-80 ppm. Fish were maintained indoors with natural light throughout the study. Approximately 2,000 fry (from each spawn) were randomly divided into three 300 L tanks with an initial density of approximately 700 swim-up fry per 79.2-gallon tank. To maintain optimal

fish densities as biomass increased, fish were culled during the study. Feeding started at 9 days post-fertilization at 2% of body ratio twice daily and was continued for 150 days. Throughout the project, we used 4 types of catfish diets, based on pellet size, and labeled D00, D01, D02, D03 (Purina Aquamax with 50% protein content in all formulas).

Sampling collection and DNA extraction.

Samples were taken at the following developmental stages: fertilized eggs, sac fry (at 1 and 3 dph), swim up fry (at 6, 9, and 15 dph), pre-fingerlings (at 21, 28, and 60 dph) and fingerling (at 90, 120, and 150 dph). Ten individuals per spawning (x2) were collected at each sampling time. Fish were euthanized using buffered MS222 (Finquel MS 222, Sigma Inc.), dissected and digestive tracts were aseptically removed. Eggs, sac fry and swim up fry were homogenized as whole individuals' due to their small size before DNA extraction. Fish were dipped in ethanol and washed three times in sterile water to reduce contamination by commensal bacteria associated with external surfaces prior homogenization. Additionally, 1 L of water from all aquatic environments (parents' tank-spawn#1, parents' tank-spawn#2, hatchery recirculating system, and G-system) were collected and filtered onto a 0.22 μm 47 mm Nitrocellulose membrane (Merck Millipore Ltd., Ireland). Feed samples were collected from each feed type (Figure 2-1).

DNA extractions were performed using the DNeasy Blood & Tissue Kit (Qiagen CA, USA) following the Gram-positive bacterial DNA extraction and Purification of Total DNA from Animal Tissues (Spin-Column) protocol following manufacture's instructions, with minor modifications for fertilized eggs. Briefly, eggs were homogenized into 200 µl of enzymatic lysis buffer containing 10 µl of lysozyme (20 mg/ml) and incubated at 37°C for 30 minutes. Afterwards, 10 µl of proteinase K (20 mg/ml) was added, vortexed and incubated at 60°C for 1

h. The product was centrifuged at 13,000 rpm for 3 min before continuing with the standard protocol.

DNA was extracted from water samples (1 L) using the PowerWater ® DNA Isolation kit and following the manufacturer's instructions (MO BIO Laboratories, Quiagen, CA, USA). Feed pellets (45 g) were grinded into a powder that was processed following the Gram-positive protocol from the manufacture using the Qiagen DNeasy Blood and Tissue Kit. Extracted DNA was quantified using the Nanodrop 2000 (Thermo Scientific, Rochester, USA). DNA quality was confirmed by using universal primers against the 16S rRNA gene as previously described [51]. Samples were kept at -20°C until sequencing.

PCR Amplification and Sequencing.

A total of 24 samples were submitted to MR DNA® (Shallowater, TX, USA) for PCR amplification and Next- Generation Sequencing using Illumina MiSeq platform targeting the 16S rRNA gene V4 variable region. Universal bacterial primers 515 F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') with a barcode on the forward primer were used to generate a 300 bp amplicon. The HotStarTaq Plus Master Mix Kit (Qiagen, USA) was used under the following PCR conditions: an initial denaturation for 3 minutes at 94 ℃ followed by 28 cycles of denaturing at 94 ℃ for 30 s, annealing at 53 ℃ for 40 s, extension at 72 ℃ for 1 min and a final elongation step for 5 min at 72 ℃. The PCR products were run through a 2% agarose gel to verify successful amplification and relative band intensity of the target DNA. Multiple samples were pooled together and purified using calibrated Ampure XP beads to prepare the Illumina DNA library. The samples were sequenced as paired-end reads on the Illumina MiSeq platform following the manufacturer's instructions. The sequences resulting were processed using a proprietary pipeline

(MR DNA, Shallowater, TX, USA). Sequencing data were joined, and all barcodes, ambiguous base calls, and sequences <150 bp were removed. Denoising of sequences was also performed, and operational taxonomic units (OTUs) were generated. Cut-offs for OTU assignment were defined at a 97% similarity (< 3% sequence variation) in concurrence with the prokaryotic species concept [52]. Taxonomic classifications were obtained using BLASTn against the GreenGenes database [53].

Data analysis.

Sequences were randomly selected from each sample in order to standardize sampling effort to that of the sample that returned the least number of sequences: catfish gut, n = 9,843; water, n = 35,432; feed, n = 23,181. Mothur v.1.33.3 [54] was used to generate rarefaction curves and to calculate alpha diversity statistics including Good's coverage, Shannon-evenness index (SEI), Abundance-based coverage estimation (ACE), Chao1, observed OTUs, and shared OTUs. A one-way ANOVA was performed on all diversity indices, followed by a Tukey's post hoc test when significant ($P < 0.05$), using JMP Version 12 .0.1 (SAS Institute Inc. Cary, NC, 1989-2007). Beta diversity analysis was determined using non-metric multidimensional scaling (nMDS) and analysis of similarities (ANOSIM) was run from the similarity matrix using Primer v6 (Primer-E Ltd, Plymouth, UK) to statistically quantify groupings seen in MDS plots. A genera abundance table was loaded into Primer v6 and similarity percentages (SIMPER) analysis was performed to determine the genera responsible for differences between ontogenetic stages. The cut-off for low contributions was set to the default at 90%.

Results

Fish development.

Hatching rates and fry mortality were within normal parameters (Karen Veverica, Director of the E. W. Shell Fisheries station, personal communication) for two of the three spawns. Unfortunately, the first spawn developed a fungal infection and it had to be terminated. The transferred fish from the hatchery into grow-out tanks (G-system) developed normally and maintained adequate growth throughout the study (Table 2-1). No mortality was observed and no treatments were applied to any of the tanks. Water quality was maintained within acceptable limits [35, 36].

Diversity and composition of the channel catfish gut microbiome.

A total of 1,591,655 bacterial sequences representing 3,636 operational taxonomic units (OTUs) were obtained from all combined samples in the study. After standardization, 236,232 bacterial sequences remained in the analysis comprising 2,521 OTUs. Sequence coverage was ≥ 98% for all samples analyzed (based on Good's coverage, data not shown). Total number of observed OTUs (Sobs) ranged from 205 in sac fry to 539 in fertilized eggs (Table 2-2). Overall, the bacterial community exhibited higher diversity at the fertilized egg stage followed by an abrupt reduction in OTUs that increased after the first feeding and decreased again after fish reached the fingerling stage. Some of those changes were statistically significant by both Sobs and predicted OTUs as calculated by Chao1 and ACE. Shannon-evenness indices were not significantly different across life stages indicating the diversity was equally even across all life stages.

The channel catfish gut microbiome contained a diverse community representing 26 bacterial phyla and 776 genera (when all life stages were taken into account). Figure 2-2 shows

the predominant phyla: Firmicutes (38%), Proteobacteria (37%), Fusobacteria (11%), and Cyanobacteria (6%) when all life stages were taken into account. The Gamma-proteobacteria (29%) was the most abundant class within the phylum Proteobacteria followed by Alpha-proteobacteria (5%) and Beta-proteobacteria (3%). Clostridia (17%) and Bacilli (11%) were the dominant classes among the phylum Firmicutes.

At the genus level, a clear shift in the microbial communities was observed between fertilized eggs and sac fry as well as between sac fry and swim up fry (Figure 2-3). Fertilized eggs were collected within 24 h of spawning and the egg-matrix was dissolved following standard hatchery protocols. Despite this chemical treatment, the eggs exhibited a rich bacterial community dominated by the genus *Vibrio* (43%) followed by *Flavobacterium* (15%), *Lysobacter* (6%), and *Sphingobium* (2%). Other bacteria (less than 1%) included members of the genera *Phylobacterium*, *Hylemonella*, *Rhodobacter* and *Tepidomonas*. After the eggs hatched, the bacterial community experienced a drastic shift and became dominated by the genus *Opitutus* (53%) (a member of the recently erected phylum Verrucomicrobia) at 1 and 3 dph (sac fry stage). Coinciding with the first feeding (between 3 and 6 dph), there was a great increase of the genus *Phyllobacterium* from 4% (at 3 dph) to 40% (at 6 dph). Throughout the swim up fry state, the community became more diverse and uniform with *Phyllobacterium* (17%), *Shewanella* (15%), *Aeromonas* (8%) as predominant genera. Between 15 dph (swim up fry) and 21 dph (pre-fingerling) the communities remained fairly similar. However, at 21 dph a new feed type was introduced and at 28 dph pre-fingerlings underwent another drastic change in their communities with *Candidatus* Arthromitus representing 40% of the community. At 60 dph there was a spike in the number of *Vibrio* OTUs that represented 31% of the community, followed by *Lactobacillus* (22%) and *Candidatus* Arthromitus (12%). This was the first sample in which the

gastro intestinal tract was excised and processed. Until that point, the whole animal had to be homogenized due to their small size. Once fish reached 90 dph, the composition of the community remained fairly stable and was dominated by the almost equally represented genera: *Clostridium*, *Cetobacterium*, and *Turicibacter* (Figure 2-3).

Microbiome differences across life stages.

Analysis of similarity (ANOSIM) confirmed the OTU composition between life stages was more dissimilar than within life stages. This was supported by strong R-values and statistically significant P-values (Table 2-3). To better visualize the similarity among samples, an MDS plot was generated with all replicates and sampling points clustered by life stage (Figure 2-4). Sac fry microbiomes formed a tight cluster that did not overlap with other samples. Fertilized eggs also displayed a significantly different microbial community although closer to those exhibited by swim up fry. Swim up fry and pre-fingerling microbial communities overlapped although significant differences were still found between each stage. The gut microbiome in pre-fingerlings progressively transitioned into the final gut microbiome observed in fingerlings.

One-way SIMPER analysis (Table 2-4) indicated that, based on phylum composition, sac fry, swim up fry and pre-fingerling were the most similar life stages with similarities of 65.04%, 65.04% and 61.70, respectively. Conversely, fertilized egg and fingerlings were the least similar (51.91% and 59.16%). The phylum Proteobacteria prevailed across all developmental stages with the Gamma-proteobacteria being the dominant class. The phylum Firmicutes contributed the most to the dissimilarity between all ontogenesis stages. At the genus level, the most abundant genus at each life stage was also responsible for the significant differences in the bacterial communities throughout ontogenesis (Table 2-4). The dominant genus at each stage was: *Vibrio*

(fertilized egg), *Opitutus* (sac fry), *Phyllobacterium* (swim up fry), *Candidatus* Arthromitus (pre-fingerling), and *Cetobacterium* (fingerling).

Water microbiome: diversity and composition.

Sequencing the 16S rRNA gene from all water samples (parents' tank-spawn#1, parents' tank-spawn#2, hatchery, and G-system) resulted in a total of 173,894 bacterial sequences representing 2,711 OTUs. For analysis, sequences from parent's tank #1 and #2 were combined in one single sample since both tanks were part of the same recirculating aquaculture system. After standardization, 141,728 bacterial sequences remained in the analysis comprising 2,634 OTUs. Sequence coverage was higher than 90% in all samples analyzed. Diversity indices including Sobs, ACE and Chao1 showed that the aquatic environment in the hatchery presented the lowest species richness followed by both parents' tanks, and the G-system (Table 2-5). The Shannon-evenness index for hatchery water was the lowest among water samples indicating specific species dominance. The total number of shared OTUs between all water samples was 388, with more shared OTUs between the parent's aquatic environments (pond water) than between the hatchery and G-systems (both of them recirculating systems started with dechlorinated city water). Each water sample contained unique groups of OTUs (Figure 2-5).

The water microbiome contained a diverse community representing 26 bacterial phyla and 656 genera, when all water samples were analyzed. The predominant phyla were: Proteobacteria (39%), Bacteroidetes (17%), Actinobacteria and Cyanobacteria (14%), and Verrucomicrobia (6%). Gamma-proteobacteria (17%) was the most abundant class followed by Alpha- proteobacteria and Beta-proteobacteria (10%). Actinobacteria (14%) was the dominant class among the phylum Actinobacteria and Cyanobacteria (14%) was among the phylum Cyanobacteria. Sphingobacteriia (9%) was the dominant class among the phylum Bacteroidetes.

The parents' tank water samples exhibited the most diverse bacterial composition at genus level, which included: *Candidatus* Planktophila (16-24%), *Cyanobacterium* (5%), *Verrucomicrobium* (5-7%), *Polynucleobacter* and *Singulisphaera* (3-4%), *Synechococcus* (3-6 %), Planktothrix (3-5%) and *Opitutus* (2-3%). The hatchery water samples were dominated by *Aquimonas* (57%), *Pedobacter (*10%), and *Bacteroides* (5%). In the G-system, the bacterial community in water was dominated by *Cyanobacterium* (22%), *Flexibacter* (5%), *Pedobacter* (3%), *Geofilum* (3%) and *Sphingobacterium* (3%) (Table 2-6).

Feed microbiome diversity and composition.

Sequencing of the 16S rRNA gene from feed samples resulted in a total of 328,939 bacterial sequences representing 1,698 OTUs. After standardization, 139,086 bacterial sequences remained in the analysis comprising 1,309 OTUs. Sequence coverage was ≥ 98% for all samples analyzed (data not shown). Rarefaction curves showed that the different feeds used in the study exhibited considerable differences in species richness. The starter feed D00 contained nearly double the OTUs found in the second type of feed administered to the fish while the larger pellets (D02 and D03) were fairly similar in terms of bacterial diversity (Table 2-5).

The feed microbiome contained a diverse community representing 20 bacterial phyla and 480 genera. The predominant phyla were: Cyanobacteria (60%), Firmicutes (21%), Proteobacteria (8%), Tenericutes (6%), and Fusobacteria (3%). Overall, OTUs ascribed to the genus *Halospirulina* was present in all feeds representing 18 to 60% of all OTUs. *Anoxybacillus* represented 40% of the community in feed D02 while *Candidatus* Phytoplasma represented 12% and 11% in D01 and D02, respectively (Table 2-7).

Interactions between microbial communities from fish, feed, and water.

Total number of Sobs (observed OTUs) was higher in fish (n = 2,522) than in water (n = 1,932) and feed samples (n = 860). A total of 337 OTUs were shared among the microbial communities in fish, water, and feed, which represents approximately 6% of total OTUs. Gut samples were the most unique with up to 1,108 OTUs only found in association with fish samples. The number of shared OTUs between fish and water was higher (n = 1,008) than those found in both fish and feed (n = 743). ANOSIM confirmed that samples clustered by sample type (fish, water, and feed) and that those groups were significantly different ($R = 0.800$, $P < 0.01$). However, MDS plot showed that the microbial communities from fish and feed had some overlap and that those communities were more similar to each other than they were to the microbial community present in water samples (Figure 2-5). One-way SIMPER analysis (at the genus level; data not shown) confirmed this finding.

After fish reached the fingerling stage (> 90 dph) more than 60% of their gut OTUs were comprised of members of the genera *Clostridium*, *Cetobacterium*, and *Turicibacter* (Figure 2-3). Those three genera significantly increased from the pre-fingerling to the fingerling stage. To determine the source of those bacteria, we compared the percent abundance of each genus over time in all the samples analyzed. All three genera were present at relatively high abundance in water samples taken from the parents' tanks (Figure 2-6A). Percentages decreased in hatchery water and slightly increased in the G-system where fish were transferred at the swim-up fry stage and where they remained for the duration of the study. Percent abundance of *Turicibacter* was low in all feeds used but *Cetobacterium* and, more strikingly, *Clostridium* were present in higher numbers in the D03 feed, which was the one administered to fingerlings (Figure 2-6B).

Discussion

The presence of a complex and dynamic gut microbiome in teleosts has been recognized as an important component of the host [26] and is key in maintaining host health [55, 56]. The composition of the microbial community in the gastrointestinal tract of fish is a mixture of aerobic, facultative anaerobic, and obligate anaerobic bacterial that varies due to external and internal factors such as water temperature, salinity, age, diet, farm practices, and genetics [1, 24, 56, 57]. The development of the gut microbiome in fish is considered a complex process and a reflection of the microbial composition of the aquatic environment and diet [58]. Recent studies have focused on the impact of environmental microbes (from feed and water) on the development of the gut microbiomes in commercially important species such as tilapia [59] and channel catfish [60]. In terms of phylum abundance, our results agree with those previously reported from channel catfish [51, 60] with the exception of Verrucomicrobia and Tenericutes (not reported by previous studies). Proteobacteria and Firmicutes were the most predominant phyla across all channel catfish life stages and they have been reported as the main phyla in Antarctic fish [61], grass carp [26, 62] and surgeonfish [63]. However, it was necessary to explore the bacterial diversity at the genus level in order to characterize patterns associated with each life stage.

The few studies that characterized bacterial development in fish species all exhibited decreased gut microbiome diversity as fish matured [33, 64, 65]. Our results support this general conclusion but by taking samples at each ontogenic stage, we were able to characterize specific details at each stage. The observed alpha diversity in fertilized eggs was the highest among all fish samples. Fertilized eggs seemed to have a high affinity for members of the genus *Vibrio* and *Flavobacterium* both of which are known to rapidly colonize and form biofilms on fish eggs [66,

67]. However, after the eggs hatched, both genera basically disappeared and the fry microbiome become dominated by members of the genus *Opitutus*. Our results were different from those presented by Bledsoe et al. [60] that showed *Bradyrhizobium* (Class Alpha-proteobacteria) as the most abundant genus at 3 dph. However, it needs to be noted that in the Bledsoe et al. (2016) study, eggs were disinfected using povidone-iodine solution while we chose not to disinfect eggs to allow for a more natural colonization. *Opitutus* was detected in hatchery water and in fertilized eggs but its abundance was low (< 0.2%). During the early development stages of bony fishes, nutrition occurs through the consumption of the egg yolk sac present in the fertilized egg. It is expected that the early colonizers come from the egg-associated bacteria and then subsequently from the surrounding environment and diet [24, 57, 58, 68]. Interestingly, *Opitutus* was found at 2.4% abundance in water samples collected from the parent's tanks, which would explain how the eggs were colonized by this genus. Clearly, *Opitutus* was favored during the transition from fertilized eggs to sac fry; however, the factors contributing to this shift in the bacterial population are unknown. *Opitutus* has been detected in freshwater and marine environments, hot springs, soils, and termite hindguts and plays a role in the nitrogen cycle in aquatic environments [69-72], but it has never been reported in association with fish embryos. It is intriguing why an obligate anaerobe would find favorable conditions on fish embryos but little is known about this new bacterial genus [70, 73].

The increase in *Phylobacterium*, *Shewanella* and *Halospirulina* at the swim-up fry stage was correlated with the first feeding event. This is a critical time in which endogenous and exogenous feeding has been documented in channel catfish [48, 74]. Several studies targeting coho salmon, rainbow trout, and zebrafish have also documented drastic shifts in the composition of bacterial communities during this transitional period [34, 65, 75]. It has been

postulated that, once feeding starts, the intestinal microbiome derives mostly from ingested feed rather than the aquatic environment [34, 57, 76]. *Phylobacterium* has been associated with plants, including soybeans, and has been detected in the gastric mucosa of yellow catfish [25] as well as in the intestinal contents of Atlantic Salmon [77]. It is plausible *Phylobacterium* reaches the fish as a carryover from plant proteins (e.g. soybeans) present in the feed. However, its presence in the fish was short-lived and was barely detected in the samples after 15 dph. Similarly, *Halospirulina* (and other members of the family Sprirulinaceae) are commonly used in fish feeds as nutritional supplements [78, 79]. Although the label of Purina AquaMax does not list cyanobacteria as one of the ingredients, we speculate the presence of *Halospirulina* in swim up fry and fingerlings correlated with its high abundance in feeds (> 18%).

During the pre-fingerling stage (21-60 dph), the communities were dominated by *Halospirulina* (phylum Cyanobacteria) and *Candidatus* Arthromitus (phylum Firmicutes). *Candidatus* Arthromitus has been found in the gut of insects and rainbow trout, but its origin and role has yet to be defined. Bledsoe et al. (2016) reported *Plesiomonas* and *Streptococcus* as the main genera at 65 dph but failed to find any cyanobacteria in their fish samples. Discrepancies between their results and ours could be due to different feeds (their study did not specify the commercial brand of feed used) or to the fact that they only sampled two times until fish reached the pre-fingerling stage (at 3 dph and 65 dph). It is likely their study missed what seems to be a hypervariable period for channel catfish ontogenesis, in terms of bacterial colonization. Another interesting discrepancy with the study of Bledsoe et al. (2016) was the significant increase of the number of vibrios at 60 dph, which was surprising because they seemed to disappear after eggs hatched. Vibrios have been previously reported from channel catfish gut and, although the genus contains fish pathogens, many species have been found in mutualistic associations with aquatic

animals [80, 81]. As Bledsoe et al (2016) we did observe an increase in the abundance of *Plesiomonas* after 60 dph that remained stable till the end of the study. There was a significant increase of *Lactobacillus* at 60 dph that later disappeared from the community. Members of the genus *Lactobacillus* have been extensively used as probiotics in fish and reported as normal component of the microflora in teleost [38], but they seemed to have a stronger presence before fish reached the fingerling stage.

At fingerling stage (90 to 150 dph) the most abundant genera were *Clostridium*, *Cetobacterium* and *Turicibacter*. Our findings are consistent with previous studies [18, 60, 82]. *Clostridium* is typically associated with soils but has also been reported from the gastrointestinal tract of animals, including humans, rainbow trout, and carp [22, 26, 83]. Its ability to ferment different carbon sources, particularly cellulose, likely helps the host to digest plant sources. *Clostridium* was present at relatively high abundance in water (G-system) but more importantly in feed. *Cetobacterium* has been isolated from mammalian intestinal tracts but has also been reported as one of the main components of the gut microbiota in freshwater fishes, including channel catfish, where it seems help fish by providing vitamin B12 and other co-enzymes [14, 51, 58, 60, 83]. The main source for *Cetobacterium* appeared to be feed. *Turicibacter* is commonly found in the gut of animals [84, 85] but its origin and role are still unclear. It was barely detectable in feed samples but was present (albeit in low numbers) in water.

Although we speculate that many of the genera found in the gut microbiome were transmitted to channel catfish from feedstuffs present in the feed (either from plant, algae, or fish derived nutritional components), we have to be careful to not over-interpret our results since sequences corresponding to inert or dead bacteria could be part of our dataset. Starter feeds used in fish are typically sinking pellets (like the ones used in this study) and are subject to a thermal

process that theoretically, reduces microbial loads to provide a stable and safe product. Commercial fish feeds guarantee the nutritional components and the physical characteristics of the pellet feed to ensure consistency and appropriate feed nutrition but feed companies do not disclose microbial parameters, at least in the US. It has been shown in other animal pelleted feeds that many sporulated and non-sporulated bacteria are capable of surviving the thermal process [86]. Recent studies used Fluorescent In Situ Hybridization (FISH) to accurately identify viable bacteria in foods but further researcher is needed in fish feeds [87]. Another point to consider is that in our study, we chose to analyze the gut content and not the gut mucosa in order to compare our data with previous studies on channel catfish [88]. It has been shown that bacteria in gut content exhibit a more transient nature than those attached to the mucosal epithelium [89]; therefore, we could have overestimated the contribution of feed to the gut microbiome if those bacterial signatures were transient in nature.

Microbiomes are displayed temporal and spatial variability across host developmental stages [41]. The beta diversity displayed through MDS graph and quantified by ANOSIM indicated that each ontogenic stage presented its own microbiome although some overlap existed between groups likely due to some fish maturing faster than others. Previous studies on catfish ontogenesis reported that the digestive system starts forming at 5 dph and is completed around 35 dph [90, 91]. Based on our results, the best window to influence the gut microbiome in channel catfish is between 6 dph (at the time of first feeding) and 60 dph, after which their microbial communities clearly become stabilized. Our data showed that each ontogenic stage exerted a selective pressure on the bacteria that were able to colonize the animals. The most abundant OTUs at each ontogenic stage did not correlate with the most abundant OTUS in water or feed samples. However, water seemed to have a stronger effect on the communities prior to

the first feeding while feed appeared to have a stronger effect between the first feeding through the pre-fingerling stage. Once fish approached the adult stage, their gut microbiome became more specific and less related to the bacterial communities' present in water and feed. In conclusion, from a management point of view, we hypothesize that the best time to apply pre- or probiotics to manipulate the gut microbiome is from first feeding to the pre-fingerling stage although further studies are needed to validate this hypothesis.

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Table 2-1. Sampling points and fish weight throughout the experimental period. Numbers averaged 30 individuals.

Developmental stage	Sampling Time	Weight (g) ± SD
Fertilized eggs	24 hpf[a]	0.020±0.002
Sac fry	1 dph[b]	0.017±0.002
	3 dph	0.036±0.010
Swim-up fry	6 dph	0.026±0.002
	9 dph	0.032±0.005
	15 dph	0.069±0.020
Pre-fingerling	21 dph	0.140±0.051
	28 dph	0.355±0.074
	60 dph	2.423±0.312
Fingerling		
	90 dph	4.348±0.357
	120 dph	7.610±1.478
	150 dph	8.702±1.957

a, hours post fertilization
b, days post hatch

Table 2-2 Diversity indexes as calculated by Mothur (ver. 1.33.2). Operational taxonomic units (OTUs) were defined at 97% sequence similarity. Significance among total values for each developmental stage was determined by one-way ANOVA followed by Tukey's post hoc test. Within a column, different superscripts indicate significant differences ($p<0.05$).

Development stage	Sobs*	Chao1	ACE†	Shannon-Evenness
Fertilized egg	539.00 ± 57.74^{a}	892.70 ± 51.79^{a}	1103.38 ± 78.08^{a}	0.4802 ± 0.150^{a}
Sac fry	205.25 ± 40.82^{c}	440.26 ± 105.78^{c}	765.20 ± 222.70^{ab}	0.4650 ± 0.085^{a}
Swim up fry	464.17 ± 33.33^{ab}	708.51 ± 39.80^{ab}	750.50 ± 61.42^{ab}	0.6448 ± 0.039^{a}
Pre-fingerlings	419.67 ± 102.76^{ab}	693.30 ± 76.36^{ab}	794.41 ± 133.83^{ab}	0.5823 ± 0.112^{a}
Fingerlings	331.50 ± 75.43^{bc}	566.40 ± 116.00^{bc}	632.71 ± 153.10^{b}	0.5315 ± 0.042^{a}

*, Sobs, total number of OTUs observed in the community
†, ACE, abundance-based coverage estimation

Table 2-3. One-way ANOSIM results for global test and pairwise comparisons of OTUs separated by development stage.

One-way ANOSIM of fish microbiome		
Pairwise tests	**R-value**	**p –value**
Global	0.881	0.001
Fertilized Egg vs. Sac fry	0.893	0.067
Fertilized Egg vs. Swim up fry	0.906	0.036
Fertilized Egg vs. Pre-fingerlings	0.865	0.036
Fertilized Egg vs. Fingerlings	1	0.036
Sac fry vs. Swim up fry	0.933	0.005
Sac fry vs. Pre-fingerlings	0.948	0.005
Sac fry vs. Fingerlings	1	0.005
Swim up fry vs. Pre-fingerlings	0.385	0.006
Swim up fry vs. Fingerlings	0.981	0.002
Pre-fingerlings vs. Fingerlings	0.874	0.002

Table 2-4. One-way SIMPER analysis comparing gut microbial from different life stages. Only genera accounting for at least 2% of dissimilarity between ontogenetic stages are noted. Percent abundance at each ontogenic stage and percent contribution to dissimilarity from each genus are listed.

Ontogenetic Stage	Genus	Stage 1	Stage 2	Contribution to dissimilarity (%)
1. Fertilized egg	*Opitutus*	0.55	50.68	28
2. Sac fry	*Vibrio*	32.54	0.54	18.13
	Flavobacterium	21.16	0.67	11.57
	Lysobacter	8.74	6.22	5.3
	Corynebacterium	0.13	4.83	2.63
	Staphylococcus	0.12	4.06	2.2
	Acinetobacter	0.36	3.96	2.2
Ave. diss.=89.53				
1. Sac fry	*Opitutus*	50.68	0.04	28.36
2. Swimming up fry	*Phyllobacterium*	1.49	14.77	8.13
	Shewanella	0.13	13.92	7.72
	Halospirulina	1.62	8.7	4.57
	Aeromonas	1.64	7.98	3.91
	Lysobacter	6.22	0.26	3.35
	Xylella	0.32	5.06	2.89
	Acinetobacter	3.96	1.99	2.47
	Corynebacterium	4.83	0.59	2.37
	Vagococcus	0.01	4.12	2.3
	Ureibacillus	0.03	3.98	2.22
Ave. diss.=89.30	*Staphylococcus*	4.06	0.69	2.07
1.Swimming up fry	*Candidatus* Arthromitus	0.03	17.53	11.58
2. Pre-fingerlings	*Phyllobacterium*	14.77	0.03	9.75
	Halospirulina	8.7	15.55	8.76
	Shewanella	13.92	2.2	8.59
	Vibrio	0.27	9.99	6.45
	Lactobacillus	1.55	9.02	5.59
	Aeromonas	7.98	2.23	4.27
	Xylella	5.06	0.02	3.34
	Vagococcus	4.12	0.45	2.51
	Ureibacillus	3.98	0.85	2.38
Ave. diss.=75.58	*Plesiomonas*	0.92	3.37	2.02

Table 2-4 continued

Ontogenetic Stage	Genus	Stage 1	Stage 2	Contribution to dissimilarity (%)
1. Pre-fingerlings	*Cetobacterium*	0.78	27.53	14.98
2. Fingerlings	*Clostridium*	1.97	26.22	13.59
	Turicibacter	1.57	20.95	10.93
	Candidatus arthromitus	17.53	0.02	9.81
	Halospirulina	15.55	0.55	8.4
	Vibrio	9.99	0.17	5.53
	Plesiomonas	3.37	11.24	5.22
	Lactobacillus	9.02	0.1	5
Ave. diss.=89.25	*Ureibacillus*	0.85	4.88	2.3

Table 2-5 Diversity indices as calculated by Mothur (ver. 1.33.2). Operational taxonomic units (OTUs) were defined at 97% sequence similarity.

Sample type	Source	Sobs*	ACE†	Chao1	Shannon-evenness
Feed	D00	702	1405.9	1110.2	0.606
	D01	378	1576.2	812.8	0.623
	D02	566	1205.6	958.2	0.486
	D03	525	1030.2	795.5	0.466
Water	Hatchery	1096	2023.5	1715.2	0.419
	G-system	1571	2135.4	2176.9	0.691
	Parent's spawn 1	1380	1852.3	1870.8	0.711
	Parent's spawn 2	1405	2493.1	2085.0	0.683

*, Sobs, total number of OTUs observed in the community

†, ACE, abundance-based coverage estimation

Table 2-6. Bacterial composition in water samples represented as percent abundance (top-ten genera).

Parent's tank	%	Hatchery	%	G-System	%
Candidatus Planktophila	20.5	*Aquimonas*	56.5	*Cyanobacterium*	21.8
Verrucomicrobium	6.1	*Pedobacter*	10.0	*Flexibacter*	4.8
Cyanobacterium	5.3	*Bacteroides*	4.5	*Pedobacter*	3.1
Synechococcus	4.0	*Nitrospira*	2.5	*Geofilum*	3.1
Planktothrix	3.9	*Bdellovibrio*	2.0	*Sphingobacterium*	3.0
Singulisphaera	3.8	*Candidatus* Kuenenia	2.0	*Clostridium*	2.4
Polynucleobacter	3.7	*Acinetobacter*	1.4	*Chitinophaga*	2.1
Sediminibacterium	2.5	*Algorimarina*	1.4	*Haliscomenobacter*	1.9
Opitutus	2.4	*Saccharospirillum*	1.0	*Aeromonas*	1.8
Kocuria	2.3	*Pseudomonas*	0.9	*Acidovorax*	1.5

Table 2-7. Bacterial composition in feed samples represented as percent abundance (top-ten genera).

D00	%	D01	%	D02	%	D03	%
Halospirulina	34.7	*Halospirulina*	18.1	*Anoxybacillus*	39.5	*Halospirulina*	60.3
Corynebacterium	8.3	*Candidatus* Phytoplasma	12.1	*Halospirulina*	22.3	*Anabaena*	9.6
Anabaena	5.0	*Acinetobacter*	9.9	*Ureibacillus*	10.6	*Clostridium*	5.2
Acetobacter	4.6	*Pasteurella*	9.6	*Candidatus* Phytoplasma	10.5	*Fusobacterium*	4.7
Candidatus Phytoplasma	3.9	*Wohlfahrtiimonas*	7.5	*Fusobacterium*	3.7	*Candidatus* Phytoplasma	3.1
Streptococcus	3.7	*Anoxybacillus*	6.2	*Anabaena*	3.0	*Photobacterium*	2.9
Lactobacillus	3.0	*Anabaena*	3.4	*Planomicrobium*	1.0	*Lactobacillus*	2.4
Bacillus	3.0	*Fusobacterium*	3.2	*Clostridium*	0.8	*Cetobacterium*	2.2
Staphylococcus	2.6	*Streptococcus*	2.8	*Cetobacterium*	0.6	*Anoxybacillus*	1.7
Sporosarcina	2.6	*Lactobacillus*	2.6	*Photobacterium*	0.6	*Shewanella*	1.6

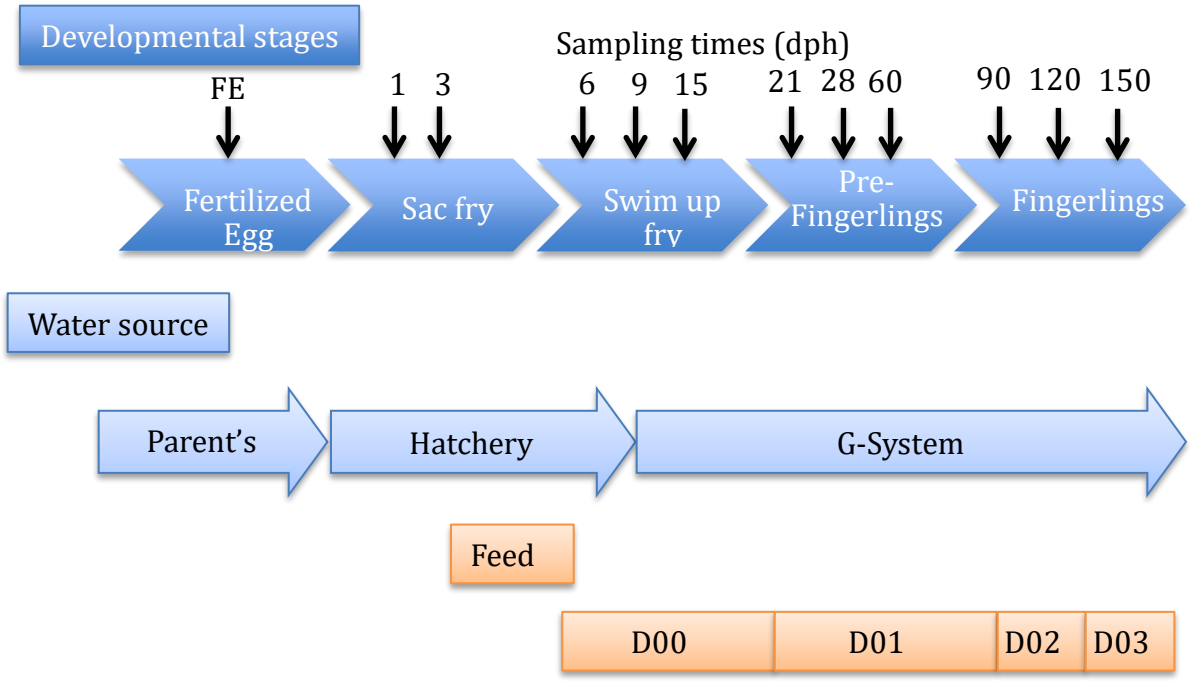

Figure 2-1. Experimental design and catfish development showing important developmental events (top) and husbandry events (bottom) during the course of the study. The sample time-points is shown.

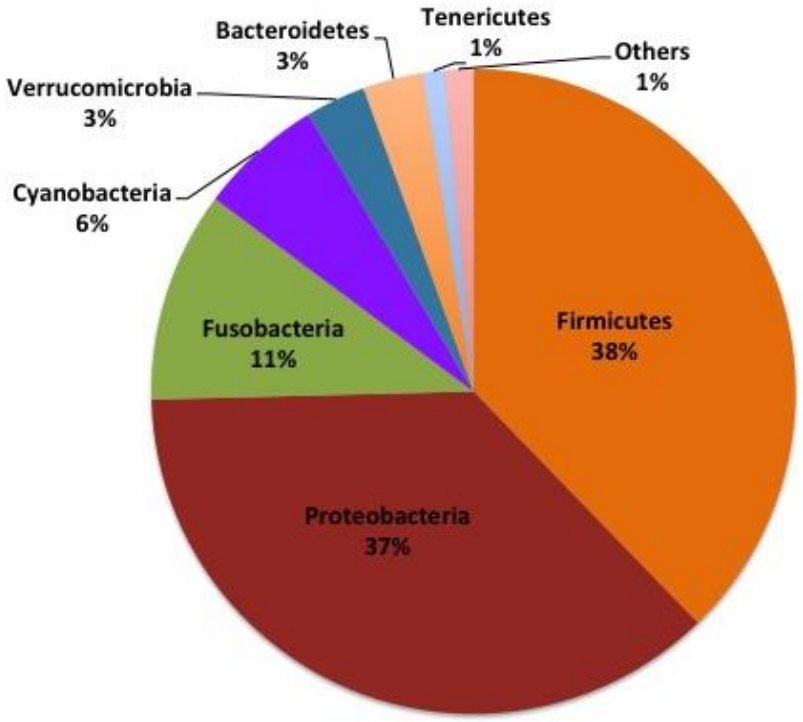

Figure 2-2. Percentages reflect abundance of phyla taking account all life stages microbial communities. All phyla present in abundance of < 0.1% are included as other.

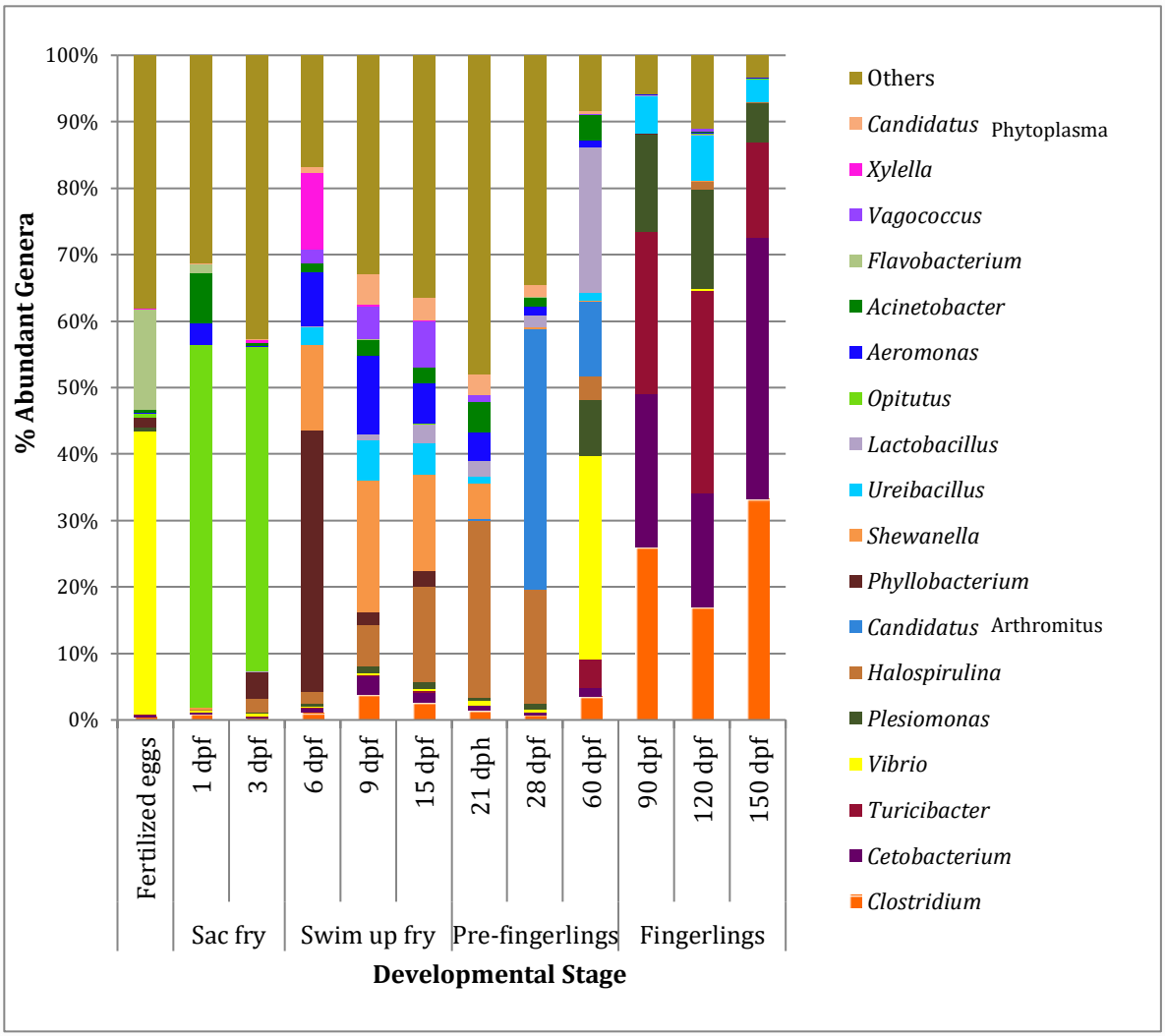

Figure 2-3. Percentage abundance at genus level. In the Y axis is represented the percentage of abundance and X axis represent the development stages and the time points.

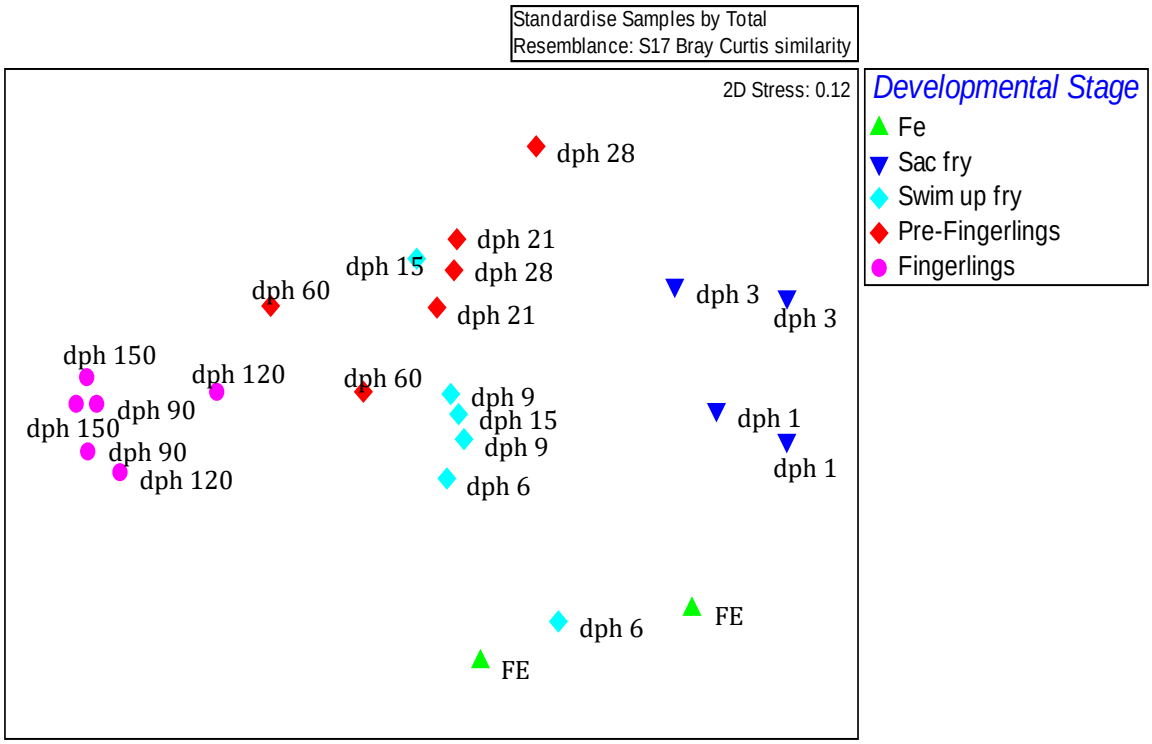

Figure 2-4. MDS from all developmental stage. Each life stage is labeled with a different color.

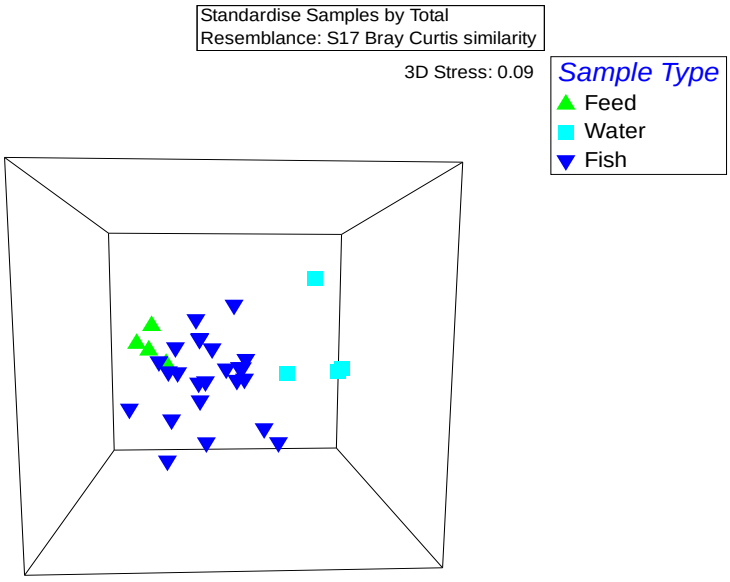

Figure 2-5. MDS comparison of fish, feed and water samples. Each group is labeled with different color.

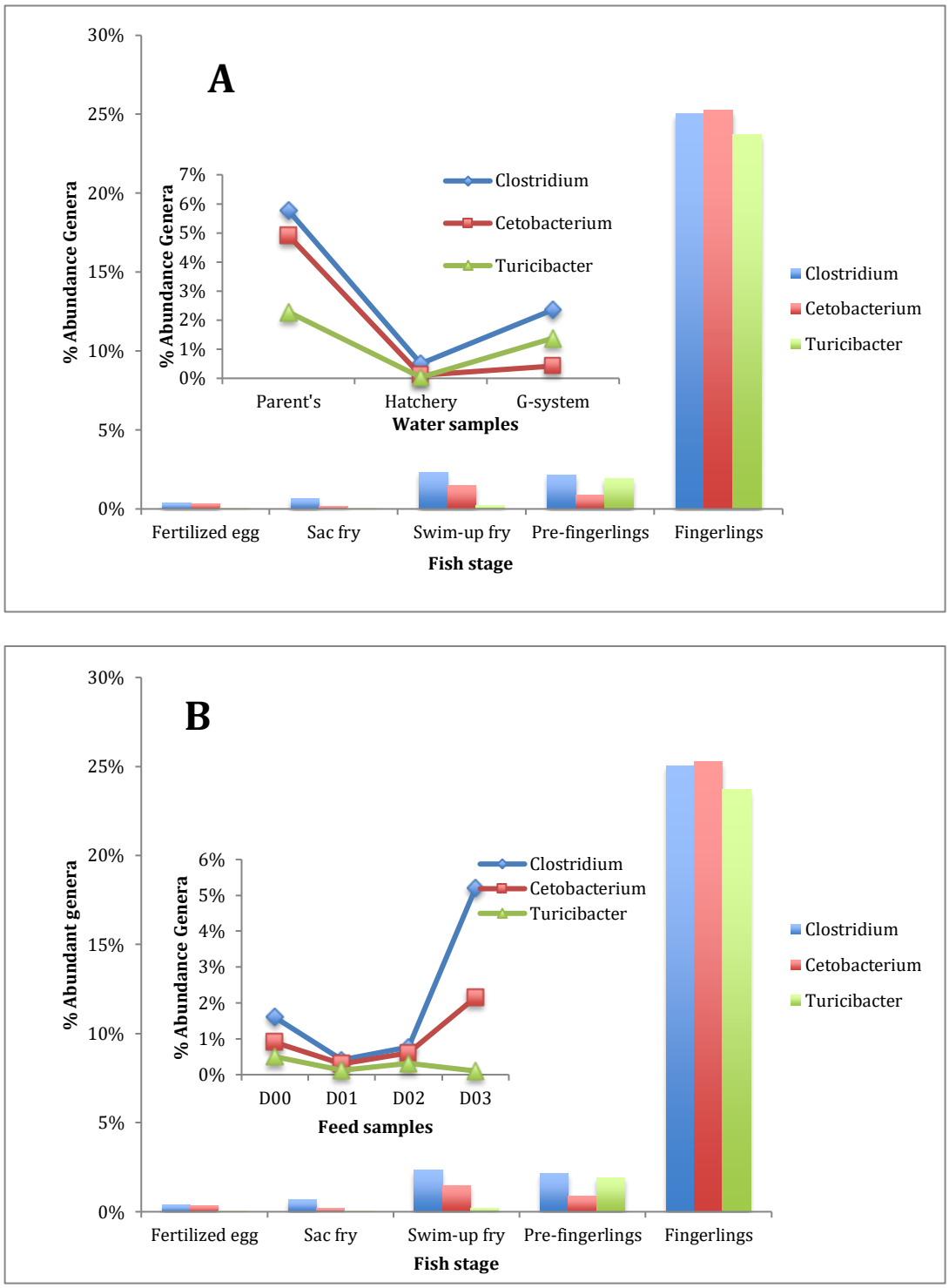

Figure 2-6. Percent abundance of the three main genera comprising more than 60% of the fingerling gut microbiome at each developmental stage and their abundance in water samples (A) in each of the systems used and in feed (B)

CHAPTER 3. EFFECT OF MEDICATED FEED IN THE GUT MICROBIOME OF ZEBRAFISH

Abstract

The gut microbiome of fish is composed by a complex bacterial community living in a symbiotic relationship with their host. When the equilibrium between the host and its gut microbiome symbiosis is disrupted (i.e., dysbiosis), bacterial diversity tends to decrease while opportunistic pathogens increase. In aquaculture, bacterial infections are often controlled by using feed supplemented with antibiotics (medicated feed). Florfenicol (FFC, commercially knows as AQUAFLOR) is a broad-spectrum antibiotic widely used to treat fish disease. The objective of this study was to evaluate the effect of FFC-medicated feed on the gut microbiome of adult zebrafish to determine i) if therapeutic doses of FFC-medicated feed induced dysbiosis, and ii) if fish with altered gut microbiome were more susceptibility to the common opportunistic bacterial pathogen, *Aeromonas hydrophila (Ah)*. Four treatments were compared in the study: a) System control (regular feed & not challenged); b) Treatment I (regular feed & challenge with *Ah*); c) Treatment II (FFC-medicated feed & challenged with *Ah*) and Treatment III (FFC-medicated feed & not challenged). After FFC treatment, fish were allowed to recover for 15 days. High-throughput Illumina MiSeq DNA sequencing of the V4 domain of the 16S rRNA gene was used to analyze changes in the gut microbiome during the experiment. qPCR was used to measure the expression levels of specific genes related to pro-inflammatory activities to

understand the effect of the medicated feed had on intestinal inflammation and homeostasis. Characterization of the microbiome using 16S rRNA gene sequencing confirmed the disruption of the gut microbiome by FFC-medicated feed, with a decrease in bacterial diversity, accompanied with a marked bloom of Proteobacteria and drastic reduction of *Mycoplasma* and *Cetobacterium*. After the withdrawal period, the communities did not fully recover and the zebrafish treated with FFC-medicated feed exhibited a significantly higher mortality rate when they were exposed to *Ah*. All genes (CCL20, IL1β, IL-8, and TNFα) were equally expressed in treated and untreated zebrafish, thus inflammation caused directly by the antibiotic did not seem to be a factor.

Introduction

The fish gut microbiome is a complex bacterial community living in a mutualistic relationship with their host [1-4]. The gut microbiome provides the host with complementary genetic resources including genes involved in energy harvesting, production of essential vitamins, bio-elements, metabolites [5-7]. In addition, the gut microbiome supports the development of host's immune system by safeguarding it against pathogen colonization and invasion [8, 9]. Failure to maintain a balanced equilibrium between host and its gut microbiome results in dysbiosis. Many studies in humans have shown that antibiotic administration induced dysbiosis with adverse effect on patient health [10-13]

In the USA and the European Union, among other countries, the use of antibiotics in fish intended for human consumption is tighly regulated and the few antibiotics approved for use in fish farms are controlled by veterinary feed directive (VFD)[14, 15]. Florfenicol is one of three antibiotics that are approved for use in channel catfish, the main aquaculture species in the USA. Florfenicol (FFC) is commercially available for fish farmers under the trade name Aquaflor® (Merck & Co., Inc.) as a pre-mix medicated feed containing 50% of florfenicol [16]. FFC is only approved to treat columnaris disease and edwardsiellosis in channel catfish (caused by *Flavobacterium columnare* and *Edwardsiella ictaluri*, respectively) [17, 18]. However, as a broad-spectrum antibiotic that inhibits Gram-positive and Gram-negative bacteria growth [19], FFC is likely to eliminate commensal members of the gut microbiome in fish resulting in an unbalanced gut microbiome[13].

FFC was the last antibiotic to be approved by FDA to treat channel catfish and it rapidly became the drug of choice by producers' due to its efficacy [20-23]. He et al. (2010) investigated the effect of FFC on the intestinal microbiome of tilapia and found a significant reduction in bacterial diversity in fish fed with medicated feed. However, their study had a limited scope due to the use of a fingerprinting-based method to characterize the bacterial communities [24]. Therefore, the full effect of FFC on the fish gut microbiome, when used at the recommended therapeutic doses, has not been investigated yet. In addition to examine changes in diversity and population structure of the gut microbiome, we were interested in testing if an inflammatory response ensued as result of the treatment as has been seen in mouse animal models [8]. We chose zebrafish as our animal model as it has been widely used to study infection, immunity, and

inflammation in vertebrates [25-27]. Studies on chemically induced enterocolitis [28-31] and antibiotic administration [12, 32-35] in zebrafish have shown a strong effect of pharmacological agents on the gut microbial communities. Flemming et al. [36] described how changes in the microbial structure during gut colitis were associated with up-regulation of pro-inflammatory cytokines markers accompanied by morphological changes in the intestinal tract including the thickening of the villi, increased infiltrated eosinophils, mucus production, and enlarged goblet cells [28, 37, 38]. Lastly, we wanted to test if antibiotic-induced dysbiosis could predispose the fish to common opportunistic pathogens such as *Aeromonas hydrophila* that have an enteric colonization route [39-41].

Material and Methods

Zebrafish husbandry.

Fish were purchased from Aquatic Bio-Tech (Sun City Center, FL, USA) as adult unsexed (> 5 months old, ZDR wild line; n = 720, with an average weight 0.17 ± 0.7 g and length 25.90 ± 2.45 cm). Upon arrival, the fish were quarantined in a stand-alone unit (270 L) for 15 days and inspected for parasites and bacteria pathogen by the Southeastern Cooperative Fish Parasite & Disease Laboratory, Auburn University (Case ID#: FL16_1). After the quarantine period, fish were stocked into 20 tanks, 37 L each, at a stocking rate of 40 fish per tank and maintained as previously described [42]. They were fed once daily to approximately 2% body weight per day with commercial catfish feed containing 32% crude protein (Alabama Catfish

Feed Mill, L.L.C, Uniontown, Al). Water quality was monitored daily, and parameters were maintained at 80 ppm alkalinity, 150 ppm hardness, 27.6 ± 0.50 ˚C, pH: 7.84 ± 0.15 (mean ± SD), ammonia and nitrites were kept at non-detectable levels with 12:12 h photoperiod throughout the experiment. The animal protocol was approved by the Auburn University Institutional Animal Care and Use Committee (IACUC number 2016-2946).

Presence of Aeromonas hydrophila in Zebrafish gut.

Aeromonas hydrophila has been previously reported as one of the typical opportunistic pathogens that are present in the intestinal tract of healthy fish. To determine if the numbers of *A. hydrophila* in zebrafish fish intestine prior to challenge, the intestinal tract and its content of 5 animals was pooled, homogenized and resuspended in 1 ml of brain heart infusion (BHI, BD GmbH-Germany). Aerobic plate counts were done on selective Aeromonas agar (AA) (LAB Neugene, Lancashire, UK) and incubated at 28 ˚C. Putative colonies of *A. hydrophila* were purified and confirmed using the API 20E identification system (API 20E, BIOMÉRIEUX, Durham, NC, USA). Susceptibility to FFC of the *A. hydrophila* isolates was carried out using a standard agar diffusion susceptibility test. Discs containing 30-µg florfenicol (Becton Dickinson, Heidelberg, Germany) were used for disc diffusion test following the outlines in the National Committee for Clinical Laboratory Standards (NCCLS). The zone of inhibition was measured according to the NCCLS manual and should between 32-44 mm [43]. Isolates that grew inside the zone of inhibition were recorded and recovered.

Resistance to FFC in gut bacteria.

We determined the degree of FFC resistance among culturable bacteria in zebrafish gut prior starting FFC treatment. Briefly, gut of 5 animals was aseptically excised, pooled, homogenized and resuspended in 1 ml of sterile 0.9% saline solution. Standar plate counts were done on Trypticase soy agar (TSA, BD GmbH-Germany) and on TSA supplemented with 25 μg/ml of FFC (Sigma-Aldrich, Darmstadt, Germany). Plates were incubated at 28 ˚C by 24 hours before colonies were counted.

Experimental design.

Experimental design consisted of 4 treatments with 5 replicates each (replicate = tank) as follows: a) System control (regular catfish feed); b) Treatment I (regular catfish feed & challenged); c) Treatment II (Medicated feed & challenged) and d) Treatment III (Medicated feed & Not bacterial challenged) (Figure 1). Tanks were randomized and assigned blindly to each treatment. During the 10-day acclimation period, all fish were fed with regular 32% catfish diet at 2% body weight (BW) (Alabama Catfish Feed Mill, L.L.C, Uniontown, Al). After acclimation, FFC-medicated catfish feed (Alabama Catfish Feed Mill, L.L.C, Uniontown, Al) was administered to fish in treatment II and III, at a dosage rate of 15-mg/kg of BW per day while regular feed was administered to the control fish (System Control and Treatment I). After 10 days of FFC treatment, all fish returned to the normal feed regime. Throughout the study, medicated and regular catfish diets were coated with fish oil (Menhaden Fish oil, Jandell Fishing Product, Tx.) to increase palatability.

Bacterial challenge.

Aeromonas hydrophila ML09-119 strain, a channel catfish epidemic strain, was used for experimental infection. Strain was cultured in brain heart infusion (BHI, BD GmbH-Germany) and incubating at 28 ˚C under shaking for 15h. Fish in treatments I and II were exposed to *A. hydrophila* by immersion for 5 hours (final concentration of the pathogen in the immersion bath was 2.65 x 10^7 CFU/ml) as described previously [44]. Fish from System control and Treatment III were sham challenged using sterile BHI broth as inoculum. After challenge, animals were returned to their respective tanks and maintained under standard husbandry conditions. Fish were observed for clinical signs of disease and mortalities were recorded twice a day for 14 days. Putative *A. hydrophila* colonies were recovered from anterior kidneys of moribund and dead fish and isolates were confirmed by qPCR.

DNA and RNA extractions from gut samples.

The entire intestine of 10 fish per tank were sampled, pooled (5 intestines for DNA and 5 intestines for RNA extraction) and snap-frozen in liquid nitrogen and stored at -80 ℃ until DNA and RNA extraction was carried out. Samples were taken at day 21 (t21 = day after medicated period end), at day 35 (t35 = at day 15 within withdrawal period and three days before the challenge) and day 52 (t52 = from the survivors at the end of the experiment). In addition, two fish per tank were fixed in formalin at each sampling time for histopathological analysis (pending).

DNA was extracted using DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions (Total DNA from Animal Tissues, Spin column Protocol) with modifications including double digestion with proteinase K and pre-treatment with

lysozyme for lysis of Gram-positive bacteria and RNase A treatment. DNA was eluted with 100-µl elution buffer/ RNA was extracted using TRizol (Invitrogen, Carlsbad, CA, USA) and liquid nitrogen method according to the manufacturer's instructions. DNA and RNA samples were quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Nanodrop Technologies, Wilmington, DE, USA), and normalized into 20 ng/µl for DNA and 100 ng/µl for RNA with ddH2O.

PCR Amplification and Next Generation Sequencing.

DNA samples were submitted to MR DNA® (Shallowater, TX, USA) for PCR amplification and Next- Generation Sequencing using Illumina MiSeq platform targeting the 16S rRNA gene V4 variable region. Universal bacterial primers 515 F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') with a barcode on the forward primer were used to generate a 300 bp amplicon. The HotStarTaq Plus Master Mix Kit (Qiagen, USA) was used under the following PCR conditions: an initial denaturation for 3 minutes at 94 ℃ followed by 28 cycles of denaturing at 94 ℃ for 30 s, annealing at 53 ℃ for 40 s, extension at 72 ℃ for 1 min and a final elongation step for 5 min at 72 ℃. The PCR products were run through a 2% agarose gel to verify successful amplification and relative band intensity of the target DNA. Multiple samples were pooled together and purified using calibrated Ampure XP beads to prepare the Illumina DNA library. The samples were sequenced as paired-end reads on the Illumina MiSeq platform following the manufacturer's instructions. The sequences resulting were processed using a proprietary pipeline (MR DNA, Shallowater, TX, USA). Sequencing data were joined, and all barcodes, ambiguous

base calls, and sequences < 150 bp were removed. Denoising of sequences was also performed, and operational taxonomic units (OTUs) were generated. Cut-offs for OTU assignment were defined at a 97% similarity (< 3% sequence variation) in concurrence with the prokaryotic species concept [45]. Taxonomic classifications were obtained using BLASTn against the Green Genes database [46].

Quantification of pro-inflammatory genes by qPCR.

To understand the effect of antibiotics on the intestinal inflammation and homeostasis, the expression of selected genes associated with pro-inflammatory response (chemokine ligand 20 (CCL20), interleukin 1 beta (IL1β), interleukin 8 (IL8), and tumor necrosis factor alpha (TNF-α)) were investigated at the 3 specific sampling points previously mentionated. cDNA was synthesized from the total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). qPCR was carried out with Power SYBR Green qPCR Master Mix (Applied Biosystems, Woolston, Warrington, UK) in an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA), using the following cycling conditions: 95 ℃ for 1 min, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. Each reaction was performed in triplicate, and a melting curve analysis was performed at the end to confirm the specificity of the reactions. The elongation factor gene, ef1α, was used as internal control. The average ΔCT was calculated by subtracting the average ef1α CT from the average target gene CT. The ΔΔCT was calculated by subtracting the control ΔCT from the treated ΔCT. The relative quantity of mRNA was calculated as 2- (ΔΔCT). Quantification of the targeted genes was conducted as described by Oehlers et al [28].

Data Analysis.

For data analysis, samples were subdivided based on treatments and sampling time into a total of 8 groups (see Figure 1). Group A (A), samples from Control System & Treatment I at t21; Group B (B), samples from Treatment II & III at t21; Group C (C), samples from Treatment II & III at t35; Group D (D), samples from Control System & Treatment I at t35; Group E (E), samples from Treatment I at t52; Group F (F), samples from the Control System at t52; Group G (G), samples from the Treatment II at t52; Group H (H), samples from the Treatment III at t52. Pairwise comparisons between control and medicated-feed fish were conducted at each sampling time since we knew, from previous experiments, that the microbial communities of fish changed over time when animals are kept under artificial, experimental conditions [47].

Mortality data were analyzed using analysis using Analysis of variance (ANOVA) followed by Tukey's student Range (HSD) test for all-pairwise comparisons to determine significant ($P < 0.05$) differences between the mean mortality of the different treatments using JMP Version 12 .0.1 (SAS Institute Inc. Cary, NC, 1989-2007). Rarefaction curves, observed OTUs, shared OTUs, ACE, CHAO1, good coverage and Shannon-evenness index (SEI) were calculated using Mothur v.1.39.5 [48] after standardization of each sample type to the sample yielding the least number of total sequences. One-way Anova was used followed by Tuckey's test for all-pairwise comparison to determine differences in diversity index between the groups. The PCoA (Principal coordinate analysis) using the variables treatment, time, and groups were performed using optimized position to visualize the similarities or dissimilarities of the samples. OTU and Genera tables including all samples were loaded into PRIMER (Primer E Ltd,

Plymouth, UK) for clustering using group average and ANOSIM analysis (group, treatments, sampling time). To accompany the results of the ANOSIM, a PERMANOVA statistical test was used to analyze the diversity across groups sampled. Besides, a PERMDISP test, was conducted under the null hypothesis of no differences of within-group multivariate dispersion across sampling time points, to give insight on within- and between - group dispersion to enable more accurate interpretation of the PERMANOVA and ANOSIM results [49]. PERMANOVA, ANOSIM, and PERMIDISP test were conducted using 9,999 permutations of data, following the recommendation of Clarke and Gorley [50]. A Pairwise test was done when statistical tests identified significant effects within main test ($P \leq 0.05$) to determine the difference between and within groups across sampling events. Similarity percentage (SIMPER) analysis on a Bray-Curtis similarity matrix assembled from a Genus level was conducted to determine specific taxonomical differences between communities. The relative abundance at phylum and genus level comparison was using One-way ANOVA.

Comparison of qPCR gene expression analysis was carried from the five pro-inflammatory targeting genes was carry out through Pair wise Fized Reallocation Randomization test [51] using Relative Expression Software Tool, Version 1.

Results

Zebrafish health check.

Routine protocols were followed to examine a few selected zebrafish before the experiment. Fish looked normal, did not present any exoparasites, and were negative for bacterial culture from internal organs. Numbers of *Aeromonas spp*., in zebrafish gut were approximately 4.5 x 10^5 CFU/g of tissue. The number of culturable heterotrophic bacteria resistant to florfenicol was 6.25 x 10^2 CFU/g. Weight and length of the fish did not significantly change during the study. Total length (cm) and weight (g) were reported as mean ± SD (see Table 3-1). After challenged, fish infected and colonized by *A. hydrophila* exhibited the typical clinical signs of motile aeromonads septicemia (MAS) including external hemorrhages and petechial in the abdomen and visceral cavity. Anecdotally, fish in-group G (antibiotic + challenged) exhibited an increase of feces after challenge.

FFC-medicated feed alters bacterial community composition and diversity.

A total number of 1,402,164 bacterial sequences representing 2,046 baterial OTUs were obtained from the study. After sample normalization, to the lowest read (n = 13,408) a total number of 536,320 bacterial sequences and 1,866 OTUs were included in the analysis. Sequence coverage was ≥ 98% in all sequenced samples (Good's coverage, Table 3-2).

Rarefaction curves (Figure 3-2) from all groups showed that the medicated treatment had more diversity compared with controls at t21 and t235 but diversity decreased overtime in all treatments. Group B (medicated feed) at 21 showed the highest diversity while group F (system control) at t52 had the least diverse bacterial population. During the transition from t21 to t35 (withdrawal period) diversity between controls Group A and D was very close, while medicated feed Group C reduce their diversity considerably compared with Group B at the same period.

Group E and H showed similar diversity at t52. Figure 3-3 shows the unique OTUs for each group as well as those shared between control & medicated-feed groups at t21 and t35 in addition to the pairwise comparison between control/medicated-feed and challenge/non-challenge groups (Table 3-3).

Over sampling time, the most significant changes were detected in treated groups regarding observed OTUs and evenness at t21 and t52, while control groups remained relatively constant over time. The microbial community richness seen through the total number of observed OTUs (Sobs) on group B was significantly higher compared with group E and F. The total expected richness as calculated by ACE and Chao1 didn't show any significant difference between and within groups. Shannon-evenness index reported a substantial difference between group A & B, also between G & H (Table 3-2)

These changes in diversity were accompanied by significant changes at phylum composition in-group F and E compared with the other groups, and OTU composition between groups post-treatment, except in the challenged group E and G wich not significance was observed between them (Figure 3-4 and Figure 3-5). When bacterial sequences were ascribed at the phylum level, a total of 19 phyla were observed, and each group returned a unique bacterial composition. Proteobacteria, Tenericutes, Firmicutes, were most abundant phylum presented between all groups. Proteobacteria accounted for 53% of the total sequences obtained, whereas, Tenericutes and Firmicutes represented the 21% and 15% respectively of the entire sequences. Fusobacteria notably represented only 7% and other less common phyla like Bacteroidetes and Planctomycetes formed 1%, and the rest 13 phyla were less than 1% of the total sequences. The

phylum Proteobacteria was the most predominant phylum in six groups and comprised the majority of all sequences, with trend to increase over time mostly at medicated groups than the control groups (48 % in A, 66% in B, 64% in C, 46% in D, 76% in G and 71% in H). The abundance of Proteobacteria was significant reduced to 27% at group F. Contrarily; Tenericutes were significantly higher in control groups, especially in groups E and F that showed the highest abundance at 54% and 49%, respectively, except for group G at 19%. On the other hand, Firmicutes showed reduction in bacterial abundance over time in the control groups from 19% to 5% with the lowest value in the challenged group E. Meanwhile, on medicated group the abundance of Firmicutes were held relatively constant (~20%) over time with the exception of challenged group G that showed a drastic reduction (3%). Conversely, Fusobacteria was mostly present in all non-medicated groups A at 10.7%, D at 23,2%, E at 6,7%, and F at 14.8% respectively, but significantly scarce (< 1%) in medicated groups B, C, G, H. Other less common phyla, like Bacteroidetes, were presented in groups D, C (> 1%) but scarce in the rest of the groups. Planctomycetes were detected in groups B, C, D and limited in the other groups. Actinobacteria and Chlamydia were presented at 1% in A and B respectively, meanwhile were less than that in the other groups. The rest of the phyla were lower than 1%.

The gut microbiome of all groups was composed of a total of 526 genera. Only the most dominant genus presented (> 0.5%) in all the groups were listed in Table 3-4. *Aeromonas* and *Pseudomonas* (Gamma-proteobacteria, phylum Proteobacteria) were the most abundant bacterial sequence presented in all groups with the highest prevalence in group H; *Aeromonas* was significantly higher in group H compared with the rest groups. Interestingly, *Mycoplasma*

(Mollicutes, phyla Tenericutes) and *Cetobacterium* (Fusobacteriia, phylum Fusobacteria) were the most abundant in control groups A, D, E, F while scarce in all medicated groups B, C, G, H. *Mycoplasma* was significantly higher in group F and E compared with the rest groups; while *Cetobacterium* was in group D compared with the treated groups. Furthermore, *Ruminiclostridium* (Clostridia, Firmicutes) was most abundant in-group A, C, and H and lowest in B, D, E, G, F. *Geobacillus* and *Bacillus* (Bacilli from the phylum Firmicutes) were present in A, B, C, D, E, F. *Phyllobacterium* (Alfa-proteobacteria, Phylum Proteobacteria) was most abundance in-group A and scarce in the rest of the groups, while *Shinella* (Alfa-proteobacteria, Phylum Proteobacteria) was in medicated group B. *Stenotrophomonas* (Clostridia, Phylum Firmicutes) and *Burkholderia* (Beta-proteobacteria, Phylum Proteobacteria) was most abundant (> 2%) at group A and B and scarce in the rest of the group.

Modulation of post- antibiotic, bacterial community reassembly.

Visual representation of the beta diversity using PCoA gut microbiome samples based on group ascription at OTUs level shows the great grouping of control and medicated individual samples across sampling events with some overlap between groups (Figure 3-5).

ANOSIM and PERMANOVA directly compared the cluster based on the following variables: treatment (I through III & Control System), time (t21, t35, t52), and group (A to H) (Table 3-5). Samples clustered significantly ($P \leq 0.05$) by all factors considered, although there was some overlap among them, however, the separation was most significant when samples were assigned to the cluster based on the group with an R-value of 0.21. These global R-values indicate that clusters are significantly correlated with all the factors although group (group =

treatment + time combined) was the most significant variable and played the primary role determining the change in the composition of the gut microbiome. The R- values for treatment and time were 0.159 and 0.191, respectively.

Further, statistical analysis of beta diversity across sampling events shows a significant divergence of the microbial communities' present between gut group than within group at OTUs level, as both PERMANOVA and ANOSIM main test indicate a significant difference ($P \leq 0.001$; Table 3-6a). Additionally, PERMDISP test ($P \geq 0.05$, Table 6a) indicated that within-group dispersion was homogenous; therefore, the results of the PERMANOVA can be interpreted as real differences in the multivariate composition of microbial communities. Complementary, the pairwise PERMANOVA and ANOSIM results were in agreement that significant shifts occurred between microbial communities every sampling time point ($P \leq 0.05$, Table 3-6b), except for the post-challenged groups E & G (t52), which failed to show significant differences ($P = 0.243$ and $P = 0.122$, Table 3-6b). The highest difference in microbial composition was detected between group F & H (non-challenge groups), as this comparison showed the largest ANOSIM and PERMANOVA test statistic (R-value 0.416 and pseudo-value 2.00, respectively). Furthermore, the paired comparison within treatments and across sampling time shows non-significant changes between all control groups, but significant differences between medicated groups C & G (PERMANOVA, data not shown).

Temporal variability over the sampling time.

To further explore microbial differences in groups, Similarity Percentage (SIMPER) analysis was used to evaluate within-group similarity and among-group dissimilarity. SIMPER

analysis by bacterial genera between replicates (within each group) showed higher similarities within medicated than control groups. Furthermore, within-groups similarities increased across sampling events in medicated B, C, G, and H (39.8%, 55.7%, 62.91%, 63.8%), while in control groups A, D, E, and F similarities were very close ranging from 40.85% to 45.8%. Conversely, SIMPER analysis showed high pairwise dissimilarities between groups (Table 3-7). The main dissimilarities between groups were due to the different relative abundance of the genera *Mycoplasma*, *Aeromonas*, *Cetobacterium*, and *Pseudomonas*. Based on genus composition, SIMPER analysis indicated that A and B were the most dissimilar (70.22%), followed by F & H (63.98%), while E & G were the least different (49.08%).

Susceptibility to the opportunistic pathogen A. hydrophila.

The mean cumulative percent mortality after challenge is shown in the (Figure 3-6). Only treatment II (medicated feed & challenged) reported mortalities, meanwhile System Control (Control feed & not challenged), Treatment I (Control feed & challenged) and Treatment III (medicated feed & not challenged) did not show any mortality throughout the experiment. Fish treated with medicated feed and challenged (Treatment II) had a mean percent mortality of 13.33 ± 0.577(SD), which was significantly different ($P < 0.05$) from the control challenged (Treatment I) which did not show any mortality. *Aeromonas hydrophila* was isolated from kidney and brain of dead and moribund fish and confirmed by PCR. Mortality persisted for four days with the majority of fish death at the two and four days post-challenge. The study was concluded after fourteen days with ten consecutive days without mortalities.

Gene expression of inflammatory response.

None of the four genes related to pro-inflammatory response was significantly up- or downregulated in any of samples analyzed, which suggested that a cytokine-mediated inflammatory response was not induced by florfenicol.

Discussion

Fish maintained under controlled conditions or captivity has been seen tend to reduce the diversity of their bacterial community [47]. In this study, we observed that over time, control fish that were not given medicated feed or were exposed to a pathogen, exhibited a reduction in their bacterial diversity. The same trend was observed in fish that received medicated feed. This factor needs to be taken into account when performing these types of studies in where fish are mantained for longer periods of time under artificial environments with low bacterial loads (i.e., no sediments, controlled biofiltration, and very low suspended solids).

Overall, our results were consisted with the studies realized in animal models treated with antibiotics which reported diminish in the diversity with lost of crucial microbial members within the gut microbiome that long lasted after the cessation of antibiotic treatment [52-58]. Interestingly, the most sensible genera to medicated feed were *Mycoplasma*, *Ruminiclostridium, Phyllobacterium,* and *Cetobacterium*. From all of these only *Mycoplasma* has been reported susceptible to FFC [59], probably due to their lack of cell wall [60, 61].

As we hypothetized, once the FFC-antibiotic selecetive pressure ends, the alterations in the gut microbiota composition remained. This disruption in microbiome structure was correlated

with a significant increase in mortality of zebrafish treaed with FFC-medicated feed (13.3%) compared to those with intact gut microbiome (0%) after pathogen exposure. Hence, gut microbial dysbiosis significantly increased zebrafish susceptibility to MAS casused by *A. hydrophila* suggesting that medicated treatment weakened the host-microbiota mutual benefit associations. Our mortality results were in accordance with the OLA-altered gut microbiota reported by He et al. [35] in zebrafish that shows an increase in the susceptibility to *A. hydrophila* infection. Freshwater Western mosquitofish (*Gambusia affinis*) with altered antibiotic microbiome reported dying more rapidly than control fish when they were challenged with *Edwardsiella ictaluri* [57]. The lack of crucial organisms and the immediate increase of opportunistic bacteria could shift the homeostatic mechanism toward potentially unfavorable outcomes. The bloom of Proteobacteria and diminish of *Cetobacterium* observed in the groups exposed to a broad spectrum antibiotic in this study, has been previously reported by He et al. [35] in gut microbiome of adult zebrafish using Olaquindox medicated feed. Proteobacteria phyla has been identified as part of the gut microbiome in zebrafish [62, 63], but their increase until become the dominant phyla over time as respond to antibiotic and chemical treatment has been previously reported in zebrafish gut dysbiosis studies [29, 35]. It has been proposed that increase in the prevalence of phylum Proteobacteria into the gut microbiome after a perturbation success can be considered as a potential diagnostic-signature of dysbiosis and risk of disease [7, 29]. *Cetobacterium* has been reported as common genus present in zebrafish [64] and other fish gut [65, 66]. One of the functional roles of this genus identified is providing critical biomolecules including Vitamin B required in critical function into the host.

The timeline in the establishment of zebrafish infection with *A. hydrophila* in our study was consistent with the reported by Saraceni [67] using zebrafish larvae to study the *A. hydrophila* pathogenesis. As well, our low mortalities rate of 13.3 % in the medicated-challenged group was consistent with the low accumulative mortalities of 33% reported by them using bath immersion mimicking the natural route of infection. *A. hydrophila* is naturally present in the gut of microbiota of zebrafish [62], it can generate an acute infection on adults but requires a pre-existing condition in the host health to cause the disease [44, 68]. Even though a shift in the microbial community was observed in control groups during the study, the microbiome did not succumb to the pathogen. It may be due to those fish had a stable gut microbiome. A balanced gut microbial community shows colonization resistance by the commensal microbiota promoting pathogen clearance [69-71].

Interestingly, in our study the groups challenged with the opportunistic pathogen *A. hydrophila* showed that dominant Proteobacteria was substituted by Tenericutes (*Mycoplasma*) in E & G (control & medicated, respectively), while the member of Fusobacteria (*Cetobacterium*) was reduced in-group E. A thorough literature review revealed no antagonism between any Fusobacteria member (mainly *Cetobacterium*) and *A. hydrophila* or any synergism with any Tenericutes member (mainly *Mycoplasma*). Our finding, suggest that virulent *A. hydrophila* can affect the biology of the host to discriminate between beneficial autochthonous microbes and harmful pathogens during colonization.

The variable group (Group = treatment + time combined) was the most influential factor affecting the gut microbiome composition. Each group presented a significant distinct

microbiome with a relatively low sample-to-sample variability within each group. Groups subject to only one treatment as F (control) and H (FFC-medicated feed) revealed that the microbiome contains a different but stable microbial community. However, in the treated fish, although most of the microbiome return to pre-treatment level once the medicated period end, several bacteria taxa failed to recover and were lost until the end of the experiment, indicating that changes in the microbiota persisted after cessation of antibiotic treatment. However, it is unclear if the loss of these members of the community could last indefinitely. By contrast, after two treatments (diet type and pathogen) few changes were observed in the microbial community in control, with a particular increased of Tenericutes phylum while Firmicutes decreased. The gut microbiome structure in control and medicated challenged groups G & E were strikingly similar to the no-bacterial challenged groups sampled at the same time. The introduction of a single new microbe in numerically inferior numbers into the bacterial microbiome during a broad community disturbance has the potential to significantly alter the subsequent reassembly of the bacterial community as it recovers from that disturbance to initial conditions [72].

Over time, a bloom of dominant Proteobacteria phylum was observed within medicated groups regardless of the composition at earlier time points. The increase in Gamma-proteobacteria was primarily due to an increase in the abundance of *Aeromonas* and *Pseudomonas* revealing their role as an opportunistic and fast-growing (K-type) species. The microbiome in Treatment II (medicated) was dominated by the genera *Aeromonas, Pseudomonas*, *Ruminiclostridium*, *Geobacillus*, and *Bacillus* before the challenge at t35 (C) and *Aeromonas*, *Mycoplasma*, *Pseudomonas*, *Shinella*, and *Ruminiclostridium* at t52 (G). The

microbiome in Treatment I (*A. hydrophila* treatment) was dominated by the genera *Aeromonas*, *Cetobacterium*, *Mycoplasma*, *Pseudomonas*, and *Geobacillus* before challenging at t35 (D) and by *Mycoplasma*, *Aeromonas*, *Pseudomonas*, *Cetobacterium* and *Ruminiclostridium* at t52 (E). This substantial difference in genera abundance between FFC-medicated treated zebrafish compared with untreated fish may have determined the increased susceptibility to *A. hydrophila* infection. Our findings support the hypothesis that an unstable gut microbial community, characterized by an abundance of Gamma-proteobacteria, may represent an active feature, rather than a passive consequence, of metabolic disturbance [73] and can be a definite sign of gut dysbiosis. Has been suggested that individuals that have pre-existing conditions such as an altered-gut microbiome, once they face a un-identified environmental factor such as viruses, bacterial infection, trauma or stress [74] could lead to an apparition of another clinical condition in the host. However, further studies under field conditions are needed to the full understand the resilience of fish gut microbiome to FFC-medicated feed in aquaculture ponds. Furthermore, should be specific studies on the interaction between *Mycoplasma*, *Cetobacterium* and virulent strain of *A. hydrophila*.

Based on our data, the microbiome shifts, as well as pathogen infection, occurred in the absence of intestinal inflammation, as the pro-inflammation genes studied CCL20, IL1β, IL-8, and TNFα were not significantly overexpressed in antibiotic-treated fish. Studies on enterocolitis and *A. hydrophila* pathogenesis using zebrafish model revealed a marked induction of pro-inflammatory cytokines interleukin-1β (Il1β), tumor necrosis factor–α (TNFα), IL-8, and Interleukin-10 (Il10) as mucosal and intestinal epithelium responded to bacteria perturbation [28,

32, 35, 44, 67, 75]. Studies in pharmacological dysbiosis and inflammation revealed that antibiotic treatment ameliorated the inflammation, since zebrafish co-treated with kanamycin and ampicillin did not initiate the transcription of pro-inflammatory cytokines IL-1 β, TNFα, CCL20, and CXCL8-l1 (CXCL8a) pathways in gut epithelial homeostasis and inflammation [28], but it is something that has not been studied with FFC. Has been demonstrated that high virulent factor aerolysin-related cytotoxic enterotoxin (Act) and the gene encoding for the surface layer protein in *A. hydrophila* stimulated the production of TNFα and up-regulated the expression of genes encoding IL-1 β and IL-6 [76, 77]. The possible lack of this virulent factors in the strain used in this study could be the cause of non-induction in inflammation respond, as natural transformation is a general property of Aeromonas isolates [78], but is something that requires being more studied.

In conclusion, our results proved that FFC-medicated feed, commonly used in aquaculture to treat bacterial diseases produced a dysbiosis state on healthy adult zebrafish that was characterized by decrease on diversity, an increase of the abundance of gamma-proteobacteria (a class containing many opportunistic fish bacterial pathogens) while diminishing members of proven beneficial bacteria. Is clear that some members of the gut microbiome on healthy fish are more susceptible to disturbed environment in the GI tract. The inference of *Aeromonas* on certain key members of gut microbiome is something that requires more attention. Dysbiotic fish that survived the challenged, contained higher numbers of them compared with the control group and could remain more susceptible to subsequent opportunistic pathogens in the event of a new disturbance. Nevertheless, our results cannot unequivocally confirm that the

FFC-altered gut microbiome was causative rather than consequential in increasing susceptibility to *A. hydrophila* infection. Further studies involving fecal transplants of dybiotic gut microbiomes into healthy (untreated) fish are required to further test this hypothesis. However, we did prove that FFC- microbial disruption is a long-term event, and the gut microbiome did not recover after FFC pressure was removed. Additional research should investigate if the addition of probiotics to the diet during the withdrawal period would prevent or diminish host susceptibility to opportunistic pathogens.

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Table 3-1. Total length (cm) and Weight (g) through the experimental period. Mean ± SD of 12 individuals are presented.

Group	Feed provided	Sampling time	Weight (g) ± SD	Length (cm) ± SD
A	1	t21	0.30 ± 0.03	2.84 ± 0.22
B	2	t21	0.32 ± 0.02	2.79 ± 0.19
D	1	t35	0.24 ± 0.06	2.85 ± 0.23
C	2,1	t35	0.26 ± 0.07	2.88 ± 0.21
F	1	t52	0.26 ± 0.04	2.88 ± 0.24
H	2,1	t52	0.24 ± 0.14	3.05 ± 0.29
E	1	t52	0.30 ± 0.09	3.10 ± 0.33
G	2,1	t52	0.29 ± 0.03	3.02 ± 0.13

1 Non-medicated feed

2 FFC-medicated feed during the 10d treatment

Table 3-2. Diversity indexes as calculated by Mothur (v.1.39.5). Operational taxonomic units (OTUs) were defined at 97% sequence similarity. Significance among total values for each developmental stage was determined by one-way ANOVA followed by Tukey's post hoc test. Within a column, different superscripts indicate significant differences ($P < 0.05$).

Group	Coverage	Sobs*	ACE**	Chao1	Shannon-evenness
A	0.995	886^{ab}	1711^{a}	1442^{a}	0.529^{ab}
B	0.994	1143^{a}	2102^{a}	1678^{a}	0.621^{a}
D	0.995	859^{ab}	1773^{a}	1383^{a}	0.504^{ab}
C	0.994	990^{ab}	1734^{a}	1409^{a}	0.513^{ab}
F	0.995	695^{b}	1460^{a}	1176a	0.429^{b}
H	0.995	805^{ab}	1641^{a}	1332^{a}	0.418^{b}
E	0.995	807^{b}	1537^{a}	1206^{a}	0.453^{b}
G	0.995	796^{ab}	1648^{a}	1232^{a}	0.455^{ab}

* Sobs, the total number of secies observed in the community

** ACE, abundance-based coverage estimation

Table 3-3. Number of unique and shared OTUs and relative abundance of shared OTUs between and within groups.

Between Group	Number of shared OTUs	% Abundance of shared OTUs
A & B	671	49.4
C & D	614	49.7
F & H	471	45.7
E & G	506	46.1
Within control group		
A & D	590	41.3
D & F	488	34.2
D & E	549	38.4
E & F	469	32.9
Within medicated-group		
B & C	719	43.7
C & H	583	35.4
C & G	556	33.7
G & H	512	31.1

Table 3-4. Genus identity of sequences represented by percentage from the total sequences. Only genera accounting for more than 0.2% of sequences in at least one group are displayed.

Genera	A	B	D	C	F	H	E*	G*
Aeromonas	11.8	14.2	22.0	36.3	13.4	41.7	18.2	35.8
Mycoplasma	19.5	0.9	15.1	1.9	49.4	5.1	54.1	19.4
Pseudomonas	8.7	18.6	13.3	12.3	7.0	19.7	6.8	19.5
Ruminiclostridium	13.5	2.6	2.0	15.2	4.4	19.0	3.2	2.4
Cetobacterium	10.7	0.3	23.2	0.2	14.8	0.2	6.7	0.2
Geobacillus	2.7	10.2	7.1	7.0	2.7	0.6	0.7	0.5
Phyllobacterium	11.1	0.7	0.3	0.7	0.5	0.4	0.1	0.4
Shinella	0.7	3.2	0.5	0.7	0.5	1.4	0.7	5.5
Bacillus	1.5	1.4	1.1	2.2	1.0	1.0	0.5	0.3
Shewanella	0.6	0.5	0.7	2.1	0.4	1.6	0.8	2.0
Stenotrophomonas	2.8	4.4	0.2	0.5	0.5	0.3	0.4	0.2
Burkholderia	2.2	5.4	0.2	0.5	0.2	0.3	0.2	0.3
Others	14.1	37.5	14.3	20.5	5.3	8.7	7.6	13.7

* Challenged group at t52

Table 3-5. Analysis of similarities (ANOSIM) values obteined from OTUs were asrobed to the variables tested in the study.

ANOSIM				PERMANOVA		
Variable	Global R	P-value	# Significant pairwise comparisson	Test statistic	P-value	# Significant pairwise comparisson
Treatment	0.159	0.009	1 out 6	2.8778	0.0003	4 out 6
Time	0.191	0.006	2 out 6	2.0872	0.0147	1 out 3
Group	0.21	0.0002	14 out 28	2.1698	0.0003	16 out 28

Table 3-6. Main (A) and pairwise test results (B) based multivariate statistical analysis of Bray-Curtis similarity of the intestinal microbiome of zebrafish (Danio rerio) by treatment across sampling time. All statistical tests were conducted using a fixed factor of fish treatment, across all 3 sampling times (t21, t35, t52). Test statistics were calculated using up to 9,999 permutations; data structure dictated the number of possible permutation.

(a) Main Test Across Treatment

Statistical test	Test Statistic	P-value	Possible permutation
PERMDISP	1.9982	0.28	9999
PERMANOVA	2.1698	* 0.0003	9875
ANOSIM	0.21	* 0.0002	9999

(b) Pairwise Test Between Treatment over time

Statistical test	Groups Compared	Test Statistic	P-value	Possible permutation
PERMANOVA	A & B	1.4907	* 0.0392	126
	C & D	1.6201	* 0.0301	126
	E & G	1.1054	0.2436	126
	F & H	2.0045	* 0.0093	126
ANOSIM	A & B	0.248	*0.05	126
	C & D	0.336	* 0.024	126
	E & G	0.048	0.22	126
	F & H	0.416	* 0.016	126

∗ Superscript indicate significant difference

Table 3-7. SIMPER analysis between groups showing pairwise dissimilarities and main genera contributing to dissimilarity.

Average dissimilarity between groups (%)	Bacteria genus	GROUP I AVERAGE ABUNDANCE	GROUP II AVERAGE ABUNDANCE	% CONTRIBUTION TO DISSIMILARITY
A & B (70.22)	*Mycoplasma*	19.74	0.9	13.41
	Aeromonas	13.11	15.99	9.89
	Ruminiclostridium	13.99	2.95	9.6
	Cetobacterium	13.25	0.36	9.2
	Geobacillus	2.5	12.53	8.79
	Pseudomonas	9.24	17.5	7.78
	Phyllobacterium	8.75	0.81	6.34
A & C (61.04)	*Aeromonas*	13.11	34.55	18.22
	Mycoplasma	19.74	2	14.75
	Ruminiclostridium	13.99	14.59	13.93
	Cetobacterium	13.25	0.24	10.66
	Phyllobacterium	8.75	0.7	7.24
	Pseudomonas	9.24	14.12	5.53
B & C (56.50)	*Aeromonas*	15.99	34.55	21.8
	Geobacillus	12.53	6.42	11.66
	Ruminiclostridium	2.95	14.59	8.08
A & D (55.28)	*Cetobacterium*	13.25	19.3	16.74
	Mycoplasma	19.74	16.31	13.49
	Aeromonas	13.11	23.13	13.12
	Ruminiclostridium	13.99	2.14	12.13
	Geobacillus	2.5	9.71	8.24
	Pseudomonas	9.24	13.62	8.02
	Phyllobacterium	8.75	0.29	7.85
B & D (64.59)	*Cetobacterium*	0.36	19.3	14.7
	Aeromonas	15.99	23.13	13.98
	Mycoplasma	0.9	16.31	11.93
	Geobacillus	12.53	9.71	11.74
	Pseudomonas	17.5	13.62	9.68
C & D (56.49)	*Aeromonas*	34.55	23.13	17.66
	Cetobacterium	0.24	19.3	16.88
	Mycoplasma	2	16.31	12.75
	Ruminiclostridium	14.59	2.14	11.47
	Pseudomonas	14.12	13.62	9.27
	Geobacillus	6.42	9.71	8.49

Table 3-7 continued

Average dissimilarity between groups (%)	Bacteria genus	GROUP I AVERAGE ABUNDANCE	GROUP II AVERAGE ABUNDANCE	% CONTRIBUTION TO DISSIMILARITY
A & E (58.49)	*Mycoplasma*	19.74	34.03	23.72
	Aeromonas	13.11	25.28	13.78
	Cetobacterium	13.25	9.03	13.68
	Ruminiclostridium	13.99	2.08	11.65
	Phyllobacterium	8.75	0.16	7.4
B & E (70.05)	*Mycoplasma*	0.9	34.03	23.64
	Aeromonas	15.99	25.28	13.69
	Geobacillus	12.53	1.54	8.89
	Pseudomonas	17.5	10.32	7.49
	Cetobacterium	0.36	9.03	6.26
C & E (59.58)	*Mycoplasma*	2	34.03	27.19
	Aeromonas	34.55	25.28	15.91
	Ruminiclostridium	14.59	2.08	11.04
	Cetobacterium	0.24	9.03	7.4
	Pseudomonas	14.12	10.32	6.36
D & E (55.09)	*Mycoplasma*	16.31	34.03	26.53
	Cetobacterium	19.3	9.03	17.89
	Aeromonas	23.13	25.28	15.32
	Pseudomonas	13.62	10.32	9.25
	Geobacillus	9.71	1.54	8.62
A & F (56.26)	*Mycoplasma*	19.74	41.78	27.54
	Cetobacterium	13.25	16.4	14.37
	Ruminiclostridium	13.99	4.62	12.47
	Aeromonas	13.11	16.26	11.02
	Phyllobacterium	8.75	0.4	7.8
	Pseudomonas	9.24	8.73	6.51
B & F (75.14)	*Mycoplasma*	0.9	41.78	27.2
	Aeromonas	15.99	16.26	10.8
	Cetobacterium	0.36	16.4	10.79
	Pseudomonas	17.5	8.73	8.49
	Geobacillus	12.53	3.69	8.38
C & F (66.78)	*Mycoplasma*	2	41.78	29.85
	Aeromonas	34.55	16.26	17.01
	Cetobacterium	0.24	16.4	12.16
	Ruminiclostridium	14.59	4.62	9.57
	Pseudomonas	14.12	8.73	7.48

Table 3-7 continued

Average dissimilarity between groups (%)	Bacteria genus	GROUP I AVERAGE ABUNDANCE	GROUP II AVERAGE ABUNDANCE	% CONTRIBUTION TO DISSIMILARITY
D & F (54.80)	*Mycoplasma*	16.31	41.78	30.35
	Cetobacterium	19.3	16.4	18.02
	Aeromonas	23.13	16.26	14.88
	Pseudomonas	13.62	8.73	10.28
	Geobacillus	9.71	3.69	0.58
E & F (53.40)	*Mycoplasma*	34.03	41.78	32.89
	Cetobacterium	9.03	16.4	16.97
	Aeromonas	25.28	16.26	16.44
	Pseudomonas	10.32	8.73	8.19
A & G (58.65)	*Aeromonas*	13.11	36.28	20.31
	Mycoplasma	19.74	19.74	14.33
	Ruminiclostridium	13.99	2.23	11.62
	Cetobacterium	13.25	0.21	11.11
	Pseudomonas	9.24	19.76	8.97
	Phyllobacterium	8.75	0.37	7.42
B & G (59.56)	*Aeromonas*	15.99	36.28	21.14
	Mycoplasma	0.9	19.74	15.93
	Geobacillus	12.53	0.52	10.15
	Pseudomonas	17.5	19.76	5.63
C & G (48.64)	*Mycoplasma*	2	19.74	18.84
	Aeromonas	34.55	36.28	18.4
	Ruminiclostridium	14.59	2.23	13.56
	Pseudomonas	14.12	19.76	8.21
	Geobacillus	6.42	0.52	6.07
D & G (51.48)	*Cetobacterium*	19.3	0.21	18.54
	Aeromonas	23.13	36.28	17.87
	Mycoplasma	16.31	19.74	17.22
	Pseudomonas	13.62	19.76	11.81
	Geobacillus	9.71	0.52	8.98
E & G (49.08)	*Mycoplasma*	34.03	19.74	29.73
	Aeromonas	25.28	36.28	18.98
	Pseudomonas	10.32	19.76	9.98
	Cetobacterium	9.03	0.21	9
F & G (57.20)	*Mycoplasma*	41.78	19.74	29.28
	Aeromonas	16.26	36.28	19.85
	Cetobacterium	16.4	0.21	14.2
	Pseudomonas	8.73	19.76	11.74

Table 3-7 continued

Average dissimilarity between groups (%)	Bacteria genus	GROUP I AVERAGE ABUNDANCE	GROUP II AVERAGE ABUNDANCE	% CONTRIBUTION TO DISSIMILARITY
A & H (61.55)	*Aeromonas*	13.11	45.81	26.57
	Ruminiclostridium	13.99	12.73	14.05
	Mycoplasma	19.74	6.42	12.22
	Cetobacterium	13.25	0.23	10.59
	Pseudomonas	9.24	20.36	10.31
	Phyllobacterium	8.75	0.34	7.06
B & H (60.55)	*Aeromonas*	15.99	45.81	26.13
	Ruminiclostridium	2.95	12.73	10.41
	Geobacillus	12.53	0.61	9.96
	Pseudomonas	17.5	20.36	9.46
C & H (41.98)	*Aeromonas*	34.55	45.81	23.23
	Ruminiclostridium	14.59	12.73	19.96
	Pseudomonas	14.12	20.36	13.79
	Geobacillus	6.42	0.61	6.93
	Mycoplasma	2	6.42	6.42
D & H (54.30)	*Aeromonas*	23.13	45.81	21.92
	Cetobacterium	19.3	0.23	17.58
	Pseudomonas	13.62	20.36	13.07
	Mycoplasma	16.31	6.42	12.05
	Ruminiclostridium	2.14	12.73	11.46
	Geobacillus	9.71	0.61	8.46
E & H (54.43)	*Mycoplasma*	34.03	6.42	27.24
	Aeromonas	25.28	45.81	21.25
	Pseudomonas	10.32	20.36	11.61
	Ruminiclostridium	2.08	12.73	11.56
	Cetobacterium	9.03	0.23	8.13
F & H (63.98)	*Mycoplasma*	41.78	6.42	28.59
	Aeromonas	16.26	45.81	23.37
	Cetobacterium	16.4	0.23	12.7
	Pseudomonas	8.73	20.36	12.08
	Ruminiclostridium	4.62	12.73	10.16
G & H (37.26)	*Mycoplasma*	19.74	6.42	22.02
	Aeromonas	36.28	45.81	19.3
	Ruminiclostridium	2.23	12.73	16.77
	Pseudomonas	19.76	20.36	12.8

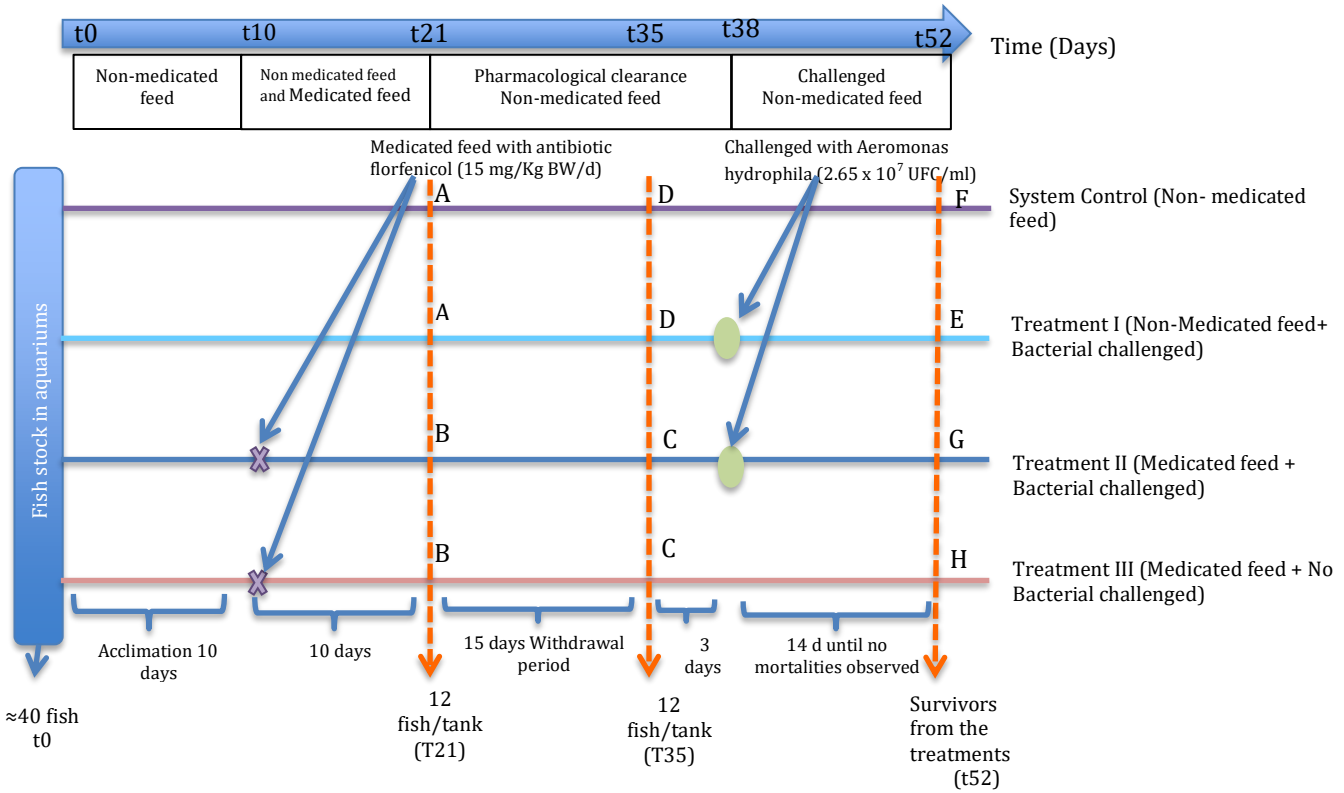

Figure 3-1. Experimental design with the different treatments and time points used in the study 4 treatments: System control = Non-medicated feed & Not challenged; Treatment (I)= Non-Medicated feed & Bacterial Challenged; Treatment (II) = Medicated feed and challenged; Treatment (III)=Medicated and Not bacterial challenged. Letters A-H represent the samples taken from each treatment at specific sampling point.

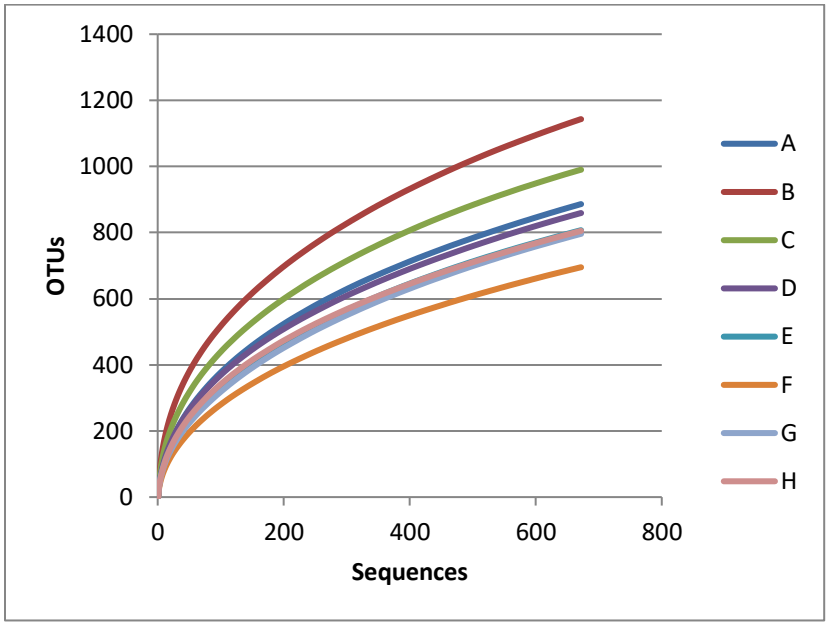

Figure 3-2. Rarefaction curves of all groups in the study. The letter and color represent each group by sampling time.

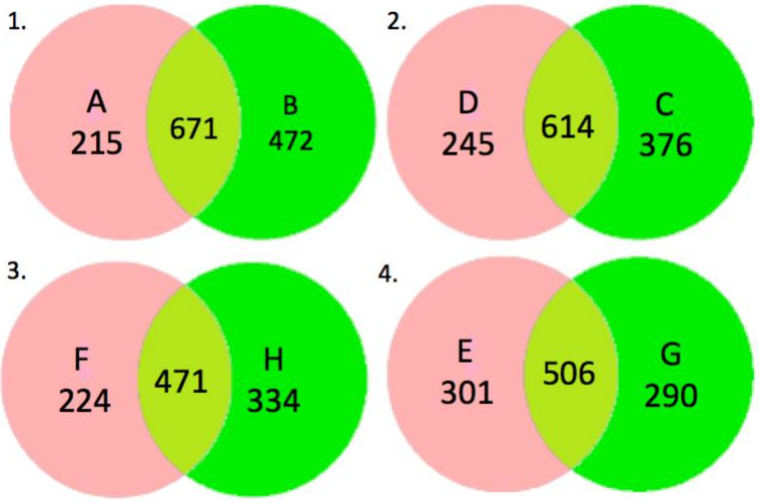

Figure 3-3. Venn diagram representing the core microbiome shared between groups during the study. Control groups are pink colored and medicated groups are green colored. 1) Group A & B at t21; 2) Group C & D at t35: 3) Non-challenged Group F & H at t52; 4) Challenged Groups E & G at t52.

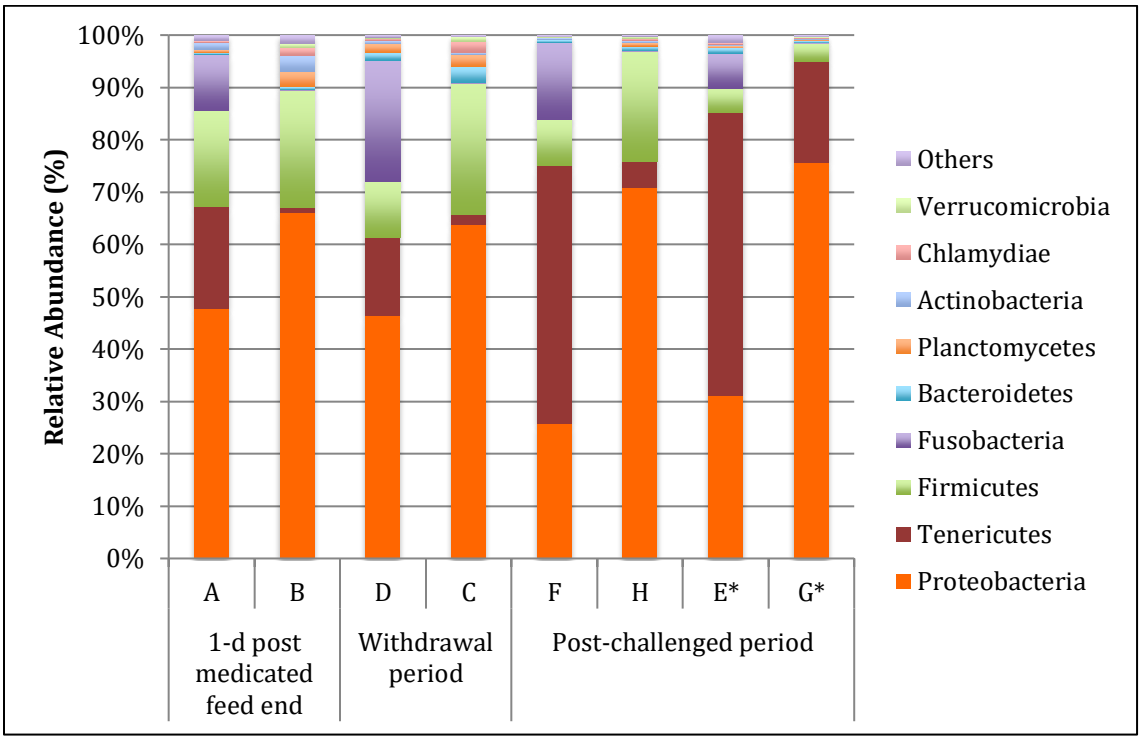

Figure 3-4. Bacterial diversity at the phylum based on sequencing of 16S rRNA gene showing the differences in the gut microbiome structure between groups and the percent of detected sequences belonging to the different bacterial phyla in each group. * Groups that were challenged with *Aeromonas hydrophila*.

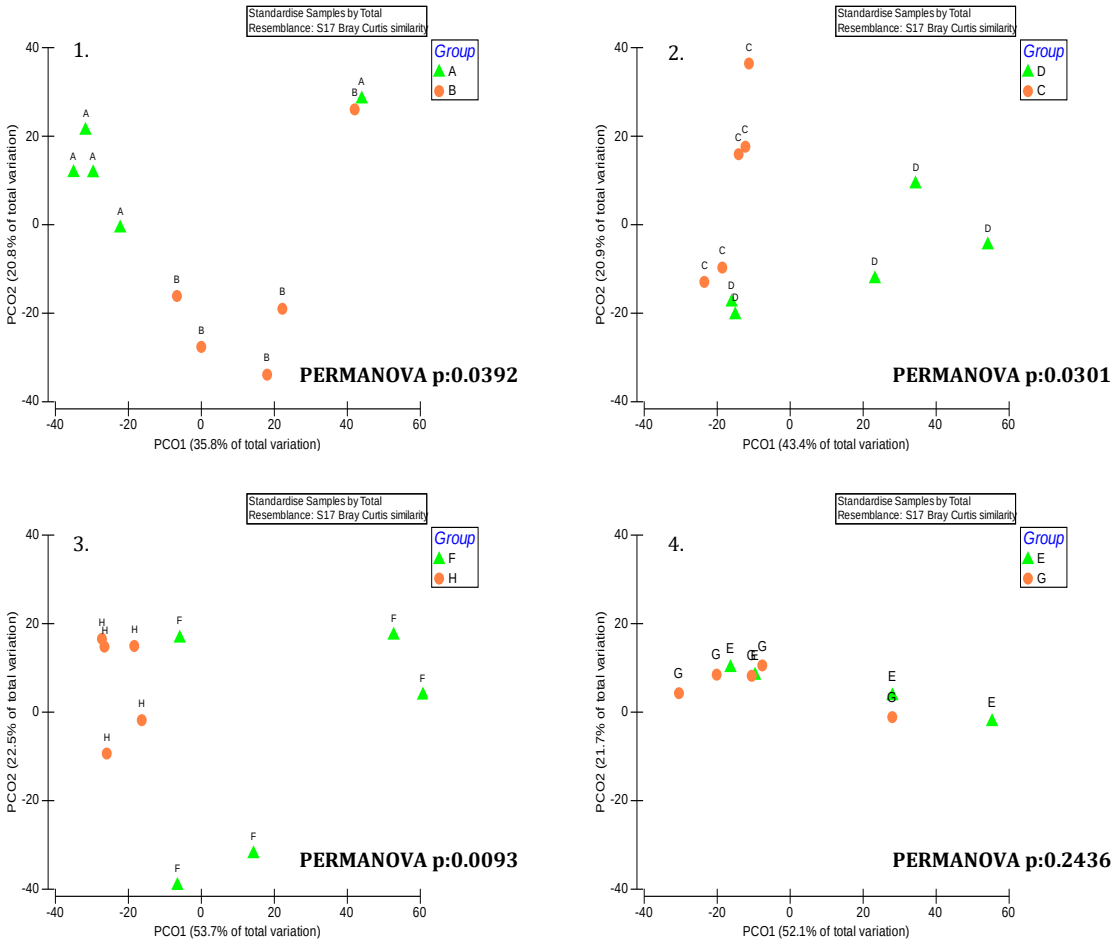

Figure 3-5. Principal coordinates analysis (PCoA) plots of Bray-Curtis distances obtained from the intestinal microbiome of zebrafish. The PCo plots compare control (green triangle) and medicated groups (orange dots) at specific sampling point. 1) Group A & B at t21; 2) Group C & D at t35: 3) Group F & H at t52; 4) Challenges Groups E & G at t52. t21, t35, t52. Points represent individual samples.

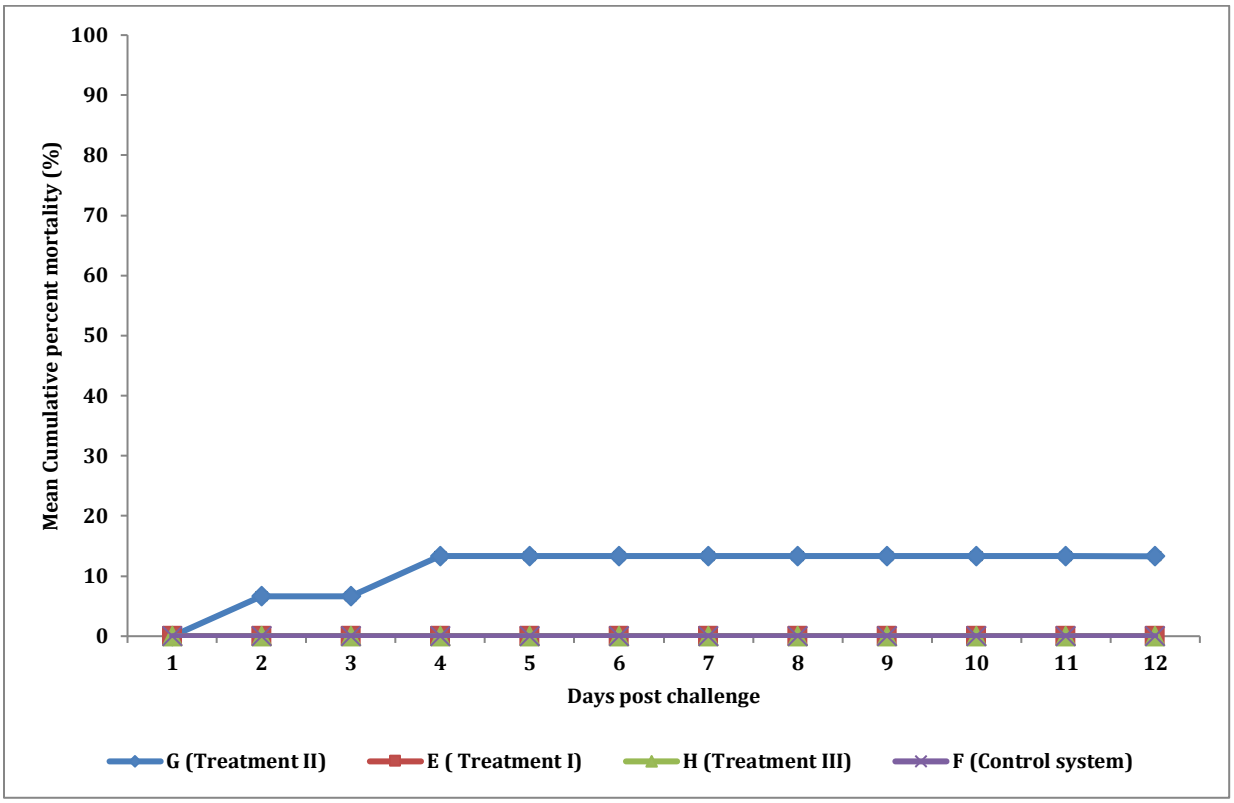

Figure 3-6. Mean cumulative percent mortality of zebrafish challenged with *Aeromonas hydrophila*. Treatments: F-Control system = Not medicated & Non-challenged; H (Treatment III) = Medicated & Non-challenged; E (Treatment I) = Control & challenged, and G (Treatment II) = Medicated & challenged (Note: control system, treatment I, III had 0% mortality, so the mortality curves are superimposed).

CHAPTER 4. EFFECT OF MEDICATED FEED IN THE GUT MICROBIOME ON CHANNEL CATFISH (*ICTALURUS PUNCTATUS*)

Abstract

Channel and hybrid catfish (*Ictalurus punctatus*) are one of the top ten freshwater-farmed raised fish in the United States. Their aquaculture intensification has led to the emergence of several bacterial diseases. The use of medicated feed is a common practice to control bacterial outbreaks in commercial farms. The gut microbiome is composed of a collection of microbes living in mutualistic relationship with their host. Antibiotics are known to disrupt the balance of the bacterial composition leading to a dysbiotic state, which has been associated with the onset of disease. The objective of this study was to determine if the use of Aquaflor® induced dysbiosis in the gut microbiome of channel catfish under industry practices. The experiment was carried out under field conditions and compared: 1) control fish fed with regular feed, and 2) fish treated with Aquaflor® (florfenicol-medicated feed). The microbial composition of the two groups was characterized using next generation sequencing of the region v4 of the gene 16S rRNA gene. Medicated feed did not induce significant changes in the gut microbial community regarding microbial diversity or species composition. There was a significant difference in species evenness between control and antibiotic-treated fish with medicated fish having a more even community. Uncultured α-proteobacteria Group 1, *Clostridium*, *Cetobacterium*, and *Aeromonas*

were the most abundant genus observed with varying abundances between treatments. Uncultured α-proteobacteria Group 1 sequences had a sequence identify of only ~82% with previously deposited sequences. It is very likely represent a species of a new lineage within the class Alphaproteobacteria.

Introduction

Catfish is the top ten U.S finfish aquaculture species. The increasing demand of catfish products is directly related to characteristics and attributes of the product including price, taste, and flavor [1]. One of the most significant negative economic impacts that catfish producers face is bacterial infections caused by, primarily, opportunistic bacteria [2].

Medicated feed is mainly used to control bacterial disease outbreaks in cultured fish. The U.S. Food and Drug Administration (FDA) has approved only four antibiotics for use in food fish. From them, only three antibiotics are commercially available, including, Terramycin (Terramycin 200® for fish), Romet (Romet 30® and Romet TC) and Florfenicol (Aquaflor®, FFC)[2]. Aquaflor® is a feed premix containing antibiotic florfenicol at a concentration of 50% (w/w). Florfenicol is a broad-spectrum antibiotic with bacteriostatic and bactericidal properties and is active against a variety of Gram-positive and Gram-negative bacteria. FFC has been approved by the FDA for use on several fish species to control mortality associated with a variety of bacterial diseases, including furunculosis [3], enteric septicemia [4], columnaris [5], mortality associated with *Streptococcus iniae* [6] and with *Francisella noatunensis* subsp [7]. Additional studies have investigated the effect of FFC-medicated feed on growth promoter in channel catfish (*Ictalurus punctatus*) [8], tilapia (*Oreochromis sp*) [9], sunshine bass [10], as

well as its physiological effects on zebrafish [11]. To date, there are no studies that characterize the impact of FFC on the intestinal microbiome composition of channel catfish.

Several studies have shown that the use of antibiotic leads to a series of profound alterations in the intestinal microbiota of the fish [12, 13]. Host-microbiome interactions are based on a dynamic equilibrium that if disrupted could lead to a pathological state (called dysbiosis) and result in the deregulation of the host immune homeostasis with a concomitant increase to disease susceptibility [14, 15]. It has been previously reported, that bacterial community decreases in evenness when healthy microbiomes is disrupted. Dysbiotic microbiomes can lead to a pro-inflammatory stage in the host, mostly due to the reduction or lack of critical biochemical compounds and lack of detoxification mechanism with the subsequent cell deterioration and erosion on the immune system [14, 16, 17].

In this study, we investigated the effect of medicated feed containing the broad-spectrum antibiotic florfenicol on the composition of the intestinal microbiota of healthy channel catfish.

Material and Methods

Fish care and maintenance.

A stock of the juvenile mixed sex channel catfish (Jubilee strain) with an average weight ~ 180 ± 7 g were housed in two 264 L raceways (with approximately 400 fish each) supplied with flow-through pond water at the E.W. Shell Fisheries Center, Auburn University, Alabama. Animals were reared under natural ambient temperature and natural photoperiod. The animal protocol was approved by the Auburn University Institutional Animal Care and Use Committee (IACUC number 2016-2946).

Experimental design.

The experimental design consisted of 2 treatments: Aquaflor®-medicated feed (hereafter, medicated feed) and control (regular feed), both diets at 32% protein commercial catfish feed (SouthFresh, Alabama, USA). For ten days, animals were fed to apparent satiation either with medicated feed or with control feed. Treatment period was followed by a withdrawal period of 28-days in which both groups were fed control feed. Temperature and oxygen dissolved (DO) were monitored twice a day, parameters were maintained from 14 to 26.8 °C, and DO was maintained above 4.0 ppm at all times.

Sampling and DNA extraction.

After the withdrawal period of 28 d, ten animals from each treatment (n = 10) were collected and transported to the laboratory. Animals were euthanized using MS-222, and individual gut samples (n = 10) were taken aseptically as described by Larsen et al. [18] from each treatment. Total lengths (mm) and weight (g) of sampled fish are shown in Table 4-1. DNA was extracted using DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions (Total DNA from Animal Tissues, Spin column Protocol) with modifications including double digestion with proteinase K and pre-treatment with lysozyme for lysis of Gram-positive bacteria and RNase A treatment. DNA was eluted with 100 µl elution buffer and quantified using Nanodrop 1000 spectrophotometer (Thermo Scientific, Nanodrop Technologies, Wilmington, DE, USA).

PCR Amplification and Sequencing.

A total of 20 samples were submitted to MR DNA® (Shallowater, TX, USA) for PCR amplification and Next Generation Sequencing using Illumina MiSeq platform targeting the 16S rRNA gene V4 variable region. Universal bacterial primers 515 F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') with a

barcode on the forward primer were used to generate a 300 bp amplicon. The HotStarTaq Plus Master Mix Kit (Qiagen, USA) was used under the following PCR conditions: an initial denaturation for 3 minutes at 94 ℃ followed by 28 cycles of denaturing at 94 ℃ for 30 s, annealing at 53 ℃ for 40 s, extension at 72 ℃ for 1 min and a final elongation step for 5 min at 72 ℃. The PCR products were run through a 2% agarose gel to verify successful amplification and relative band intensity of the target DNA. Multiple samples were pooled together and purified using calibrated Ampure XP beads to prepare the Illumina DNA library. The samples were sequenced as paired-end reads on the Illumina MiSeq platform following the manufacturer's instructions. The sequences resulting were processed using a proprietary pipeline (MR DNA, Shallowater, TX, USA). Sequencing data were joined, and all barcodes, ambiguous base calls, and sequences <150 bp were removed. Denoising of sequences was also performed, and operational taxonomic units (OTUs) were generated. Cut-offs for OTU assignment were defined at a 97% similarity (< 3% sequence variation) in concurrence with the prokaryotic species concept [19]. Taxonomic classifications were obtained using BLASTn against the Green Genes database [20].

Data Analysis.

Rarefaction curve, observed OTUs (sobs), shared OTUs, ACE, CHAO1, Good Coverage and Shannon-evenness index (SEI) were calculated using Mothur v.1.39.5 [21] after standardization of each sample type to the sample yielding the least number of total sequences (n = 9,734). A Student's t-test was used to determine differences in diversity index between treatments using JMP Version 12 .0.1 (SAS Institute Inc. Cary, NC, 1989-2007). To show differences among samples, a cluster tree, Multidimensional scaling (MDS) and analysis of similarities (ANOSIM) were run from the similarity matrix using Primer v6 (Primer-E Ltd,

Plymouth, UK). A genera abundance table was loaded into Primer v6, and similarity percentages (SIMPER) analysis on Bray-Curtis similarity matrix assembled was performed to determine the genera responsible for differences between treatments. The cut-off for low contributions was set to the default at 90%.

Results

At the end of the study, fish weight (g) was 281.9 ± 23.9 and 272.5 ± 26.3 for control and medicated feed treatments, respectively, while length (mm) 178.9 ± 53.4 and 174.7 ± 68, for control and medicated feed treatments, respectively. No significant differences were found in weight and length between control and treatment. During the study, no disease breakouts were detected.

In total, 1,512,687 bacterial sequences representing 631 operational taxonomic units (OTUs) were obtained from all samples in the study. After standardization, 194,689 bacterial sequences remained in the analysis comprising 463 OTUs. Sequences coverage was ≥ 99% for all samples analyzed (based on Good's coverage, Table 4-2) supported by the rarefaction curves generated by Mothur (Figure 4-1).

Phyla composition (> 0.1%) was similar between both groups with the only difference being that the medicated feed group lacked the phylum Acidobacteria. Overall, Proteobacteria (58%) was the most prominent phylum, followed by Firmicutes (24%), Fusobacteria (15%), Bacteriodetes (1.5%); less abundant phyla were grouped as 'other' (< 0.1%) for graphic representation (Figure 4-2). Within the Proteobacteria phylum, the control and medicated feed groups contained mainly α-Proteobacteria (44%), followed by γ-Proteobacteria (11%). Within

the phylum Firmicutes, the most abundant class were Clostridia (9%), Bacilli (7%), and Erysipelotrichia (6%), with abundances varying between treatments.

A total of 269 genera were identified with control samples harboring 220 and medicated samples containing 215 genera. Both treatments shared 11 genera that were present at >0.01% abundance (Table 4-3). Control samples contained 5 unique genera at > 0.0001% and 47 of unique genera at < 0.01%. Medicated feed sample only contain 45 of unique genera at less than 0.01% (data not shown). Across all samples, the most abundant genera were classified as Uncultured α-proteobacteria Group 1, followed by *Clostridium*, *Cetobacterium*, *Aeromonas*, *Geobacillus*, *Turicibacter*, *Phyllobacterium*, *Vibrio*, *Pseudomonas*, *Bacteroides*, *Edwardsiella* (Figure 4-3).

The OTUs ascribed as genus Uncultured *α-proteobacteria* Group 1 were present in high abundance in both control and medicated feed samples. All OTUs belonging to this group shared more than 90% sequence similarity. All OTUs within the Uncultured *α-proteobacteria* Group 1 shared, on average, 81% sequence identity with known bacteria sequences of the genera *Litorimonas*, *Thalassococcus,* and *Algimonas,* all members of the α-proteobacteria class (family Hyphomonadaceae and Rhodobacterraceae). Due to the low percent similarity found between our Uncultured *α-proteobacteria* Group 1 and those previously deposited in GenBank and GreenGenes (similarities ranged from 78 to 84%), sequences could not be ascribed to known genera (or even family) and remained unclassified at this point.

Despite the antibiotic effect, the medicated treatment exhibited higher bacterial richness; however, there was an apparent increase/decrease within the shared bacterial sequences at phylum and genus level in medicated treatment compare with Control. Figure 4-2 & Table 4-3 show that Fusobacteria, Bacteroidetes, Verrucomicrobia increased in abundance; while

Proteobacteria and Firmicutes decreased in medicated treatment. As well in the medicated treatment, Figure 4-3 & Table 4-3 shows that Uncultured α-proteobacteria Group 1, *Clostridium*, *Phyllobacterium*, *Pseudomonas* were the genera that showed a decrease, while *Cetobacterium*, *Aeromonas*, *Turicibacter*, *Vibrio*, *Bacteroides*, *Edwardsiella*, and *Bacillus* increased.

The gut microbial richness measured by observed Sobs, ACE, and CHAO did not differ significantly between treatments. However, Shannon-evenness index was significantly higher in the medicated group than control, suggesting medicated feed reduced an even distribution of bacterial taxons within the community in favor of some that became more dominant (Table 4-2). A total of 205 OTUs (44.3 %) were detected as core microbiota, with 125 unique OTUs for the Control group and 133 for the medicated group (Fig.4-4). MDS plot showed samples within treatments clustered together but when treatment distribution was compared, samples clustered with no clear separation between them (Figure 4-5). Results from ANOSIM analysis revealed there is no significant difference at OTU level between treatments ($R = 0.114$; $P = 0.056$)

SIMPER analysis showed that control samples were more similar within their group (40.32%) compared with the medicated (28.01%) group (data not shown). The higher number of Uncultured α-proteobacteria Group 1, *Clostridium*, and *Cetobacterium* contributed (> 10%) to the difference between treatments (Table 4-4).

Discussion

Diet is one of the main factors that modulate the fish gut microbiome [22, 23]. This study showed significant differences in bacterial evenness between the gut microbial composition of the control and medicated feed groups, which is in agreement with previous studies on antibiotic dysbiosis in the fish gut [13]. It has been reported that healthy individuals contain a greater

diversity and richness than those found in sick individual [24-26]. When analyzing microbial diversity, one has to consider both the richness composition and the evenness. In this study, the species evenness was significantly higher in the medicated feed group compared with control group suggesting some dominance in the microbial consortium. However, this statistic is rarely reported in these types of studies; thus, we cannot speculate if this is a common occurrence in artificially altered gut microbial communities or not. The increase of the evenness was accompanied by a decrease in diversity, as 220 genera were detected in control and only 115 in medicated.

A study realized by Antonopoulos et al. [27] in mice model treated with antibiotics, revealed that community tends to returns to the same configuration after a small perturbation, but may shift to a different shape or equilibrium after a significant disturbance. Our results suggested that the microbial composition in the gut of fish fed medicated feed did not return to that in the control group even after a long withdrawal period.

Significant differences between samples at genus level were mainly due to varying abundances of Uncultured α-proteobacteria Group 1. The bacterial sequences from our OTUs blasted with three bacterial sequences identified as *Litorimonas sp., Thalassococcus lentus* and *Algimonas porphyrae* deposited in the GenBank and GreenGenes database. All three bacterial strains have been identified as marine bacteria associated with algae[28-32]. We hypothesize that a new genus (or genera) within α-proteobacteria class exist in freshwater algae present in catfish ponds. To determine this, research would require culturing representatives in the environment as well in catfish gut from this group in the lab, which can be extremely difficult; but due to the high prevalence of this group in aquaculture raised channel catfish may warrants further investigation.

Interestingly, the increase of the genus *Cetobacterium* in medicated catfish is something that has not been reported previously. *Cetobacterium* is a bacteria micro-aerotolerant, gram-negative rod with a fermentative metabolism that was initially reported in children with late-set autism [33]. However, it has been reported from a variety of freshwater fish species including channel catfish [18, 34]. The biological and functional role of this genus has been related to the production of vitamin B12[34, 35]. In addition, *Cetobacterium somerae* can inhibit the growth of other bacterial species which might have some benefits to the host [34]. Their increase in the medicated feed group makes it a potential candidate to be used as a probiotic after antibiotic treatments.

Previous in vitro research with pathogenic bacteria in fish indicates that majority of fish pathogens are sensitive to florfenicol, including *Edwardisella ictaluri*, *E. tarda*, *Photobacterium damsel* (subsp. Piscicida), *Vibrio anguillarum, Aeromonas hydrophila, A. salmonicida* [4, 36, 37]. In this study, we found an increase in *Aeromona*s and *Vibrios* after antibiotic administration, both of which include pathogenic and opportunistic bacteria. The increased abundance of these members suggests that there may have been a particular antibiotic resistance present within the microbial community. Several studies have reported the presence of the florfenicol resistance genes encoded in a transferable plasmid present in fish isolated [38-40]. The presence of pathogens within microbial communities is common in healthy fish. Whether such associations reflect an asymptomatic carrier stage, a preliminary colonization step before pathogenesis, or simply a commensalism- synergism relationship needs further investigation [41].

The results of our study showed that medicated feed had a little effect on channel catfish gut microbiome indicating the resilient nature of those communities. Although some effect was observed, our results did not suggest that dysbiosis was induced in FFC treated fish under field

conditions. This is a good outcome for farmers since FFC is now their antibiotic of choice to treat bacterial infections in pond.

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Table 4-1. Total length (mm) and weight (g) of individual catfish sampled for each treatment.

Treatment	Individual Number	Total Length (mm)	Weight (g)
Control	1	265	161.09
	2	280	160.68
	3	257	132.53
	4	313	246.3
	5	303	219.25
	6	265	190.78
	7	323	274.66
	8	280	166.4
	9	280	117.28
	10	255	120.32
Medicated	1	327	288.77
	2	270	156.21
	3	289	181.9
	4	276	161.71
	5	290	201.53
	6	253	125.37
	7	240	112.2
	8	246	105.93
	9	252	111.95
	10	282	170.14

Table 4-2. Diversity indexes as calculated by Mothur (v.1.39.5). Operational taxonomic units (OTUs) were defined at 97% sequence similarity. Significance among total values for each treatment was determined by T-student test. Within a column, different superscripts indicate significant differences ($P < 0.05$).

Treatment	Sobs*	Coverage	ACE†	Chao 1	Shannon-evenness
Medicated	338[a]	0.99	424[a]	455[a]	0.51[a]
Control	330[a]	0.99	411[a]	405[a]	0.37[b]

*, Sobs, total number of OTUs observed in the community
†, ACE, abundance-based coverage estimation

Table 4-3. Genera identified in Channel Catfish (*Ictalurus punctatus*) gut microbiota by percentage of total sequences. Genera accounting for ≥0.01% of sequences at least in one treatment are included. Shared genera are presented in both Control and Medicated treatment. Unique genera are present in only one treatment.

	Classification				Relative abundace (%)	
	Phylum	**Class**	**Family**	**Genus**	**Control**	**Medicated**
Shared Genera	Proteobacteria	α-Proteobacteria	Rhodobacteriales	Uncultured α-proteobacteria Group 1	51.73	30.55
	Proteobacteria	α-Proteobacteria	Phyllobacteriaceae	*Phyllobacterium*	4.23	0.09
	Proteobacteria	γ-Proteobacteria	Aeromonadaceae	*Aeromonas*	3.9	11.99
	Proteobacteria	γ-Proteobacteria	Vibrionaceae	*Vibrio*	0.02	2.8
	Proteobacteria	γ-Proteobacteria	Pseudomonadaceae	*Pseudomonas*	1.44	0.96
	Proteobacteria	γ-Proteobacteria	Hafniaceae	*Edwardsiella*	0.01	2.15
	Firmicutes	Clostridia	Clostridiaceae	*Clostridium*	23.02	9.2
	Firmicutes	Bacilli	Bacillaceae	*Geobacillus*	3.47	3.1
	Firmicutes	Erysipelotrichia	Erysipelotrichaceae	*Turicibacter*	0.64	3.75
	Fusobacteria	Fusobacteria	Fusobacteriaceae	*Cetobacterium*	5.68	24.62
	Bacteroidetes	Bacteroidia	Bacteroidaceae	*Bacteroides*	0.02	2.27
Unique Genera	Chloroflexi	Anaerolineae	Anaerolineaceae	*Longilinea*	> 0.0001	0
	Proteobacteria	γ-Proteobacteria	Ectothiorhodospiraceae	*Thioalkalivibrio*	> 0.0001	0
	Proteobacteria	β-Proteobacteria	Burkholderiales	*Tepidimonas*	> 0.0001	0
	Bacteroidetes	Chitinophagia	Chitinophagaceae	*Flavisolibacter*	> 0.0001	0
	Firmicutes	Bacilli	Carnobacteriaceae	*Carnobacterium*	> 0.0001	0

Table 4-4. One-way Simper analysis comparing gut microbial from different treatment. Only genera accounting for at least 1% of dissimilarity between treatments are noted. Percent abundance at each treatment and percent contribution to dissimilarity from each genus are listed.

Group	Bacteria genus	Group 1 Average abundance	Group 2 Average abundance	% Contribution to Dissimilarity
1. Control	Uncultured α-proteobacteria Group 1	57.33	31.08	33.2
2. Medicated	*Clostridium*	15.51	10.55	14.09
	Cetobacterium	4.73	18.72	13.54
	Aeromonas	2.98	8.55	7.3
	Geobacillus	4.54	3.39	4.36
	Phyllobacterium	5.22	0.46	4.04
	Pseudomonas	1.64	4.37	3.41
	Turicibacter	0.71	4.41	3.02
	Vibrio	0.03	2.13	1.55
	Bacillus	1.26	1.05	1.35
	Bacteroides	0.03	1.59	1.15
	Delftia	0.5	1.3	1.07
Ave.diss. = 68.54				

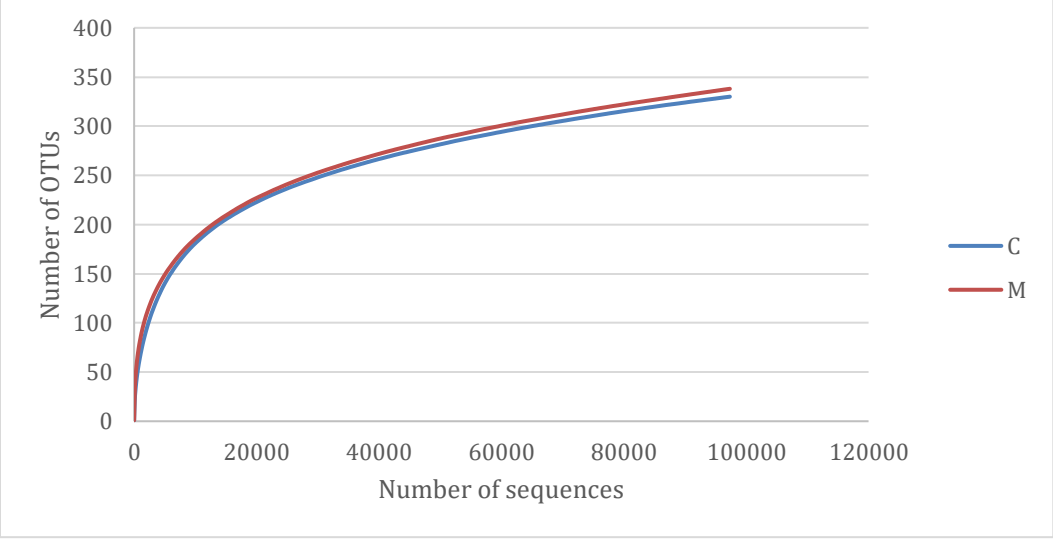

Figure 4-1. Rarefaction curve representing the Control and Medicated treatment. Sequences were standardized to equal sample sizes for direct comparison. Red, Medicated; blue, Control.

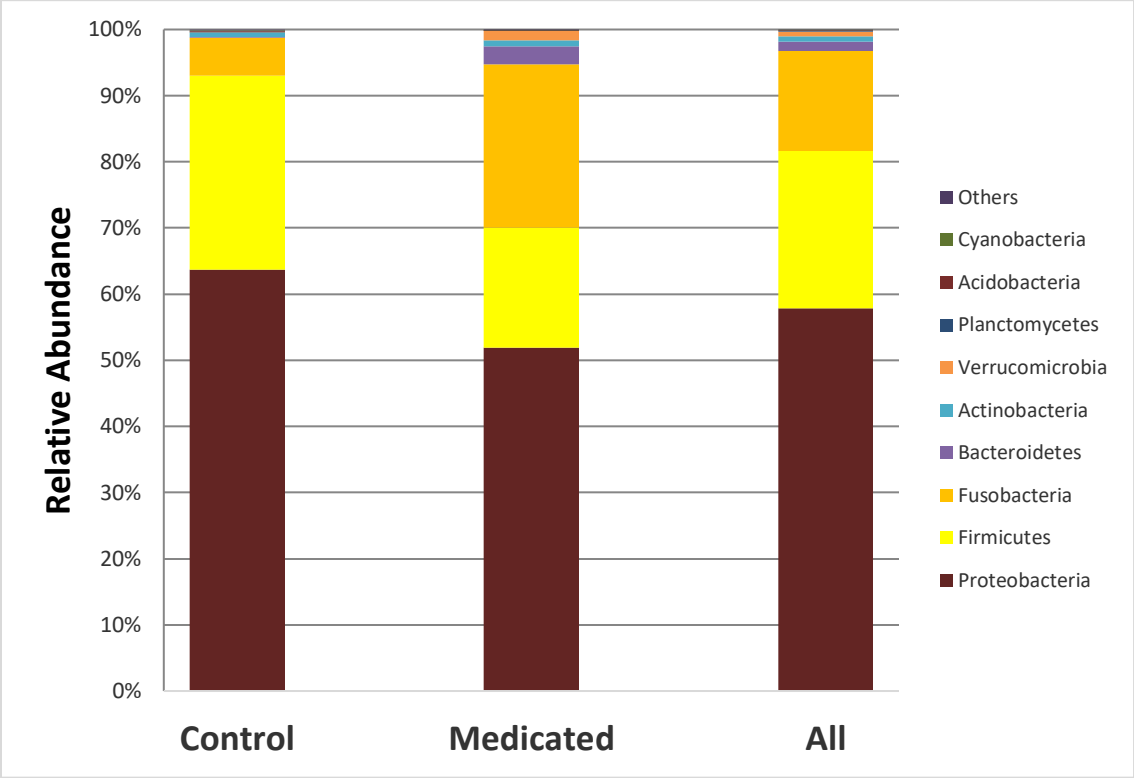

Figure 4-2. Relative abundance of Gut microbiome composition at phylum level. Composition of Control, Medicated treatments, and All samples combined is represented.

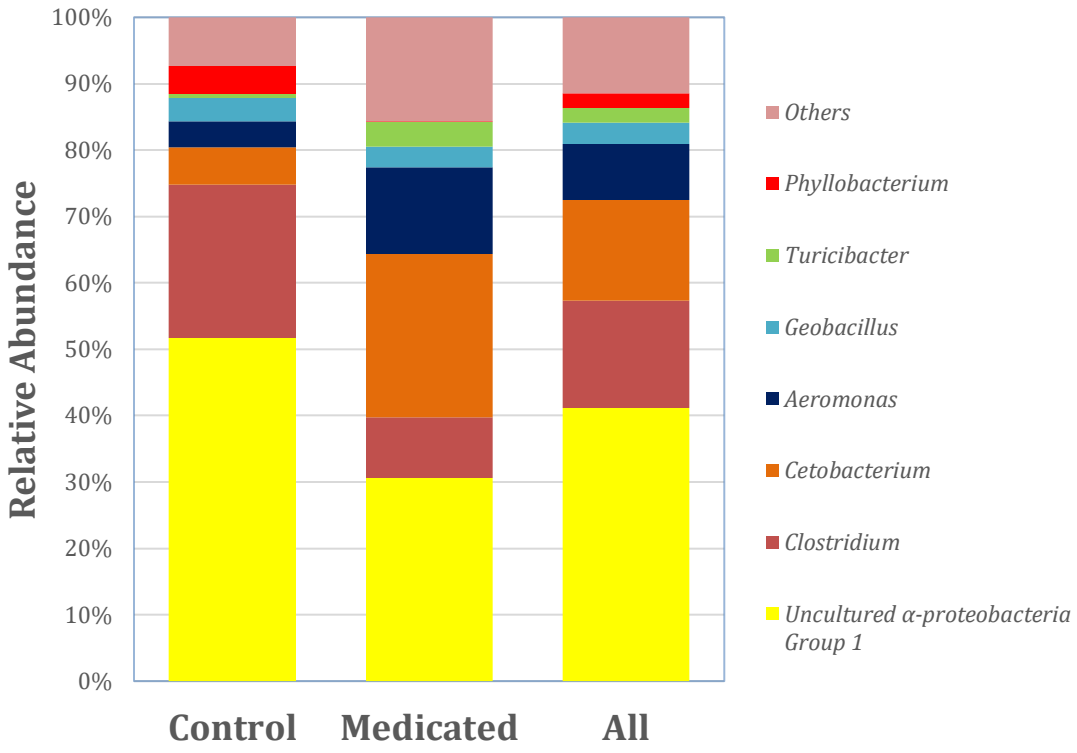

Figure 4-3. Relative abundance of Gut microbiome composition at genus level. Control, Medicated, and All samples are presented.

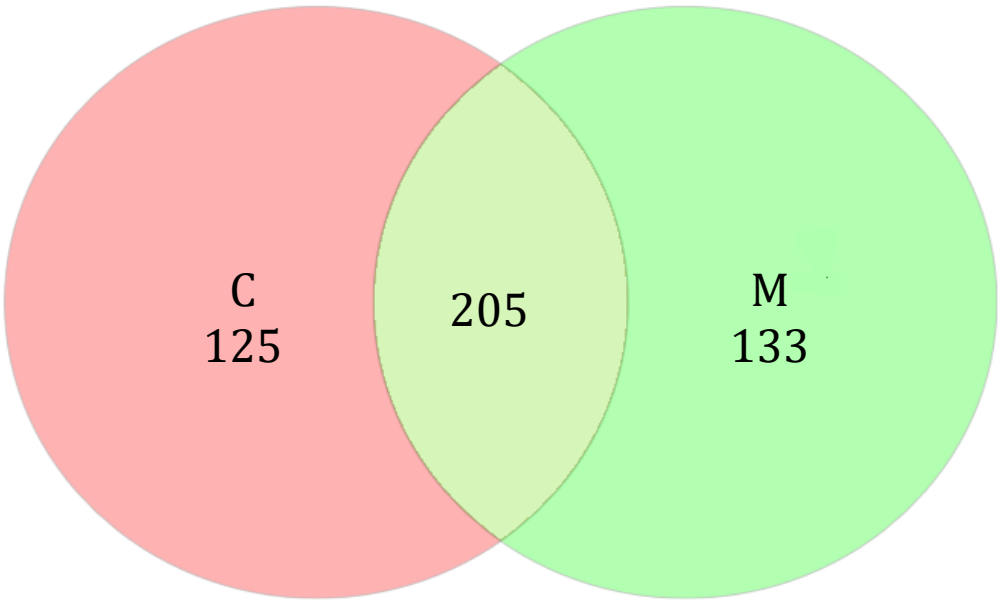

Figure 4-4. Venn diagram shows core microbiota OTUs distribution between Control (C) and Medicated (M) treatments.

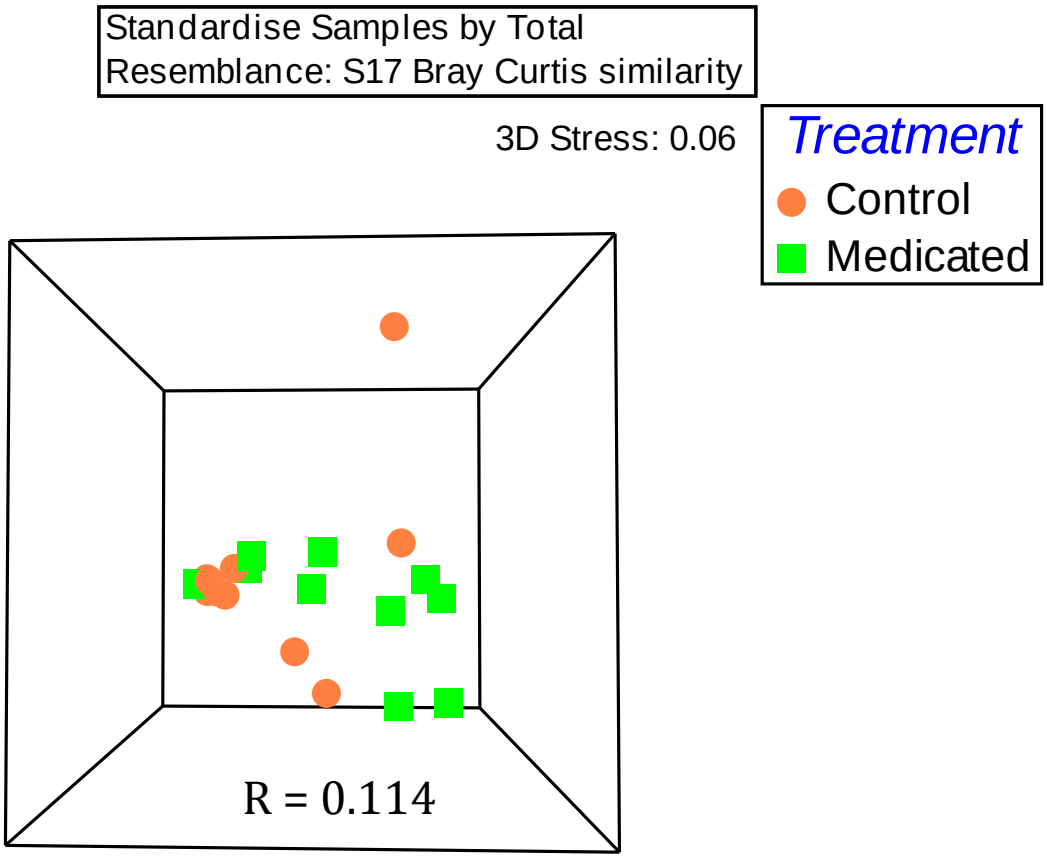

Figure 4-5. Multidimensional scaling (MDS) based on Bray- Curtis metrics between Control and Medicated fish gut samples. Each symbol represent one sample. Green color are samples from Control treatment and Orange color represent samples from Medicated treatment.

CHAPTER 5. EFFECT OF MECHANICAL INJURIES TO CHANNEL CATFISH (*ICTALURUS PUNCTATUS*) GUT MICROBIOME

Abstract

The brain-gut axis (GBA) is a bidirectional pathway between the central and the enteric nervous system that has been fairly well described in mammals. In teleost, little information exists about GBA and the role that microbial gut communities might have on the fish central nervous system and vice-versa. To address this topic, we investigated if mechanical injuries inflected to fish had an effect on their gut microbiomes (as has been proposed in murine models). We compared the gut microbial communities of channel catfish that had been subjected to fin clipping (adipose fin was removed) with those from control fish. High-throughput Illumina MiSeq DNA sequencing of the V4 domain of the 16S rRNA gene was used to analyze changes in the gut microbiome from each of the groups. The 'fin clipped' group showed a significantly different gut microbiome when compared with the control group. Most of the differences between treatments were related to the abundances of the three genera: Uncultured α-proteobacteria Group 1, *Cetobacterium* and *Geobacillus*. These results demonstrated that mechanical injury elicited a shift of the gut microbiome composition. As in the previous chapter, Uncultured α-proteobacteria Group 1 sequences shared on average 82% of bacterial identity with members belong to the class α-proteobacteria suggesting belonging to a new species within the class. Mechanical injuries influenced the gut microbiome with an increased of Proteobacteria and decreased of Fusobacteria.

Introduction

Studies in humans and mouse models have generated insight into the gut-brain crosstalk, revealing a complex communication system that not only ensures the proper maintance of gastrointestinal homeostasis [1], but is likely to have multiple effects on affect, motivation, and higher cognitive functions[1, 2]. The complexity of these interactions is enclosed in the denomination of "gut-brain axis" (GBA). Its role is to monitor and integrate gut functions as well as to link emotional and cognitive centers of the brain with peripheral intestinal functions and mechanisms such as immune activation, intestinal permeability, enteric reflex, and entero-endocrine signaling. The mechanism underlying GBA communications involve neuro-immuno-endocrine mediators. Both clinical and experimental evidence suggest that enteric microbiome has an important impact on GBA, interacting not only locally with intestinal cells and ENS (Enteric nervous system), but also directly with CNS (Central nervous system) through neuroendocrine and metabolic pathways.

In humans, many studies have now demonstrated strong correlations between specific microbiome types and anxiety and depressive-like behaviors and, more recently, of dysbiosis in autism. In fact, autism patients present specific microbiome alterations according to the severity of the disease[3, 4]. Studies on GBA in teleost are scarce but zebrafish larvae reared as germ-free exhibited strong anxiety-related behavior when compared with larvae that were allowed to develop a normal gut microbiome[5].

Commercial aquaculture practices expose the animals to artificial environments that can be stressfull due to high densities, poor water quality, etc.,[6-8]. Quantifying physiological anxiety in fish is difficult and requires measuring thigmotaxis[5]. Instead of measuring physiological

anxiety, and as a first step to better understand GBA in fish, we utilized a physiological injury model. Fish under commercial aquaculture practices are constantly exposed to mechanical injuries through seining, aerators, etc., as well as to bites inflicted by other fish in overcroud situations. Krezalet et al., [9] and Kinross et al., [10] observed changes in the human intestinal microbiome following surgical procedures,while Earley et al.,[11] found similar response in the mouse gut microbiome after physical injuries. For this study, we removed the adipose fin on channel catfish to determine if the wound healing process would have an effect on the gut microbiome. Clipping the adipose fin is considered a non-invasive surgical method that causes no apparent harm in a healthy animal. However, it does open a port of entry for opportunistic bacteria to colonize the host that can result in septicemia [12]. We utilized a previously established challenge model to induce motile aeromona septicemia in channel catfish [12-15]

Material and Methods

Animal care and maintenance.

Approximately, 150 healthy channel catfish of the Jubilee strain with a weight of 138.9 ± 20.9 grams (g) with a length of 21.7 ± 1.5 centimeters (cm) were obtained from stocks maintained at the E.W. Shell Fisheries Center, Auburn University, Alabama and transported to the Aquatic Genetic and Genomic Laboratory, Auburn University. Animals were stocked in 50 L rectangular glass aquaria (8 fish per tank, n = 8). Upon arrival to the lab, 6 fish were randomly caught, examined following standard procedures for disease confirmation and confirmed negative for any *A. hydrophila* infection. Glass aquaria were supplied with heated dechlorinated municipal water at a rate of 0.2 L/min and aerated [16, 17]. Water quality parameters were measured daily, and the averages throughout the experiment were as follows: DO 6.70 ± 0.30

mg/L, temperature 28.7 ± 0.5 ℃, ammonia concentration 0.31 ± 0.08 mg/L, and pH 7.4 ± 0.3. Fish were fed to apparent satiation with a commercial 32% protein catfish ration (SouthFresh, Alabama, USA) while maintaining a constant photoperiod (LD 12:12) during the study. The animal protocol was approved by the Auburn University Institutional Animal Care and Use Committee (IACUC number 2015-2774).

Experimental design and Bacterial Challenge.

The study was conducted with two groups: 1) intact fish, the control group, and 2) adipose fin-clipped group (FC). Each group consisted of 6 replicates tanks, with eight fish per tank. Tanks were randomly assigned to each group. Fish from the fin-clip tanks were sedated using 100 mg/L of buffered tricaine methanesulfonate (MS-222). Once fish displayed signs of sedation (indicated by loss of equilibrium and slowing of opercula movement), the adipose fin was clipped as described previously described [18] and returned to their respective tank. For the challenge, the water flow was turned off in the tanks. Immediately after all fins were clipped, 100 mL of the bacterial culture of virulent *Aeromonas hydrophila* was added to each challenge tank with a final concentration of ~ 2.0×10^7 CFU/ml. Fish were immersed in the aerated challenge suspension for 1 h, and then, the water flow, at 0.2 L/min was restored. Fish mortality was monitored and recorded twice daily for one week post-challenge. Fish were not fed on the challenge day, but were offered pelleted catfish feed day one post-challenge and throughout the rest of the study. The confirmation of v*Ah* as the cause of death was made as previously described by Hossain et al., [19]. The experiment was terminated after there were ten consecutive days without mortalities.

Sampling and DNA extraction.

Animals, which survived the challenge, from each group (n = 10) were sampled and transported to Aquatic Microbiology Laboratory (CASIC Building, Auburn University). Fish were euthanized using buffered MS-222, and individual gut samples (n = 10) were taken aseptically as described by Larsen et al. [20] from each treatment. Total lengths (mm) and weight (g) of sampled fish are given in Table 5-1. DNA was extracted using DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions (Total DNA from Animal Tissues, Spin column Protocol) with modifications including double digestion with proteinase K and pre-treatment with lysozyme for lysis of Gram-positive bacteria and RNase A treatment. DNA was eluted with 100 µl elution buffer and quantified using Nanodrop 1000 spectrophotometer (Thermo Scientific, Nanodrop Technologies, Wilmington, DE, USA).

PCR Amplification and Sequencing.

A total of 20 samples were submitted to MR DNA® (Shallowater, TX, USA) for PCR amplification and Next Generation Sequencing using Illumina MiSeq platform targeting the 16S rRNA gene V4 variable region. Universal bacterial primers 515 F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') with a barcode on the forward primer were used to generate a 300 bp amplicon. The HotStarTaq Plus Master Mix Kit (Qiagen, USA) was used under the following PCR conditions: an initial denaturation for 3 minutes at 94℃ followed by 28 cycles of denaturing at 94℃ for 30 s, annealing at 53℃ for 40 s, extension at 72℃ for 1 min and a final elongation step at 72℃ for 5 min. The PCR products were run through a 2% agarose gel to verify successful amplification and relative band intensity of the target DNA. Multiple samples were pooled together and purified using calibrated Ampure XP beads to prepare the Illumina DNA library. The samples were sequenced as paired-end reads on the Illumina MiSeq platform following the manufacturer's

instructions. The sequences resulting were processed using a proprietary pipeline (MR DNA, Shallowater, TX, USA). Sequencing data were joined, and all barcodes, ambiguous base calls, and sequences < 150 bp were removed. Denoising of sequences was also performed, and operational taxonomic units (OTUs) were generated. Cut-offs for OTU assignment were defined at a 97% similarity (< 3% sequence variation) in concurrence with the prokaryotic species concept[21]. Taxonomic classifications were obtained using BLASTn against the Green Genes database [22].

Data Analysis.

Rarefaction curves, observed OTUs (sobs), shared OTUs, ACE, CHAO1, Good's Coverage, and Shannon-evenness index (SEI) were calculated using Mothur v.1.39.5 [23] after standardization of each sample type to the sample yielding the least number of total sequences (n = 9,734). A Student's t-test was used to determine differences in diversity index between treatments using JMP Version 12 .0.1 (SAS Institute Inc. Cary, NC, 1989-2007). A cluster tree and MDS plot were generated, ANOSIM was run from the similarity matrix using Primer v6 (Primer-E Ltd, Plymouth, UK) to quantify groupings seen in MDS plots statistically. In term of relative abundance, One-way ANOVA was used to the comparison of two groups. A genera abundance table was loaded into Primer v6, and similarity percentages (SIMPER) analysis on Bray-Curtis similarity matrix assembled was performed to determine the genera responsible for differences between treatments. The cut-off for low contributions was set to the default at 90%.

Results

Bacterial sequences and composition.

The study yielded a total of 1,269,779 bacterial sequences representing 499 OTUs, and after standardization to the lowest number (n = 27,323), a total of 546,460 bacterial sequences with 449 OTUs remained in the analysis. Sequence coverage for the groups were ≥ 99% (Table 5-1), supported by the rarefaction curves generated by Mothur (Figure 5-1).

When bacterial sequences for each group were ascribed at the phylum level, a total of 20 phyla were observed. Overall, Proteobacteria (56.5%) was the most abundant phylum between all samples, followed by Firmicutes (21.2%), Fusobacteria (19.4%) and Bacteroidetes (1.5 %) (Figure 5-2). Control group harbored 18 phyla but lacked representatives from the phylum Nitrospirae, and Aquificae, while the fin-clipped group possessed 19 phyla but lacked Chlamydiae. The class α-Proteobacteria dominated within the phylum Proteobacteria followed by γ-Proteobacteria, and β-Proteobacteria. Proteobacteria significantly increased from a relative abundance of 39.5% to 85.7% while Fusobacteria significantly decreased from 30.6% to 0.3% in the control and fin-clipped group, respectively. Firmicutes also decreased in fin-clipped fish when compared to control (from 27.7% to 10.2%) while Actinobacteria and Bacteroidetes remained similar between groups (Figure 5- 2)

Taking into account all samples, a total of 239 genera were observed with the genus Uncultured α-proteobacteria Group 1 (40.12%) dominating, followed by *Cetobacterium* (19.4%), *Turicibacter* (10.6%), *Geobacillus* (8.8%), *Pseudomonas* and *Phylobacterium* (> 4 %), and *Vibrio* (2.5%) (Figure 5-3). Interestingly, significantly higher abundance of *Cetobacterium* was observed in control group compared with the fin-clipped, while Uncultured α-proteobacteria Group 1 was significantly higher in the treated group compared with control. Additionally, *Phyllobacterium* relative abundance increased from < 0.1% in control to 11.85% in the treatment. Similarly, *Pseudomonas* also increased from 2.13% in control to 9.05% in fin-clipped

fish. Conversely, *Geobacillus* and *Turicibacter* were more abundant in control than in injured fish (Table 5-2).

Diversity.

The rarefaction curve shows a higher diversity of the OTUs in the treatment compared with the control group (Figure 5-1). The observed richness as calculated by sobs and total estimated richness as calculated by ACE and Chao1 did not differ significantly by group. There was no significant difference in evenness either. A total of 186 OTUs shared between both groups, with 116 OTUs unique in the control group and 147 OTUs in the treatment group (Figure 5-4).

The MDS plot (Fig 5-5) showed that individual samples from each group cluster together showing higher similarities within the groups than between groups; however, there were some samples from each group that overlapped. This observation was confirmated by ANOSIM, which showed a Global R-value of 0.193 but, despite the overlap, the groups were significantly different (P = 0.014). SIMPER analysis revealed that samples from the fin-clipped group were more similar (54.42%) than those in the control group (28.86%). The genera Uncultured α-proteobacteria Group 1, *Cetobacterium* and *Geobacillus* contributed the most to the dissimilarity between groups (Table 5- 4). The OTUs ascribed as genus Uncultured *α-proteobacteria* Group 1 were detected in high abundance and all shared at least 92% of similarity. Surprisingly the OTUs of Uncultured *α-proteobacteria* Group 1 shared on average 81% sequence identity with known bacterial sequences beign more closely related to members of the genera *Litorimonas*, *Thalassococcus,* and *Algimonas* within the family Hyphomonadaceae and Rhodobacterraceae. Since ascription to known genera was not possible based on low sequence similarity (78-84%) we therefore, refer to them as Uncultured *α-proteobacteria* Group 1.

Mortalities.

The initial mortalities were seen at 24 h post-challenge and lasted three days post-challenge. The mean mortality in the treatment group was higher compared with the control; however, there was no significant difference between groups (Table 5-5). Moribund fish showed the typical clinical signs of redness and lesions. *A. hydrophila* was isolated and confirmed as previously described from those fish [19].

Discussion

For decades, aquaculturists and fisheries biologists have use adipose fin clipping to mark groups of fish for breeding and conservation studies [24, 25], as well as a non-lethal method to obtain tissue samples [26]. The use of this practice is contradictory these days, while some studies report no evidence of any adverse effect on the fish, others report the contrary [27]. The main negative effects associated with adipose fin clipping were a reduction in the survival rate of the fish due to stress caused in the animal and decrease in swimming ability [28]. Clearly, the primary concern about using this technique is the physiological injury exerted that if the wound does not heal properly, which may lead to wound infections and even secondary septicemias. However, some studies have shown that adipose fin clipping is a fairly harmless procedure [29]. Histopathological studies in Atlantic salmon (*Salmo salar L.*), related with the healing process in adipose fin clippings indicated that it took 18 hours for the wound to close with rapid epidermal normalization and lacked any inflammatory reaction signs [30].

However, when fin clipping is followed by a control exposure to an opportunistic pathogen, this method increases mortality and improves the reproducibility of the challenge [18, 31, 32]. In this study, we observed a higher number of mortalities in fin-clipped fish than in

control, but these dfferences were not significantly different survival rates. Interestingly, the relatively abundance of *Aeromonas* genus was higher in control than in fin-clipped fish (0.18% to 0.09%, respectively). This finding suggests that the different gut microbiome observed in fin clipped fish did not favor the colonization of the gut mucosa by *A. hydrophila*.

SIMPER analysis revealed that the differences between treatments were due to genera belonging to Uncultured α-proteobacteria Group 1, *Cetobacterium* and *Geobacillus*. The origin of the Alphaproteobacteria in the channel catfish gut samples is unknown, but we speculate that they might be associated with the algae found in the ponds from where the fish originated prior to experimentation. The closest species to the Uncultured α-proteobacteria Group 1 were isolated from algae albeit from marine environments. Catfish are omnivorous, and algae are part of their natural diet. Additional studies are needed to further characterize the Uncultured α-proteobacteria Group 1 and determine its role in the channel catfish gut.

Cetobacterium was significantly reduced in the fin-clipped group, suggesting this genus is susceptible to a disturbance in host homeostasis. This genus has been reported in a variety of freshwater fish species including channel catfish [20, 33]. The biological and functional role of this genus has been related to the production of vitamin B12 but it also to inhibition of specific bacteria [33]. The genus *Geobacillus* has not been previously described in the gut of channel catfish and its origin is unknown. However, it has been isolated from sources on all seven continents; from hydrothermal vents, cools soils, and cold ocean sediments [34, 35]. Members of the genus frequently are contaminants of dairy production facilities and milk products [36, 37]. One feature of this genus is to form a dormant spore state, which allows them to be transported and occupy new niches. *Geobacillus thermoleovorans* is being studied as a possible candidate as a probiotic [38] and has recently been isolated as part of the gut microbiome in salmon [39].

Adipose fins are only present in a few fish including trouts, salmons, and catfishes and its function remained unclear until recently. A couple of studies [40, 41] suggested that the adipose fin increases swimming efficiency under turbulent water conditions. Later studies described the adipose fin as a precaudal flow sensor that inproves fish maneuverability in turbulent waters [42]. The presence of extensive nervous tissue, including subdermal complex of interconnected astrocyte-like cells, posit the adipose fin acts as a mechanosensor with an important physiological role [42, 43]. Removal of the adipose fin could not only result in lower swimming efficiency under turbulent conditions but could also trigger a stress response due to a wound in a neurologically sensitive area. Studies in human and mice have shown a bidirectional neuro-humoral communication pathway between the intestinal microbiota and the brain (gut-brain axis). These studies have paid particular attention to how a physical injury shifts the gut microbiome of the host affecting the host' health [2, 9-11]. The reduce abundance of *Aeromonas hydrophila* bacterial sequences in the gut of injuried fish suggest that removing this fin triggers a GBA-mediated response that significantly modified the gut microbiome. Our results revealed that mechanical injuries exert a strong influence in the gut microbiome composition with an increase of Proteobacteria and reducing the Fusobacteria.

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Table 5-1.Total length (mm) and weight (g) of individual sampled for each treatment.

Treatment	Individual Number	Total Length (mm)	Weight (g)
Control	1	260	141.2
	2	230	151.6
	3	220	149.7
	4	203	130.4
	5	210	128.7
	6	113	112.5
	7	118	117.8
	8	123	123.5
	9	149	148.6
	10	169	169.3
Fin-clipped	1	146	146.4
	2	126	126.3
	3	152	152.4
	4	125	124.7
	5	138	137.5
	6	176	176
	7	142	141.6
	8	94	94.16
	9	177	176.55
	10	129	129.3

Table 5-2. Diversity indexes as calculated by Mothur (version 1.39.5). Operational taxonomic units (OTUs) were defined at 97% sequence similarity. Significance among total values for each treatment was determined by T-student test. Within a column, different superscripts indicate significant differences ($P < 0.05$).

Treatment	Coverage	Sobs*	ACE†	Chao1	Shannon-evenness
Control	0.99	302[a]	443[a]	409[a]	0.39[a]
Fin-clipped	0.99	333[a]	428[a]	390[a]	0.35[a]

*, Sobs, total number of OTUs observed in the community
†, ACE, abundance-based coverage estimation

Table 5-3. Major genera making up channel catfish (*Ictalurus punctatus*) gut microbiota in Control and Fin-clipped treatment. Genera accounting for ≥0.01% of sequences at least in one treatment are included. Unique genera are present in only one treatment.

					Relative abundance (%)	
	Phylum	**Class**	**Family**	**Genus**	**Control**	**Fin clipped**
Shared genera	Proteobacteria	α-Proteobacteria	Rhodobacteriales	Uncultured α-proteobacteria Group 1	29.68	57.94
	Proteobacteria	α-Proteobacteria	Phyllobacteriaceae	*Cetobacterium*	30.66	0.19
	Proteobacteria	α-Proteobacteria	Phyllobacteriaceae	*Phyllobacterium*	< 0.1	11.85
	Proteobacteria	β-Proteobacteria	Burkholderiaceae	*Ralstonia*	0.1	1.11
	Proteobacteria	γ-Proteobacteria	Pseudomonadaceae	*Pseudomonas*	2.13	9.05
	Proteobacteria	γ-Proteobacteria	Vibrionaceae	*Vibrio*	3.89	< 0.1
	Firmicutes	Bacilli	Bacillaceae	*Geobacillus*	10.22	6.46
	Firmicutes	Erysipelotrichia	Erysipelotrichaceae	*Turicibacter*	16.62	0.29
	Bacteroidetes	Flavobacteria	Flavobacteriaceae	*Cloacibacterium*	< 0.1	1.33
	Bacteroidetes	Bacteroidia	Bacteroidaceae	*Bacteroides*	1.3	< 0.1
Unique Genera	Actinobacteria	Actinobacteria	Acidomicrobiaceae	*Ilumatobacter*	0	< 0.1
	Proteobacteria	α-Proteobacteria	Acetobacteriaceae	*Acidisphaera*	0	< 0.1
	Tenericutes	Mollicutes	Mycoplasmataceae	*Mycoplasma*	0	< 0.1
	Firmicutes	Bacilli	Planococcaceae	*Planococcus*	0	< 0.1
	Firmicutes	Clostridia	Peptpcoccaceae	*Desulfotomaculum*	0	< 0.1
	Actinobacteria	Actinobacteria	Streptomycetaceae	*Streptomyces*	0	< 0.1
	Firmicutes	Clostridia	Peptococcaceae	*Peptococcus*	0	< 0.1
	Bacteroidetes	Sphingobacteria	Flammeovirgaceae	*Flexithrix*	0	< 0.1
	Actinobacteria	Actinobacteria	Acidimicrobiaceae	*Ferrithrix*	0	< 0.1
	Cyanobacteria	Cyanophyceae	Leptolyngbyaceae	*Trichocoleus*	0	< 0.1

Table 5-4. One-way Simper analysis comparing gut microbial from different treatment. Only genera accounting for at least 3% of dissimilarity between treatments are noted. Percent abundance at each treatment and percent contribution to dissimilarity from each genus are listed.

Group	Bacteria genus	Control Average abundance	Fin-clipped Average abundance	% Contribution to Dissimilarity
1. Control	Uncultured α-proteobacteria Group 1	35.05	59.12	31.53
2. Fin-clipped	*Cetobacterium*	25.61	0.22	18.53
	Geobacillus	10.94	6.96	10.36
	Turicibacter	11.83	0.37	8.66
	Pseudomonas	3.57	11.03	7.07
	Phyllobacterium	0.03	6.07	4.42
	Vibrio	4.16	0.05	3.04
Ave.diss.= 68.66%				

Table 5-5. Mortality and Survival rates (%) from Control and Fin-clipped group.

Treatment	% Mortality	% Survival
Fin-clipped	16.7	83.3
Control	10	90

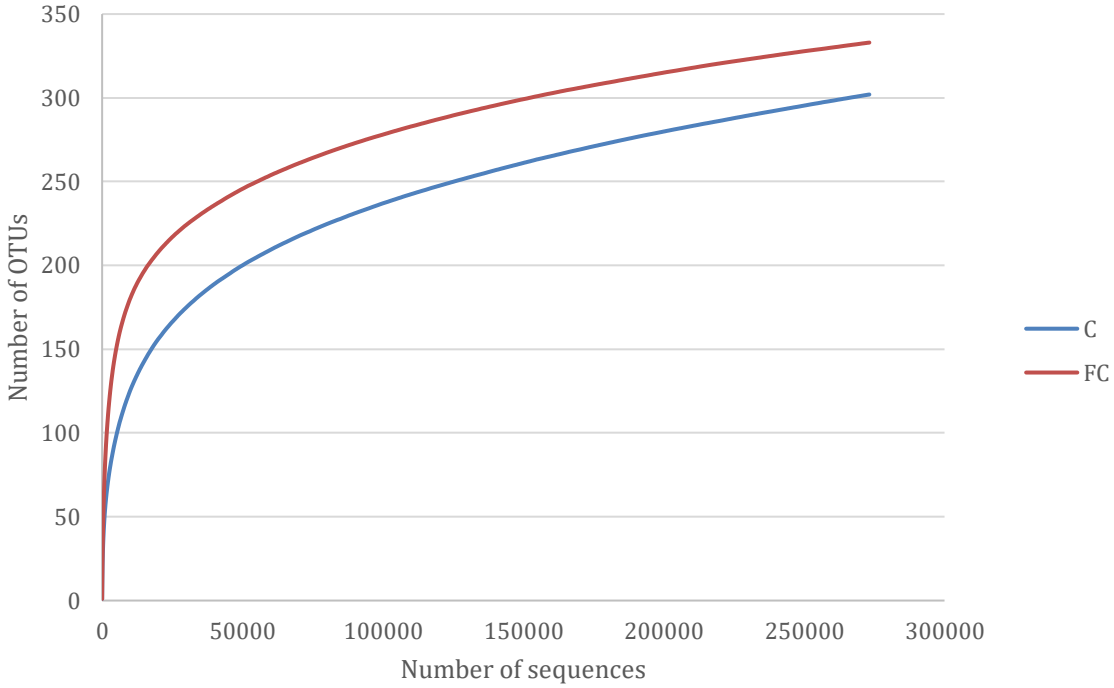

Figure 5-1. Rarefaction curve representing the Control and Fin-clipped. Sequences were standardized to equal sample sizes for direct comparison. Orange, Fin-clipped; blue, Control.

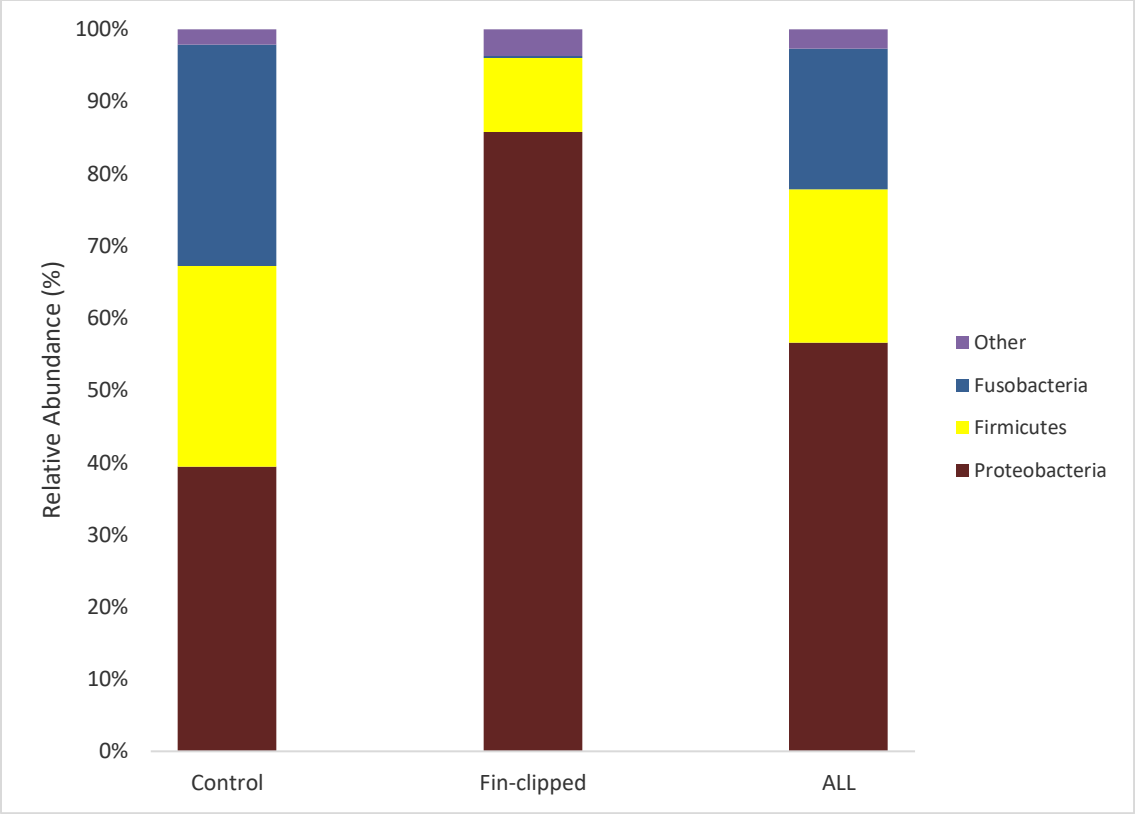

Figure 5-2. Phylum composition of Control, Fin-clipped treatments, and All samples analyzed. Each bar shows the bacteria phyla composition, representing average of all replicates obtained by Illumina MISeq.

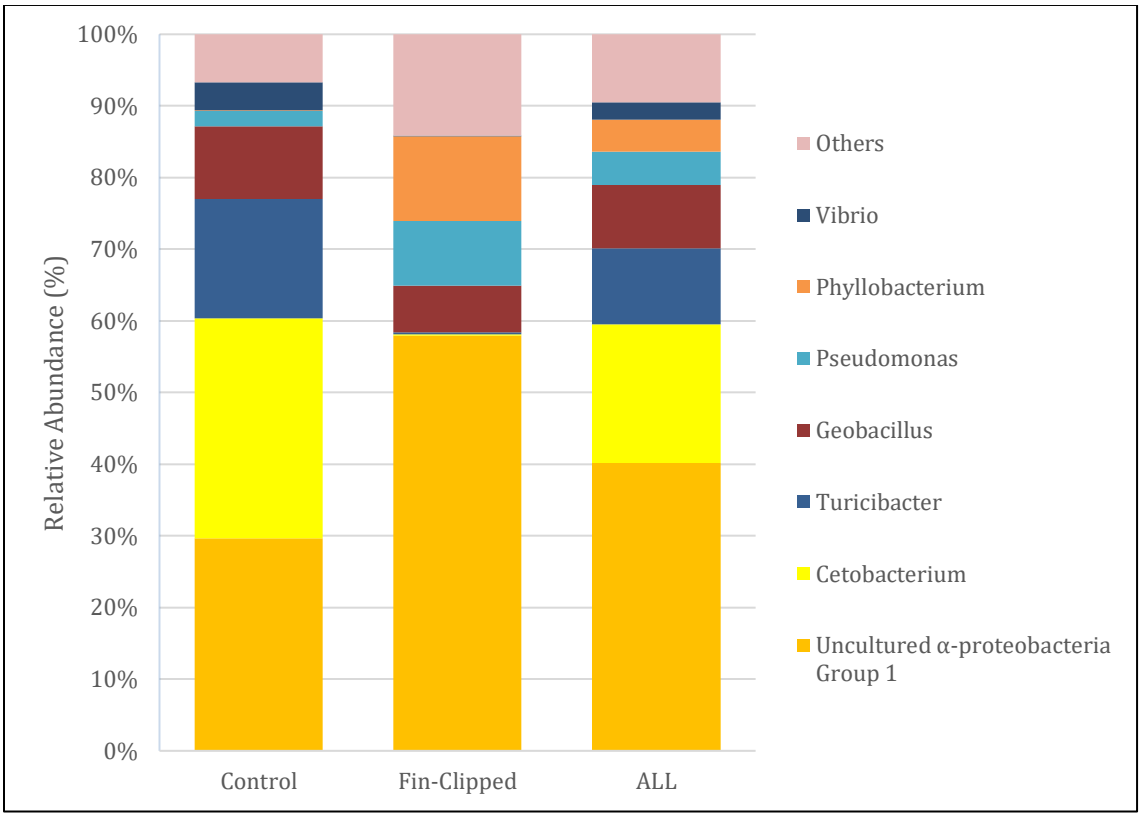

Figure 5-3. Relative abundance of Gut microbiome composition at genus level. Composition of Control, Fin-clipped treatments, and All samples combined is represented.

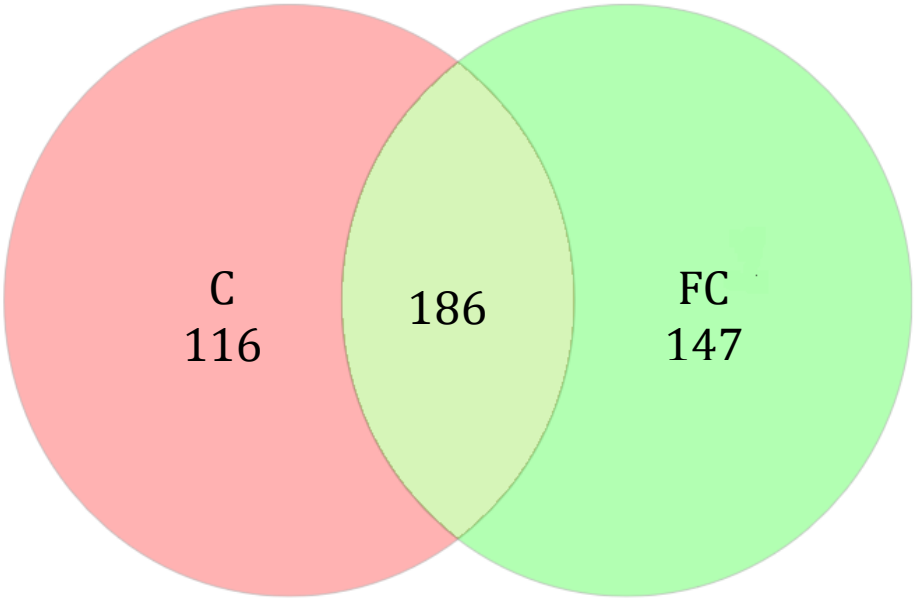

Figure 5-4. Venn diagram shared OTUs between Control and medicated treatments.

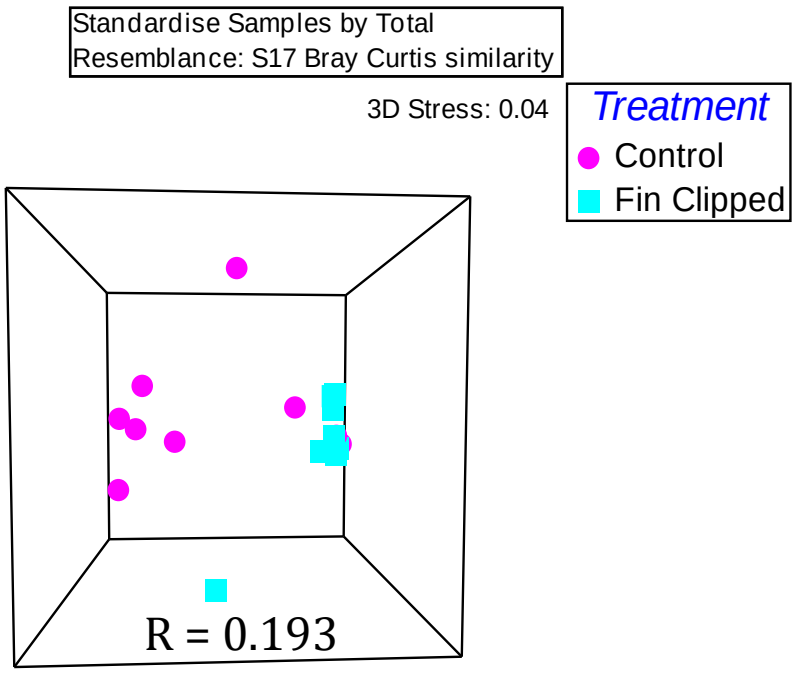

Figure 5-5. Multidimensional scaling (MDS) based on Bray- Curtis metrics between Control and Fin-clipped fish gut samples. Each symbol represents one sample. Pink colors are samples from Control treatment and Turquoise color represent samples from Fin-clipped treatment.

CHAPTER 6. EFFECTS OF A TRIVALENT VACCINE ON MICROBIOME OF CHANNEL CATFISH (*ICTALURIS PUNCTATUS*) UNDER FIELD TRIALS

Abstract

Vaccines are one of the best management tools to prevent the occurrence of infectious diseases. In catfish aquaculture, however, the use of vaccines is limited due to lack of approved vaccines and the observation that available commercial vaccines show low efficacy under field conditions. As part of a recent project aimed at testing the efficacy of a trivalent vaccine against *Aeromonas hydrophila*, *Edwarsiella ictaluris,* and *Flavobacterium columnare* in channel catfish, we investigated the gut, gill and skin microbiome of channel catfish before and after vaccination. Fish were vaccinated by bath immersion at the S6 pond at E. W. Shell Fisheries Center Auburn University and then moved to the Williamson Cattle Company farm. High-throughput sequencing revealed that the vaccine administration failed to induce significant changes in the fish microbiome. However, there was relatively a significant spatial-temporal shift in gut, gill and skin microbiome due to the time. The bacterial composition along all samples type was dominated by the phyla Proteobacteria and Firmicutes. The most abundant group was an Uncultured α-proteobacteria Group 1 that dominated all the samples. Interestingly, Uncultured α-proteobacteria Group 1 OTUs shared, on average, only 82% of sequence identity with previously known sequences suggesting those OTUs represent a new linage within the α-proteobacteria class.

Introduction

Intensification of aquaculture has lead to an increase of infectious diseases in farm which has created the need for control and preventive treatments measures. Vaccination is an effective strategy used worldwide for controlling infectious diseases in farm animals. There are commercial vaccines for more than 17 fish species that protect against more than 22 different bacterial diseases [1]. Most of them are killed vaccines that are delivered by injection or immersion. The only two commercial vaccines available for channel catfish are modified live vaccines against *Edwardsiella ictaluri* (AQUAVAC-ESC) and *Flavobacterium columnare* (AQUAVAC-COL). Although they have shown good protection in laboratory studies when used in fry as early as 7-30 days post-hatch [2, 3], their efficacy under field conditions has been questioned by farmers and their use is limited within the industry (as today, AQUAVAC-COL has been discontinued by the manufacter).

Through a collaborative effort between researchers at the School of Fisheries, Aquauclture, and Aquatic Science and USDA-ARS as well as industry partners at Kennebec River Biosciences, and the catfish farmers, we investigated the efficacy of a tri-valent vaccines against the main bacterial pathogens affecting catfish in Alabama (i.e., *F. columnare, A. hydrophila, and E. ictaluri*). The vaccines consisted of formalin-killed cells of the aforementioned bacterial pathogens mixed in equal amounts and administered by injection and immersion with and without adjuvant. We were interested in determining if the exposure to an immersion vaccine would have an effect on the microbial communities associated with mucosal epithelia (gill, skin, and gut). The role of the mucus layer and its association with the microbial communities of gut,

gill, and skin is particularly relevant to cultivated fish species. Aquacultured fish are often stocked at higher densities than in their native environments which makes the fish more vulnerable to infectious agents [4]. Mucosal microbial communities vary amongst fish species and tissue type and are influenced by endogenous and exogenous factors [5, 6], and could compromise the effectiveness of the vaccine treatment. Studies using mice revealed that the co-evolution of the microbiome with the innate immune system has resulted in a complex relationship by which both the microbiome and the host need each other to maintain mutual homeostasis [7]. This intimate cross-talk may be driven by metabolite secretion and signaling and exerts a profound influence on host immunity and physiology [8]. Vaccination elicits a strong immune response in the host, which can be counterproductive in hosts that are not immunocompetent. Farm animals often exhibit unbalanced or dysbiotic microbiomes due to commercial practices that have been associated with a decrease in immunocompetency (in mammals) [9]. There is a limited amount of studies on how vaccines could alter the host-microbiome balance. The objective of this study was to characterize if vaccination practices elicit a significant shift in mucosal microbial communities of channel catfish under field conditions.

Material and Methods

Fish husbandry and experimental design.

The study was conducted during from May 2017 to November 2017 (at the time of writing this dissertation, vaccinated animals were still maintained at a commercial farm and vaccine efficacy had not yet been evaluated). The experimental design consisted of 2 groups: Control (not-handled) and Vaccine (exposed to a trivalent killed vaccine). Approximately, 150 pounds of unsex and un-sized channel catfish from Harvest Select (Inverness Farm, Mississippi,

38753). Upon arrival to the pole barn, the 16,000 fish (at 60 lb/1000 fish) were stocked into 12 raceways, with each containing 320 lbs of fish (so ~ 5333 fish per raceways, in triplicate) located at the E.W. Shell Fisheries Center, Auburn University (Pole barn, PB-S6) and were supplied with flow-through pond-water. After 3 days of acclimation in the raceways, approximately 16,000 fish were vaccinated with trivalent vaccines containing killed *A. hydrophila*, *F. columnare* and *E. ictaluri*. Vaccines were prepared by Kennebec Inc., and their formulations were proprietary and thus not disclosed to us. Although different vaccines were tested, this study focused on fish vaccinated by immersion with the trivalent vaccine containing adjuvant. Fish in the triplicate raceways were vaccinated by crowding fish into a volume of water of ~ 175 gallons, after wich 3 L of vaccine were added. Fish were exposed to the vaccine for 15 mintues and afterwards the crowder was removed. Dissolved oxygen was maintained above 5 ppm during the vaccination by supplying oxygen via cylinder and airstones. Control fish were not handled or sham-vaccinated. 264 L of water. Control and vaccinated fish were kept in the PB-S6 location for approximately 30 days until they were moved to the Williamson Cattle Company (WCC, 4801 Al Hwy 25 Newbern, Al 36765). At WCC farm, fish were divided into three ponds that contained in-pond raceways (4 cells per treatment/raceways = 12 replicates per treatment). Each cell was randomly stocked with 1,000 trial fish, for a total of 8,000 fish per pond. Throughout the study, both vaccinated and control fish were managed identically.

Sampling.

Fish from each group were randomly sampled before vaccination (d0) and post-vaccination at day 19 (d19) at PB-S6, and at day 57 (d57) and day 173 (d173) at WWC farm (Table 6-1). At each sampling point, 4 fish per each treatment (n = 4) were sampled and

euthanized with MS-222 following standard protocol and transported to the laboratory on ice to being processed.

Prior necropsies, total lengths (mm) and weight (g) were recorded for each individual fish (Table 6-2). The middle gut, a skin explant (~1 cm next to the dorsal fin, including dermis, epidermis, and mucus) and the 2nd and 3rd gill arch were taken for DNA extraction. Samples were immediately placed into a sterile tube and set on ice. Upon arrival to the laboratory (less than 4 hours), DNA was extracted using Dneasy Blood & Tissue kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions (Total DNA from Animal Tissues, Spin column Protocol) with modifications including double digestion with proteinase K and pre-treatment with lysozyme for lysis of Gram-positive bacteria and RNase A treatment. DNA was eluted with 100 µl elution buffer and quantified using Nanodrop 1000 spectrophotometer (Thermo Scientific, Nanodrop Technologies, Wilmington, DE, USA).

PCR Amplification and Sequencing.

A total of 20 samples were submitted to MR DNA® (Shallowater, TX, USA) for PCR amplification and Next Generation Sequencing using Illumina MiSeq platform targeting the 16S rRNA gene V4 variable region. Universal bacterial primers 515 F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') with a barcode on the forward primer were used to generate a 300 bp amplicon. The DNA sequences and pipeline analysis was conducted following the protocol for PCR Amplification and Sequencing described in the previous studies in this dissertation.

Data Analysis.

Comparison of growth parameters between control and vaccine group was carried out with One-way ANOVA using JMP Version 12 .0.1 (SAS Institute Inc. Cary, NC, 1989-2007).

Rarefaction curves, observed OTUs (sobs), shared OTUs, ACE, CHAO1, Good Coverage and Shannon-evenness index (SEI) were calculated using Mothur v.1.39.5 [10] after standardization of each sample type to the sample yielding the least number of total sequences (n = 9,734 for gut; n = 765,716g for gill, and n = 10,186 for skin). One-way ANOVA was used to determine differences in diversity index between control and vaccine group, and those in the groups among different time points using JMP Version 12 .0.1 (SAS Institute Inc. Cary, NC, 1989-2007). A cluster multidimensional scaling (MDS) plot was used to further visualize variations of community member and structure between groups, also within groups among different time points and by location. Analysis of similarities (ANOSIM) was run at OTU level from the similarity matrix using Primer v6 (Primer-E Ltd, Plymouth, UK) to quantify groupings seen in MDS plots statistically. A genera abundance table was loaded into Primer v6, and similarity percentages (SIMPER) analysis on Bray-Curtis similarity matrix assembled was performed to determine the genera responsible for differences between bacterial communities. The cut-off for low contributions was set to the default at 90%.

Results

Overall animal health.

Mortality throughout the study period was low and within the range of what has been observed in the same in-pond raceways in previous sessions using similar stock densities. No disease outbreaks were reported during the study. Fish from both control and vaccinated treatments sustained weight gain and growth, shown in Table 6-2, with no significant differences between treatments during the study. Gross examination of external and internal organs from fish from both treatments appeared normal. However, as an anecdotal observation, vaccinated fish had higher abundance of belly fat covering the internal organs compared with control but no

attempt was made to quantify that observation. We did find ectoparasites in gill from both control and vaccinated fish. The parasites were identified as *Henneguya sp.* with myxospores encysted in plasmodia, but the number of polar filaments coils was not determined (parasitic identifications were made by personnel at the Southeast Cooperative Fish Parasite & Disease Laboratory, Auburn University).

Microbial diversity in gut, gills, and skin.

Gut. Overall, a total number of 3,557,393 bacterial sequences were obtained and ascribed to 5,294 OTUs from 28 gastrointestinal (GI) samples (n = 28). After standardization to the lowest read, a total number of 1,090,628 bacterial sequences remained that represent 3,848 OTUs with a coverage > 99% in all samples. The number of shared between groups increased during the study, from 286 at d0 to 1,015 OTUs at d173 in WWC. The number of unique OTUs in each group also increased throughout the sampling period and range from 437 to 893 and from 327 to 584 in control and vaccinated fish, respectively (Table 6-3a). There was a significant increase in the diversity in both groups after fish were transferred from the E.W. Shell Fisheries station to the WWC farm.

The diversity and evenness increased during the study (Table 6-4). Expected richness calculated by Chao1 and ACE was significantly higher in vaccinated group compared than in the control group at day 57 at WCC farm. Species evenness was not statistically different between control and vaccinated groups throughout the study. Regardless of the group, there was a strong significant spatial and temporal difference between samples but not within treatment groups. ANOSIM analysis confirmed that there were no significant differences between the gut microbiomes of control and vaccinated fish at any given point during the study.

Gill. A total number of 3,214,225 bacterial sequences were obtained and ascribed to 6,260 OTUs from gill samples (n = 28). After standardization to the lowest read, a total number of 765,716 bacterial sequences remained representing 4,621 OTUs with a coverage > 99% in all samples. A total number of 1,887 OTUs were shared between control and vaccine group accounting all samples with similar unique OTUs observed in both control and vaccine group (1,370 OTUs and 1,364, respectively). The number of shared OTUs increased along the time, with shared OTUs ranging from 342 at d0 to 1,101 at d173 (Table 6-3b).

Similar to what was observed in gut samples, microbial diversity in gills increased over time and communities become significantly more even at the end of the study (Table 6-5). However, ANOSIM analysis confirmed there were no significant differences between control and vaccinated gill communities at any given point.

Skin. A total number of 2,672,099 bacterial sequences representing 4,760 OTUs was yielded from skin samples. After standardization to the lowest read, a total number of 285,208 bacterial sequences remained, representing 3,103 OTUs. Observed OTUs covered moe than ≥ 98%, with an exception of one sample from the vaccinated group at d57 ($S\text{-}V^{WWC\text{-}57}$) that had a coverage ≥ 97%. There was an increased in the number of OTUs (both unique and shared) throughout the study but to a lower extend that the trend observed in gut and gill communities (Table 6-3c). The skin microbial diversity described by the estimated richness (Chao1 and ACE) showed that vaccine group had a higher bacterial richness compared with control but no significant differences were observed at the rest of the sampling points. The evenness remained similar between and within the groups at d0, d19, and d57, with the exception at d173, where vaccinated group showed significantly higher evenness compared with control. Comparison within groups along time points showed that the bacterial community richness and evenness in

the vaccinated group at d173 was significant higher compared with the same group at the other three sampling points (Table 6-6). ANOSIM analysis confirmed there were not a significant difference between samples.

Microbial Composition of Gut, Gill, and Skin.

Gut microbiota. The gut microbiome consisted of 32 bacterial phyla, and 85% of the bacterial communities were composed primarily of three phyla: Proteobacteria (α- and γ-proteobacteria), Firmicutes and Fusobacteria. Each of these phyla showed little variation in their relative abundance between control and treatment groups (on average 46% and 43% for Proteobacteria, 24% and 30% for Firmicutes, and 15% and 12% for Fusobacteria) (Figure 6-1). When bacterial sequences were ascribed into genera, up to 842 genera were identified in the gut microbiome. The phylum Proteobacteria were dominated by the genus Uncultured α-proteobacteria Group 1 while the phylum Firmicutes was represented by *Clostridium* and *Geobacillus,* and the phylum Fusobacteria by the *Cetobacterium*. *Aeromonas, Plesiomonas, Edwardsiella, Rhodopirellula,* and *Bacteroides* were also present as > 2.0 % of all sequences (Table 6-7a).

OTUs classified as Uncultured *α-proteobacteria* Group 1 were found in high abundances in gut, gill, and skin samples and they all shared at least 92% of similarity. These Uncultured *α-proteobacteria* Group 1 shared, on average, 82% sequence identity with known sequences of *Litorimonas*, *Thalassococcus,* and *Algimonas,* all belonging to α-proteobacteria (family Hyphomonadaceae and Rhodobacterraceae). Based on the low percent similarity between Uncultured *α-proteobacteria* Group 1 and previously known bacterial sequences, they are likely to represent a new lineage within the class Alphaproteobacteria.

Gill microbiome. The overall gill microbiome consisted of 30 bacterial phyla, and 90% of the bacterial communities were composed primarily of four phyla: Proteobacteria (α- and γ-proteobacteria), Chlamydiae, Firmicutes, and Cyanobacteria. Each of these phyla showed variants in their relative abundance among control and treatment groups respectively. The phylum Proteobacteria was relatively higher in control than the vaccinated fish (on average, 64.7% and 46.16 %, respectively). The second most abundant phyla Chlamydiae showed higher abundance in vaccinated fish compared with control group (on average, 29.2 % to 16.2 %). The bacterial abundances of phyla Firmicutes and Cyanobacteria were closed > 5 % in both groups (Figure 6-2). A total of 863 genera were found in the gill of channel catfish. The phylum Proteobacteria in the control and vaccinated groups was dominated by Unculture α-proteobacteria Group 1, the phylum Chlamydiae by Candidatus *similichlamydia*, the phylum Firmicutes by the *Geobacillus*, and the phylum Cyanobacteria by the *Mycrocystis*. *Aeromonas* and *Phyllobacterium* were also present and represented > 2.0 % of all sequences (Table 6-7b).

Skin microbiome. The overall skin microbiome consisted of 29 bacterial phyla. The most abundant phyla across all samples were Proteobacteria and Firmicutes accounting the 85% of the bacterial sequences. Each of these phyla varied in their relative abundance among control and treatment groups, on average 56.5% and 45.3% for Proteobacteria, and 31.7% and 34.9% for Firmicutes, (Figure 6-3). Accounting all samples, 827 genera were found inhabiting the skin microbiome. The phylum Proteobacteria in the control and vaccine group was dominated by *Uncultured α-proteobacteria* Group 1, and Firmicutes by *Geobacillus*. *Cetobacterium*, *Lautropia*, *Clostridium*, *Aeromonas*, *Edwardsiella,* and Kallotenue was also present as > 5.0 % of all bacterial sequences (Table 6-7c).

Temporal Seasonal Dynamics of microbial communities.

Gut. MDS plot based on Bray-Curtis distances at OTUs level from all samples showed sampling time had more influence on bacterial communities associated with gut than the treatment applied (group) or location (Figure 6-4). MDS plots showed there is no treatment effect on microbial community structure at the OTU level in the gut, only differences between sampling points were further analyzed. MDS plot revealed the gut samples collected at different sampling days differed significantly from each other in all the four sampling points (ANOSIM, $P < 0.05$). The global R-value for the time was 0.709 with pairwise comparison R-values ranging from 0.332 to 0.95. A total of 10.1 % OTUs were shared by at least 2 sampling points. The microbial community structure at d0 was the most-well separated within all samples (Figure 6-5), mainly due to differences in *Aeromonas*, *Edwarsiella,* and *Bacteroides*. SIMPER analysis indicated that the main difference in community structure occurred between d0 and d19 (87.95 %). *Aeromonas, Edwarsiella*, Bacteroides, and Uncultured α-proteobacteria Group were responsible for most of the variation (Table 6-8).

Gill. As in the gut microbiome, there was not a treatment or location influence in the bacterial community structure. However, it was observed that sampling time highly influenced the microbial community structure leading to a very distinctive community along the study (Figure 6-6). The global R-value for sampling time was 0.559. However, pairwise comparison revealed that there was not significant difference between the microbial communities at d19 and d57. A total of 11.2% OTUs were shared by at least 2 sampling points. The microbial community structure at d0 was the most different from all communities (Figure 6-7), mainly due to changes in *Phyllobacterium, Aeromonas, and Edwardsiella.* SIMPER analysis revealed that most differences in community structure between the fourth sampling times were between d0 and d19 (88.23 %). Specific genera abundance is listed on (Table 6-9).

Skin. As it was observed in the gut and gill, the MDS plot based on Bray-Curtis distances at OTU level showed that the microbial community structure associated with fish skin was influenced by sampling time level than the treatment (group) or location (Figure 6-8). The global R-value for the time was 0.469 with pairwise comparison R-values ranging from 0.186 to 0.857. Statistical analysis (ANOSIM) further revealed significant differences in the microbial structure at different time points ($P < 0.05$). A total of 11.0% OTUs have shared at least 2 sampling points. The microbial community structure at d0 was the most unique from all sampling points (Figure 6-9) mostly due to the abundance of *Lautropia, Aeromonas,* and *Edwardsiella*. Similarly, as gut and gill SIMPER analysis indicated that the most difference in the community structure was between d0 and d19 (88.21 %). Specific variations in genus abundance is listed on (Table 6-10).

Discussion

Microorganisms colonize external surfaces of the fish and the gut. Early studies on fish mucosal microbial community used culture-based methods to characterize the microbial diversity. Because only a small percent of bacteria could be cultured in the lab, results from those studies suggested that the external surfaces of the fish reflected the microbial communities of their surrounding environments [11, 12]. Previous studies by our group [13, 14] have demonstrated that bacterial communities associated with skin and mucus of fish were clearly different from those found in water and that host species exerted a strong influence in shaping those communities than season or geographic location.

Gammaproteobacteria has been identified as the major class of the Proteobacteria phylum identified in fish bacterial communities [14-18]. To our surprise, the bacterial community in all

our samples was dominated by alpha-proteobacteria along all samples although a few studies have shown high abundance of α-proteobacteria in skin, gut and gill samples from fish [19-21]. The presence of Uncultured α-proteobacteria Group 1 was consistent with previous studies that reported high abundance of Uncultured α-proteobacteria associated with mucosal surfaces of gibel carp and bluntnose black breamthat were co-cultured in ponds [19, 21-23].

The second most abundant bacteria group found in gill was identified as *Candidatus similichlamydia sp.* This genus has been classified as an emerging pathogen in striped trumpeter [24-26] and brown trout (*Salmo trutta*)[27]. We also identified *Mycrocystis* in gill samples. This cyanobacteria is typically associated with algae blooms [28, 29] and has been identified as a health problem in the gill of carp [30].

Skin samples were also dominated by Uncultured α-proteobacteria Group 1 followed by common gammaproteobacteria (such as *Pseudomonas*) that have previously reported from fish skin [13, 14, 21, 31]. We also identified a relatively high abundance of genus representing *Lautropia, Lysobacter,* and *Bacteroides*. Those genera have been detected as part of mucosal microbiome in the oral cavities and upper respiratory tract of humans [32-34], as well in fish mucosal surface [23, 35, 36].

Major differences between samples at genus level were largely due to varying abundances of Uncultured α-proteobacteria Group 1. The bacterial sequences from our OTUs blasted in Gen Bank, and GreenGenes databases were identified as *Litorimonas sp., Thalassococcus lentus,* and *Algimonas porphyrae*. All three bacterial strains have been identified as marine bacteria associated with algae [37-41]. We hypothesize that this group represents a new species (or several) associated with freshwater algae that warrants further investigation. Due to its high abundance and the absence of pathology, is tempting to speculate that this bacteria is

helping the host to digest or absorb specific nutrients present in the pond environment of Pole barn (PB-S6) [42]. What make this group intriguing were the sharp shifts of abundance that occurred once the fish arrived to PB-S6 and after the fish were moved to WCC farm. Fish arrived to PB-S6 with few Uncultured α-proteobacteria Group 1 but then that group become dominant while fish were kept in PB-S6, sharply decreased after fish were moved to WCC farm and nearly disappeared overtime.

Many factors have been identified to influence vaccine effectiveness, including nutrient, age, sex, and genetics [43-45]. Recently, strong evidence showed that the immune-microbiome interdependence [7, 9] play the strongest factor in the vaccine efficacy [46]. It is now fairly well established that the microbiome affects the development and function of immune cell population and vice versa and therefore could affect vaccine performance. A previous study showed that the use of a a live attenuated *Salmonella Typhi* (Ty21a) in humans failed to alter the composition, diversity, or stability of the gut microbiome [47]. Our results agreed with that previous study.

The most interesting outcome of our study was the spatial and temporal changes observed in all tissues. The drastic increase and decrease in Uncultured α-proteobacteria Group 1 suggest microbiomes (skin, gill, and gut) were not stable or that the environment exerted such as strong effect on the microbiome that the host selective pressure was overcome by what was in the environment. It could also suggest that when the hosts were moved into PB-S6 they had dysbiotic communities that were not resilient to the change of environment.

Temporal/seasonal variations in fish microbes appear to be a normal occurrence [14, 48, 49] but the changes observed in our study were drastic and occurred within the summer months thus, seasonality in terms of temperature and physiological status of the fish should not have such a strong effect on the bacterial communities. However, it has been reported that relocating

animals into a new housing facility [50] or from artificial to natural environment [51] result in significant changes in the host-microbiome. In our study, there was an obvious change in richness between the two locations in which the fish were reared. At PB-S6 pond, the microbial richness was relatively low. Once the animals were moved the WWC farm, there was a steady increase in microbial diversity. This could be due to the different rearing system in which the animals were kept. At the pole barn, fish were maintained for approximately 30 days in raceways systems supply with constant flow-through pond-water from PB-S6 pond, while at the WCC farm, fish were kept for approximately 5 months in in-pond raceways systems, under higher stocking densities, with higher aeration and higher water flow. In conclusion, there was no effect in the microbial community composition associated to standard vaccination protocol, which is positive outcome for producers and further supports the use of vaccines as one of the best tools to prevent bacterial infections.

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Table 6-1. Description of Groups ID along the study. Control and Vaccination treatment (here for treatment) described by sampling time and location. Group ID= Sub-control (SC), Control (C), Vaccine (V). Superscripts describe Location: PB (Pole barn) / WCC (Williamson Cattle farm) & Sampling time in days (d).

Group ID *	Treatment name	Sampling time (days)	Location
SC^{PB-0}	Sub-Control	0	PB
C^{PB-19}	Control	19	PB
V^{PB-19}	Vaccine	19	PB
C^{WCC-57}	Control	57	WCC
V^{WCC-57}	Vaccine	57	WCC
$C^{WCC-173}$	Control	173	WCC
$V^{WCC-173}$	Vaccine	173	WCC

* For identify the sample type, the letter G (Gut), GL(Gill), and S (Skin) accompanied the group ID, for the microbiome description

Table 6-2. Total length (mm) and Weight (g) are reported as Mean ± Standard deviation (DS) for each group by time and locations. Group IDs consisted of Group Sub-control (SC), Control (c) and Vaccine (V). Superscript represent Location: PB (Pole barn) / WCC (Williamson Cattle farm) & Sampling time (day).

Group ID	Individual number	Total Length (mm)	Weight (g)	Group Average
SC^{PB-0}	1	183	64.8	
SC^{PB-0}	2	115	15.02	
SC^{PB-0}	3	135	21.56	Length average (mm): 140.75 ± 29.4
SC^{PB-0}	4	130	18.41	Weight average (g): 29.94 ± 23.3
C^{PB-19}	1	183	44.293	
C^{PB-19}	2	194	51.731	
C^{PB-19}	3	164	31.761	Length average (mm): 167.75 ± 28.05
C^{PB-19}	4	130	18.73	Weight average (g): 36.62 ± 14.5
V^{PB-19}	1	165	29.095	
V^{PB-19}	2	163	31.732	
V^{PB-19}	3	128	14.929	Length average (mm): 152.25 ± 16.99
V^{PB-19}	4	153	26.804	Weight average (g): 25.64 ± 7.41
C^{WCC-57}	1	210	75.23	
C^{WCC-57}	2	180	50.86	
C^{WCC-57}	3	150	23.57	Length average (mm): 183.0 ± 25.21
C^{WCC-57}	4	192	52.64	Weight average (g): 50.57 ± 21.14
V^{WCC-57}	1	150	29.35	
V^{WCC-57}	2	170	40.47	
V^{WCC-57}	3	220	104.33	Length average (mm): 188.75 ± 34.24
V^{WCC-57}	4	215	88.21	Weight average (g): 65.59 ± 36.31
$C^{WCC-173}$	1	370	466	
$C^{WCC-173}$	2	240	126.35	
$C^{WCC-173}$	3	235	126.04	Length average (mm): 267.5 ± 68.61
$C^{WCC-173}$	4	225	106.2	Weight average (g): 206.15 ± 173.49
$V^{WCC-173}$	1	230	92.87	
$V^{WCC-173}$	2	280	215.17	
$V^{WCC-173}$	3	265	161.85	Length average (mm): 263.75 ± 23.58
$V^{WCC-173}$	4	280	196.26	Weight average (g): 166.53 ± 53.84

Table 6-3. Shared OTUs and Unique OTUs observed in the Gut (a), Gill (b), and Skin (c).

Sample type	Group ID	SHARED	Unique	
			Control	Vaccine
a. Gut	SC^{PB-0} & C^{PB-19}	286	437	327
	SC^{PB-19} & V^{PB-19}	336	387	349
	C^{PB-19} & V^{PB-19}	349	264	336
	C^{WCC-57} & V^{WCC-57}	682	501	774
	$C^{WCC-173}$ & $V^{WCC-173}$	1015	893	584
b. Gill	SC^{PB-0} & C^{PB-19}	342	626	448
	SC^{PB-19} & V^{PB-19}	453	515	754
	C^{PB-19} & V^{PB-19}	490	300	717
	C^{WCC-57} & V^{WCC-57}	549	399	919
	$C^{WCC-173}$ & $V^{WCC-173}$	1101	941	870
c. Skin	SC^{PB-0} & C^{PB-19}	259	446	419
	SC^{PB-19} & V^{PB-19}	279	426	399
	C^{PB-19} & V^{PB-19}	339	339	339
	C^{WCC-57} & V^{WCC-57}	406	375	707
	$C^{WCC-173}$ & $V^{WCC-173}$	419	547	565

Table 6-4. Diversity index as calculated by Mothur software (v.1.39.5). Operational taxonomic units (OTUs) are defined at 97% sequence similarity. Significance among Total values for each group was determinate by one-way ANOVA followed by Tukey's post hoc test. Group IDs consisted of Sample type (G), Group ID representing: Group Sub-control (SC), Control (c) and Vaccine (V). Superscript represent Location: PB (Pole barn) / WCC (Williamson Cattle farm) & Sampling time (day).

Diversity of the Gut Microbiome						
			# Predicted OTUs			
Group ID	**Individual number**	**# Observed OTUs (sObs)**	**Chao1**	**ACE**	**Shannon-Evenness**	**Good's Coverage**
$G\text{-}SC^{PB\text{-}0}$	1	268	704.13	526.10	0.25	0.996
$G\text{-}SC^{PB\text{-}0}$	2	401	818.11	645.92	0.54	0.996
$G\text{-}SC^{PB\text{-}0}$	3	370	828.77	673.77	0.50	0.995
$G\text{-}SC^{PB\text{-}0}$	4	214	441.04	379.37	0.36	0.997
$G\text{-}C^{PB\text{-}19}$	1	317	626.79	475.28	0.41	0.996
$G\text{-}C^{PB\text{-}19}$	2	236	590.56	458.00	0.35	0.997
$G\text{-}C^{PB\text{-}19}$	3	268	597.16	432.89	0.33	0.996
$G\text{-}C^{PB\text{-}19}$	4	274	710.31	544.48	0.36	0.996
$G\text{-}V^{PB\text{-}19}$	1	249	654.66	427.64	0.29	0.996
$G\text{-}V^{PB\text{-}19}$	2	386	879.94	655.45	0.48	0.995
$G\text{-}V^{PB\text{-}19}$	3	292	715.07	520.49	0.42	0.996
$G\text{-}V^{PB\text{-}19}$	4	258	639.10	449.75	0.34	0.997
$G\text{-}C^{WCC\text{-}57}$	1	436	926.86	694.11	0.56	0.995
$G\text{-}C^{WCC\text{-}57}$	2	286	675.18	569.83	0.34	0.996
$G\text{-}C^{WCC\text{-}57}$	3	684	1229.03	1035.78	0.57	0.992
$G\text{-}C^{WCC\text{-}57}$	4	556	1203.73	913.04	0.51	0.993
$G\text{-}V^{WCC\text{-}57}$	1	570	1309.08	1152.15	0.39	0.992
$G\text{-}V^{WCC\text{-}57}$	2	789	2079.83	1625.72	0.56	0.989
$G\text{-}V^{WCC\text{-}57}$	3	612	1464.21	1089.37	0.46	0.992
$G\text{-}V^{WCC\text{-}57}$	4	462	1235.92	897.49	0.43	0.993
$G\text{-}C^{WCC\text{-}173}$	1	794	2074.19	1468.14	0.70	0.990
$G\text{-}C^{WCC\text{-}173}$	2	874	2522.33	1684.57	0.64	0.989
$G\text{-}C^{WCC\text{-}173}$	3	905	1890.36	1529.81	0.63	0.990
$G\text{-}C^{WCC\text{-}173}$	4	721	1663.13	1198.37	0.60	0.992

Table 6-4 continued

Group ID	Individual number	# Observed OTUs (sObs)	Chao1	ACE	Shannon-Evenness	Good's Coverage
G-V$^{WCC-173}$	1	712	2580.13	1402.17	0.67	0.990
G-V$^{WCC-173}$	2	656	1978.86	1343.98	0.64	0.992
G-V$^{WCC-173}$	3	846	1929.59	1454.60	0.54	0.989
G-V$^{WCC-173}$	4	675	1868.82	1215.96	0.65	0.992
TOTALS						
G-SC^{PB-0}		313^{d}	698$^{c, d}$	556^{c}	0.41$^{b, c}$	0.99
G-C^{PB-19}		274^{d}	631^{d}	478^{c}	0.36^{c}	0.99
G-V^{PB-19}		296^{d}	722^{d}	513^{c}	0.38$^{b, c}$	0.99
G-C^{WCC-57}		490^{c}	1008^{c}	803^{c}	0.49^{b}	0.99
G-V^{WCC-57}		608^{c}	1522^{b}	1191^{b}	0.45$^{b, c}$	0.99
G-C$^{WCC-173}$		824^{a}	2037^{a}	1470^{a}	0.64^{a}	0.99
G-V$^{WCC-173}$		722^{ab}	2089$^{a, b}$	1354^{a}	0.62^{a}	0.99

Table 6-5. Diversity index as calculated by Mothur oftware (v.1.39.5). Operational taxonomic units (OTUs) are defined at 97% sequence similarity. Significance among Total values for each group was determinate by one-way ANOVA followed by Tukey's post hoc test. Group IDs consisted of Group Sub-control (SC), Control (c) and Vaccine (V). Superscript represent Location: PB (Pole barn) / WCC (Williamson Cattle farm) & Sampling time (day).

Group	Individual number	# Observed OTUs (sObs)	# Predicted OTUs		Shannon-Evenness	Good's Coverage
			Chao1	ACE		
GL-SC^{PB-0}	1	327	547.66	668.44	0.46	1.00
GL-SC^{PB-0}	2	481	751.43	1061.52	0.57	0.99
GL-SC^{PB-0}	3	368	697.64	771.29	0.28	0.99
GL-SC^{PB-0}	4	514	855.72	1000.38	0.65	0.99
GL-C^{PB-19}	1	328	512.37	696.94	0.39	0.99
GL-C^{PB-19}	2	258	381.28	590.16	0.51	1.00
GL-C^{PB-19}	3	334	608.74	757.35	0.43	0.99
GL-C^{PB-19}	4	408	681.32	869.38	0.31	0.99
GL-V^{PB-19}	1	362	598.92	876.78	0.35	0.99
GL-V^{PB-19}	2	610	966.80	1042.98	0.45	0.99
GL-V^{PB-19}	3	604	875.73	983.13	0.64	0.99
GL-V^{PB-19}	4	510	871.50	1084.83	0.38	0.99
GL-C^{WCC-57}	1	522	885.26	769.91	0.44	0.99
GL-C^{WCC-57}	2	171	319.75	447.72	0.53	1.00
GL-C^{WCC-57}	3	257	582.11	738.33	0.21	1.00
GL-C^{WCC-57}	4	526	845.51	1035.53	0.43	0.99
GL-V^{WCC-57}	1	634	1101.28	1478.27	0.48	0.99
GL-V^{WCC-57}	2	702	1251.50	1567.93	0.53	0.99
GL-V^{WCC-57}	3	561	1099.01	1319.61	0.43	0.99
GL-V^{WCC-57}	4	561	1251.76	1959.04	0.40	0.99
GL-C$^{WCC-173}$	1	1078	1553.18	1488.44	0.49	0.99
GL-C$^{WCC-173}$	2	703	1450.50	2131.11	0.76	0.99
GL-C$^{WCC-173}$	3	786	1586.46	2243.75	0.69	0.99
GL-C$^{WCC-173}$	4	784	1584.60	2407.07	0.69	0.99

Table 6-5 continued

Group ID	Individual number	# Observed OTUs (sObs)	Chao1	ACE	Shannon-Evenness	Good's Coverage
GL-V$^{WCC-173}$	1	986	2106.68	2785.93	0.71	0.99
GL-V$^{WCC-173}$	2	887	1789.71	2321.48	0.69	0.99
GL-V$^{WCC-173}$	3	724	1316.06	1987.32	0.63	0.99
GL-V$^{WCC-173}$	4	699	1337.08	1991.89	0.58	0.99
TOTALS						
GL-SC^{PB-0}		423$^{c, d}$	713$^{b, c}$	875^{d}	0.49^{b}	0.99
GL-C^{PB-19}		332^{d}	546^{c}	728^{d}	0.41^{b}	0.99
GL-V^{PB-19}		522$^{b, c}$	828^{c}	997$^{c, d}$	0.46^{b}	0.99
GL-C^{WCC-57}		369$^{c, d}$	658^{c}	748^{d}	0.40^{b}	0.99
GL-V^{WCC-57}		615^{b}	1176$^{a, b}$	1581$^{b, c}$	0.46^{b}	0.99
GL-C$^{WCC-173}$		838^{a}	1544^{a}	2068$^{a, b}$	0.60^{a}	0.99
GL-V$^{WCC-173}$		824^{a}	1637^{a}	2272^{a}	0.65^{a}	0.99

Table 6-6. Diversity index as calculated by Mothur software (v.1.39.5). Operational taxonomic units (OTUs) are defined at 97% sequence similarity. Significance among Total values for each group was determinate by one-way ANOVA followed by Tukey's post hoc test. Group IDs consisted of Group Sub-control (SC), Control (c) and Vaccine (V). Superscript represent Location: PB (Pole barn) / WCC (Williamson Cattle farm) & Sampling time (day).

Group	Individual number	# Observed OTUs (sObs)	# Predicted OTUs		Shannon-Evenness	Good's Coverage
			Chao1	ACE		
S-SC^{PB-0}	1	275	620.33	601.68	0.47	0.99
S-SC^{PB-0}	2	320	513.52	682.81	0.69	0.99
S-SC^{PB-0}	3	227	362.80	449.90	0.24	0.99
S-SC^{PB-0}	4	361	593.81	696.78	0.70	0.99
S-C^{PB-19}	1	276	424.75	543.43	0.55	0.99
S-C^{PB-19}	2	189	539.20	703.66	0.47	0.99
S-C^{PB-19}	3	353	557.19	721.09	0.50	0.99
S-C^{PB-19}	4	247	394.57	379.24	0.51	0.99
S-V^{PB-19}	1	260	444.53	592.43	0.50	0.99
S-V^{PB-19}	2	318	540.78	641.36	0.58	0.99
S-V^{PB-19}	3	254	434.27	564.34	0.48	0.99
S-V^{PB-19}	4	284	475.82	628.64	0.51	0.99
S-C^{WCC-57}	1	297	473.70	599.17	0.49	0.99
S-C^{WCC-57}	2	447	618.01	660.35	0.59	0.98
S-C^{WCC-57}	3	328	499.27	646.17	0.52	0.99
S-C^{WCC-57}	4	236	423.29	543.36	0.48	0.99
S-V^{WCC-57}	1	484	1305.67	2274.34	0.59	0.97
S-V^{WCC-57}	2	469	911.00	1179.62	0.62	0.98
S-V^{WCC-57}	3	361	797.57	933.27	0.51	0.98
S-V^{WCC-57}	4	370	732.50	823.76	0.47	0.98
S-C$^{WCC-173}$	1	408	770.07	799.10	0.52	0.98
S-C$^{WCC-173}$	2	295	625.55	972.02	0.67	0.98
S-C$^{WCC-173}$	3	289	739.85	1372.67	0.41	0.98
S-C$^{WCC-173}$	4	393	779.54	1428.98	0.75	0.98

Table 6-6 continued

Group ID	Individual number	# Observed OTUs (sObs)	Chao1	ACE	Shannon-Evenness	Good's Coverage
S-V$^{WCC-173}$	2	429	902.20	1363.01	0.85	0.98
S-V$^{WCC-173}$	3	310	575.00	999.56	0.62	0.98
S-V$^{WCC-173}$	4	424	966.52	1444.41	0.71	0.98
TOTALS						
S-SC^{PB-0}		296$^{b, c}$	523^{c}	608^{b}	0.52^{b}	0.99
S-C^{PB-19}		266^{c}	479^{c}	587^{b}	0.51^{b}	0.99
S-V^{PB-19}		279$^{b, c}$	474^{c}	607^{b}	0.52^{b}	0.99
S-C^{WCC-57}		327$^{a, b, c}$	504^{c}	612^{b}	0.52^{b}	0.99
S-V^{WCC-57}		421^{a}	937^{a}	1303^{a}	0.55^{b}	0.98
S-C$^{WCC-173}$		346$^{b, c}$	729$^{a, b}$	1143^{a}	0.59^{b}	0.98
S-V$^{WCC-173}$		367^{b}	818^{b}	1303^{a}	0.71^{a}	0.98

Table 6-7. Major Genera identified by sequencing of 16 S rRNA gene by Illumina MiSeq of channel catfish (*I. puncatus*). Samples of Gut, Gill and Skin were sampled. Only genus making up > 2.0% of total sequences were included

Sample type	**Phylum (Class)**	**Genus**	**% of sequences**
		Uncultured α-proteobacteria Group	
	Protoebacteria (α)	1	24.7
	Protoebacteria (γ)	*Aeromonas*	3.8
	Protoebacteria (γ)	*Plesiomonas*	3.4
	Protoebacteria (γ)	*Edwardsiella*	3.3
a. Gut	Firmicutes (Clostridia)	*Clostridium*	17.4
	Firmicutes (Bacillis)	*Geobacillus*	4.6
	Fusobacteria (Fusobacteriia)	*Cetobacterium*	13.4
	Planctomycetes (Planctomycetia)	*Rhodopirellula*	2.8
	Bacteroidetes (Bacteroidia)	*Bacteroides*	2.8
		Uncultured α-proteobacteria Group	
	Protoebacteria (α)	1	31.4
	Protoebacteria (α)	*Phyllobacterium*	4.3
b. Gill	Protoebacteria (γ)	*Aeromonas*	2.1
	Chlamydiae (Chlamydiia)	Candidatus *similichlamydia*	22.2
	Firmicutes (Bacillis)	*Geobacillus*	4.0
	Cyanobacteria	*Microcystis*	3.0
		Uncultured α-proteobacteria Group	
	Protoebacteria (α)	1	75.7
	Proteobacteria (β)	*Thauera*	2.3
	Proteobacteria (β)	*Lautropia*	7.9
	Proteobacteria (γ)	*Edwardsiella*	7.2
	Proteobacteria (γ)	*Aeromonas*	5.2
	Proteobacteria (γ)	*Lysobacter*	4.1
	Proteobacteria (γ)	*Pseudomonas*	3.5
c. Skin	Proteobacteria (γ)	*Plesiomonas*	2.8
	Firmicutes (Bacillis)	*Geobacillus*	64.4
	Firmicutes (Clostridia)	*Clostridium*	7.9
	Firmicutes (Bacilli)	*Bacillus*	2.4
	Fusobacteria (Fusobacteriia)	*Cetobacterium*	8.1
	Bacteroidetes (Bacteroidia)	*Bacteroides*	3.5
	Chlamydiae (Chlamydiia)	Candidatus *similichlamydia*	2.6
	Bacteroidetes (Bacteroidia)	*Proteiniphilum*	2.5
	Chloroflexi (Chloroflexia)	*Kallotenue*	5.7

Table 6-8. Similarity percentages (SIMPER) results by genera for gut samples based on sampling time. Genera contributing with > 3% of dissimilarities are included.

Sampling time (Days)	Genus	Average Abundance		Contribution to dissimilarities (%)
		1	2	
	Uncultured α-proteobacteria			
1. d0	Group 1	0.4	55.82	31.51
2. d19	*Aeromonas*	27.06	0.06	15.35
	Edwardsiella	25.75	0.05	14.61
	Bacteroides	16.13	5.06	9.68
	Cetobacterium	9.81	14.91	8.14
	Geobacillus	4.87	6.26	3.56
	Clostridium	0.45	5.42	3.09
Av.diss. (87.95 %)				
1. d0	*Clostridium*	0.45	34.71	19.58
2. d57	*Aeromonas*	27.06	0.52	15.16
	Edwardsiella	25.75	0.04	14.69
	Uncultured α-proteobacteria			
	Group 1	0.4	16.37	9.23
	Bacteroides	16.13	5.6	9.2
	Cetobacterium	9.81	16.56	6.67
	Plesiomonas	0.04	7.56	4.3
	Geobacillus	4.87	4.06	3.3
Av.diss. (87.49 %)				
1. d0	*Aeromonas*	27.06	0.18	14.84
2. d173	*Edwardsiella*	25.75	0	14.22
	Bacteroides	16.13	1.17	8.89
	Cetobacterium	9.81	5.27	6.35
	Clostridium	0.45	9.79	5.16
	Rhodopirellula	0	8.96	4.95
	Geobacillus	4.87	6.65	3.98
	Methylocystis	0.15	6.94	3.75
	Halospirulina	0.04	6.2	3.41
Av.diss. (90.54 %)				
	Uncultured α-proteobacteria			
1. d19	Group 1	55.82	16.37	33.09
2. d57	*Clostridium*	5.42	34.71	22.81
	Cetobacterium	14.91	16.56	10.75
	Plesiomonas	0.07	7.56	5.19
	Geobacillus	6.26	4.06	4.62
	Bacteroides	5.06	0.56	3.7
Av.diss. (72.12 %)				

Table 6-8 continued

Sampling time (Days)	Genus	Average Abundance		Contribution to dissimilarities (%)
	Uncultured α-proteobacteria			
1. d19	Group 1	55.82	0.06	30.46
2. d173	*Cetobacterium*	14.91	5.27	8.65
	Clostridium	5.42	9.79	6.69
	Rhodopirellula	0	8.96	4.89
	Geobacillus	6.26	6.65	4.19
	Methylocystis	0.01	6.94	3.79
	Halospirulina	0.01	6.2	3.38
Av.diss. (91.52 %)				
1. d57	*Clostridium*	34.71	9.79	18.38
	Uncultured α-proteobacteria			
2. d173	Group 1	16.37	0.06	10.48
	Cetobacterium	16.56	5.27	10.43
	Plesiomonas	7.56	0.15	4.77
	Geobacillus	4.06	0.15	4.68
	Rhodopirellula	2.48	6.65	4.5
	Methylocystis	0.58	8.96	4.12
	Halospirulina	0.08	6.94	3.94
Av.diss. (77.83 %)				

Table 6-9. Similarity percentages (SIMPER) results by genera for gill samples based on sampling time. Genera contributing with > 3% of dissimilarities are included.

Sampling time (Days)	Genus	Average Abundance 1	Average Abundance 2	Contribution to dissimilarities (%)
1. d0	Uncultured α-proteobacteria Group 1	0.45	56.48	31.75
2. d19	*Phyllobacterium*	20.13	0.03	11.4
	Aeromonas	17.38	0.79	9.58
	Candidatus similichlamydia	8.44	12.24	7.51
	Edwardsiella	12.42	0.09	6.99
	Candidatus branchiomonas	6.07	0.46	3.21
Av.diss. (88.23 %)				
1. d0	Candidatus *similichlamydia*	8.44	32.48	16.09
2. d57	Uncultured α-proteobacteria Group 1	0.45	25.03	14.45
	Phyllobacterium	20.13	4.5	13.12
	Aeromonas	17.38	0.17	10.12
	Edwardsiella	12.42	0.02	7.28
	Candidatus *branchiomonas*	6.07	0.21	3.44
Av.diss. (85.19 %)				
1. d0	*Phyllobacterium*	20.13	0.08	12
2. d173	*Aeromonas*	17.38	0.34	10.18
	Candidatus *similichlamydia*	8.44	17.09	7.68
	Edwardsiella	12.42	0	7.4
	Mycrocystis	0.01	8.77	5.22
	Halospirulina	0.17	6.94	4.11
	Comamonas	0.06	5.53	3.27
	Candidatus *branchiomonas*	6.07	1.12	3.04
Av.diss. (83.92 %)				
1. d19	Uncultured α-proteobacteria Group 1	56.48	25.03	31.86
2. d57	Candidatus *similichlamydia*	12.24	32.48	23.04
	Geobacillus	4.31	4.67	4.27
	Phyllobacterium	0.03	4.5	3.35
Av.diss. (67.14 %)				
1. d19	Uncultured α-proteobacteria Group 1	56.48	0.07	32.19
2. d173	Candidatus *similichlamydia*	1224	17.09	10.59
	Mycrocystis	0.09	8.77	4.95
	Halospirulina	0.1	6.94	3.94
	Comamonas	0.06	5.53	3.13
Av.diss. (87.63 %)				

Table 6-9 continued

Sampling time (Days)	Genus	Average Abundance		Contribution to dissimilarities (%)
1. d57	Candidatus *similichlamydia*	32.48	17.09	17.84
2. d173	Uncultured α-proteobacteria Group 1	25.03	0.07	15.86
	Halospirulina	0.01	6.94	4.4
	Mycrocystis	4.02	8.77	3.93
	Comamonas	0.06	5.53	3.48
	Geobacillus	4.67	2.72	3.35
Av.diss. (77.83 %)				

Table 6-10. Similarity percentages (SIMPER) results by genera for skin samples based on sampling time. Genera contributing with > 3% of dissimilarities are included.

Sampling time (Days)	Genus	Average Abundance		Contribution to dissimilarities (%)
		1	2	
1. d0	Uncultured α-proteobacteria Group 1	0.51	54	30.32
2. d19	*Edwardsiella*	23.37	0.18	13.15
	Lautropia	23.06	0.04	13.05
	Geobacillus	7.35	26.95	12.83
	Aeromonas	15.64	0.1	8.81
Av.diss. (88.21 %)				
1. d0	*Geobacillus*	7.35	38.41	18.82
2. d57	Uncultured α-proteobacteria Group 1	0.51	24.53	13.65
	Edwardsiella	23.37	0.06	13.19
	Lautropia	23.06	0.02	13.04
	Aeromonas	15.64	0.36	8.71
	Clostridium	0.19	6.13	3.36
	Cetobacterium	0.27	6.1	3.35
Av.diss. (88.36 %)				
1. d0	*Edwardsiella*	23.37	0	13.53
2. d173	*Lautropia*	23.06	0	13.35
	Geobacillus	7.35	19.89	10.9
	Aeromonas	15.64	0.47	8.9
	Pseudomonas	2.48	8.93	4.29
Av.diss. (86.36 %) %				
1. d19	Uncultured α-proteobacteria Group 1	54	24.53	34.07
2. d57	*Geobacillus*	26.95	38.41	24.8
	Clostridium	0.22	6.13	5.68
	Cetobacterium	0.79	6.1	5.59
	Bacteroides	0.06	3.26	3.1
Av.diss. (52.10 %)				
1. d19	Uncultured α-proteobacteria Group 1	54	0.07	32.38
2. d173	*Geobacillus*	26.95	19.89	15.01
	Pseudomonas	1.06	8.93	4.92
Av.diss. (83.28 %)				
1. d57	*Geobacillus*	38.41	19.89	20.19
2. d173	Uncultured α-proteobacteria Group 1	24.53	0.07	15.29
	Pseudomonas	0.42	8.93	5.34
	Clostridium	6.13	4.27	4.14
	Cetobacterium	6.1	1.42	4.11
Av.diss. (79.98 %)				

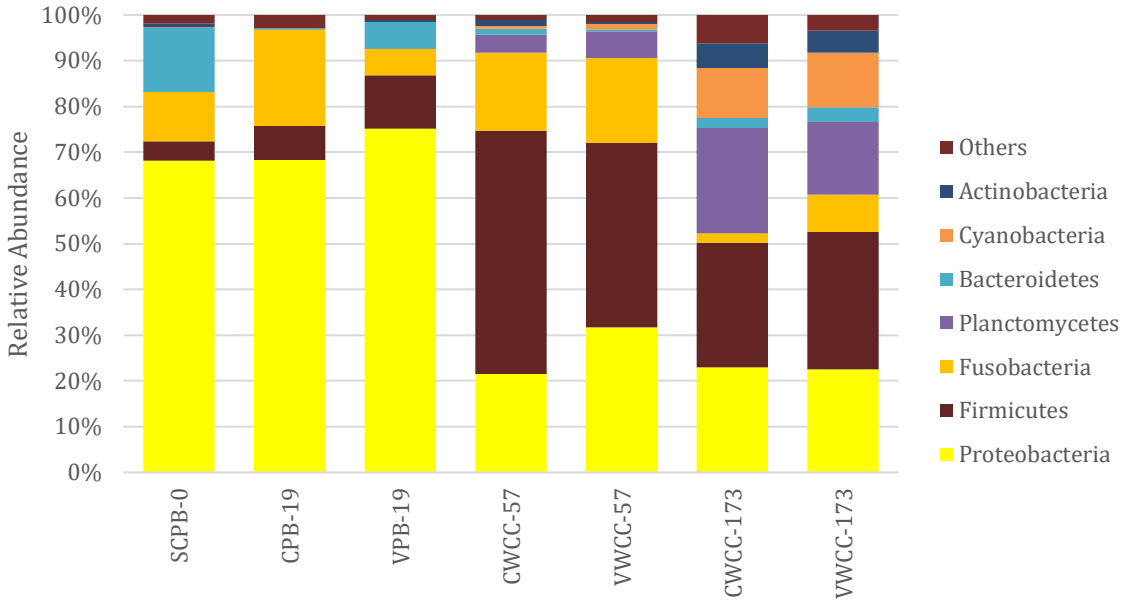

Figure 6-1. Bacterial diversity at the phylum level (bar graph) based on 16S rRNA gene sequencing for all gut samples. Only phyla making up 2.5% of total sequences were included.

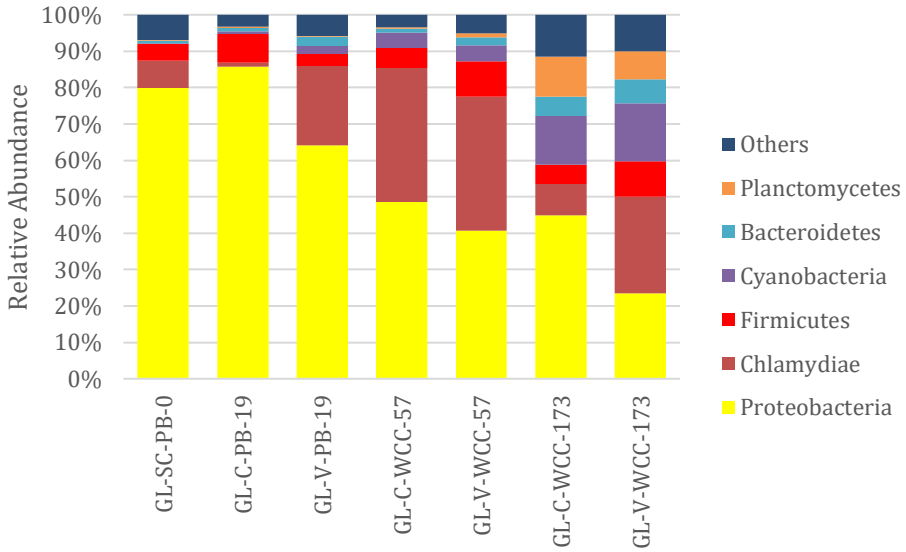

Figure 6-2. Bacterial diversity at the phylum level (bar graph) based on 16S rRNA gene sequencing for all gill samples. Only phyla making up 2.5% of total sequences were included.

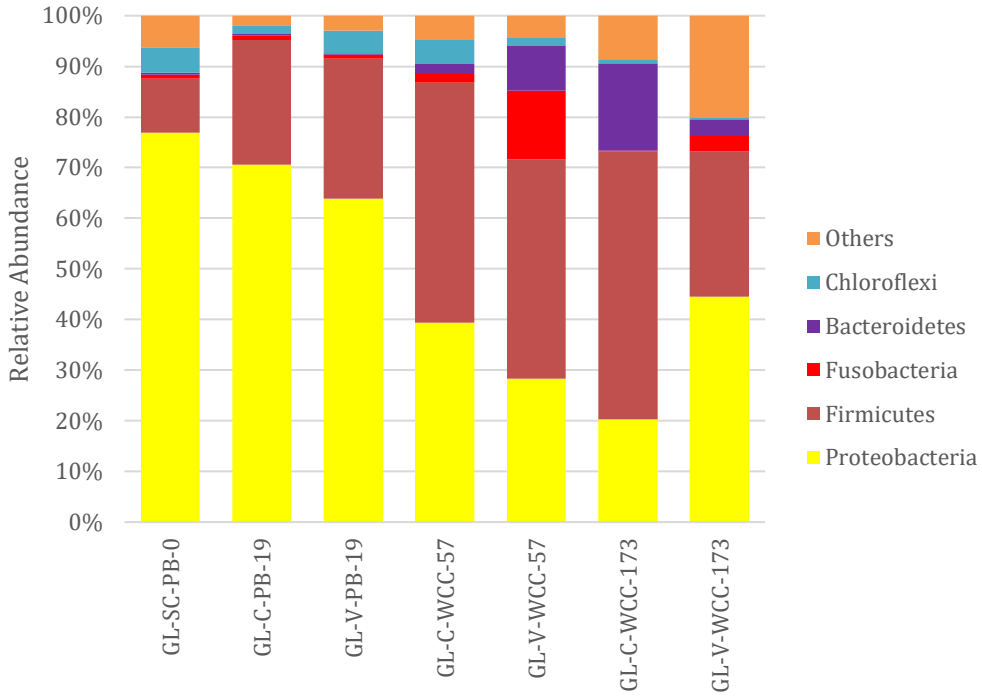

Figure 6-3. Bacterial diversity at the phylum level (bar graph) based on 16S rRNA gene sequencing for all skin samples. Only phyla making up 2.5% of total sequences were included.

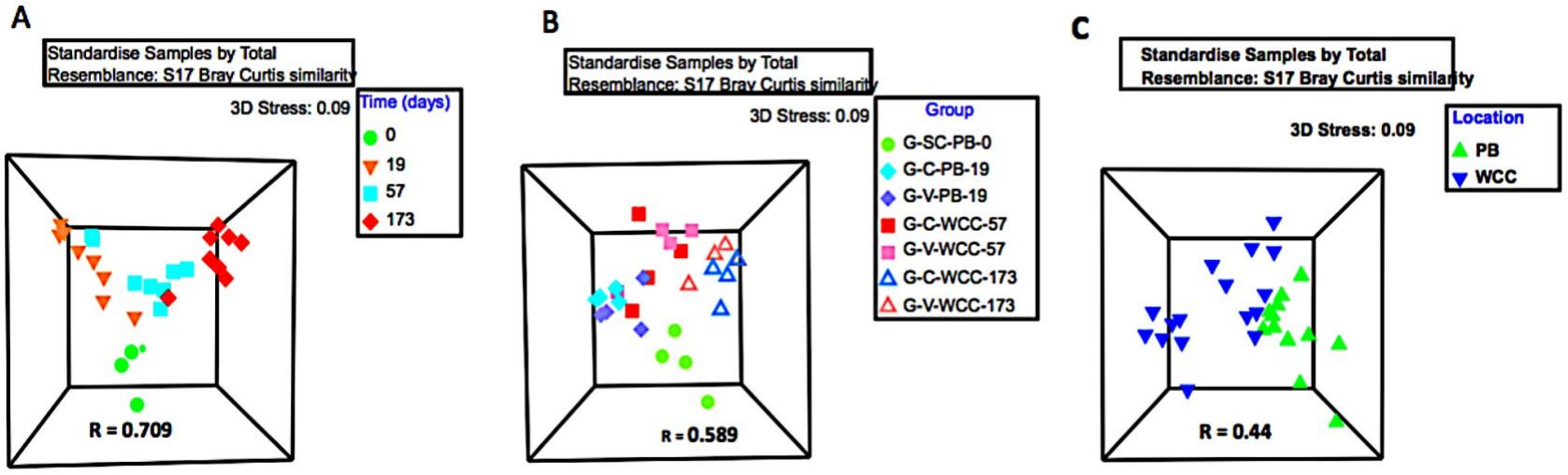

Figure 6-4. Multidimensional scaling (MDS) plot based on percent similarity in OTU abundance for gut samples based on A, sampling time; B, Group, and C, Location.

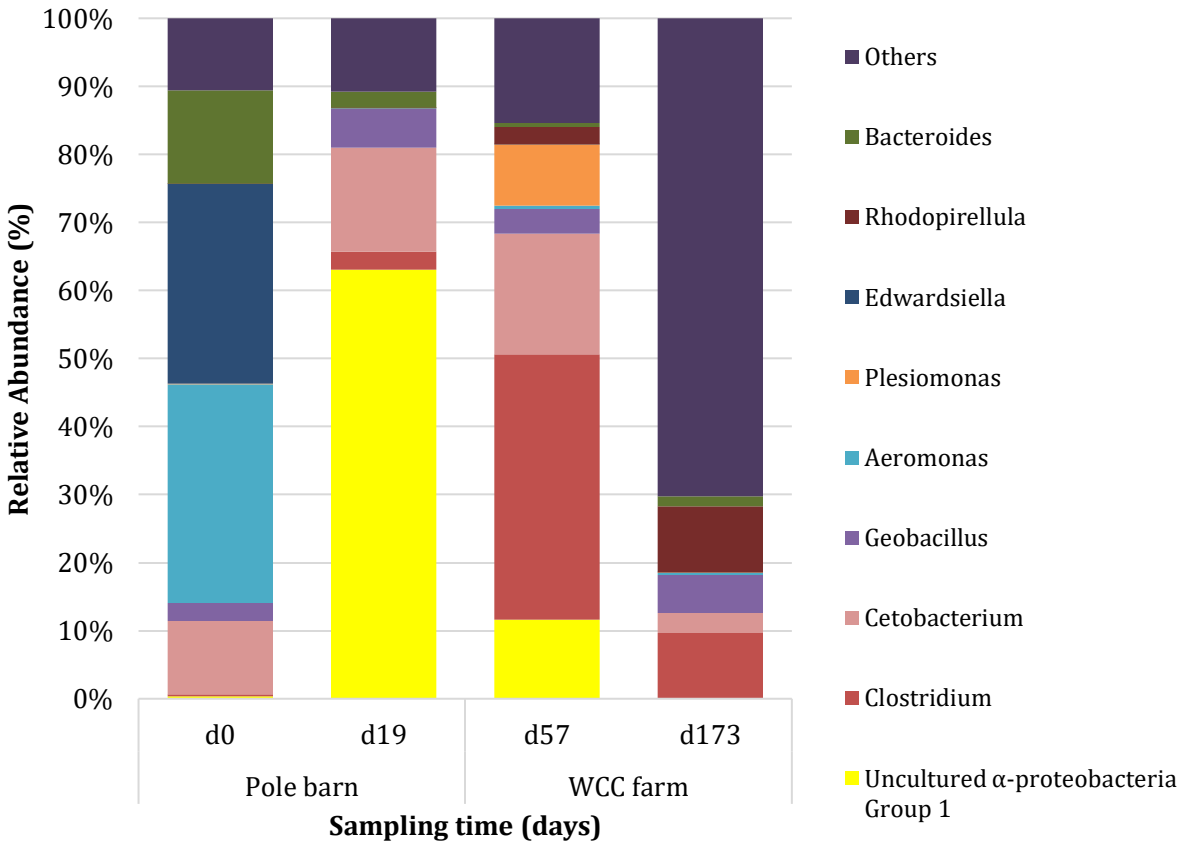

Figure 6-5. Average of the most abundant genera across all gut samples. The bar graph shows the microbial community composition along the study, based in the microbial composition at specific period of time and location.

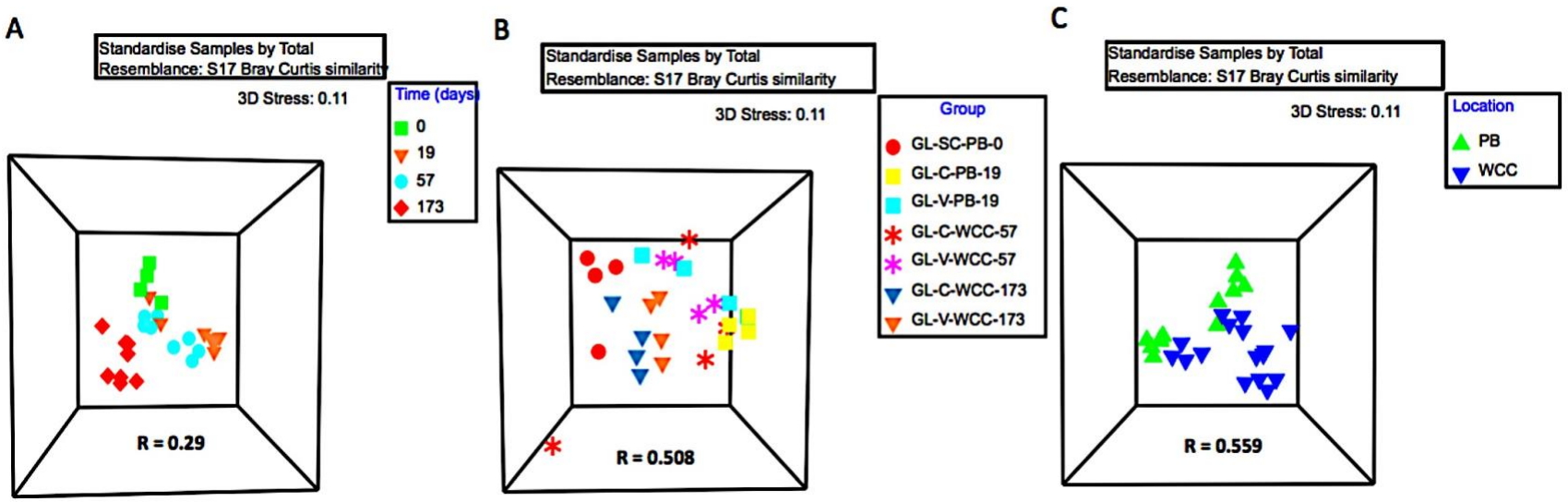

Figure 6-6. Multidimensional scaling (MDS) plot based on percent similarity in OTU abundance for gill samples based on A, Sampling time; B, Group, and C, Location.

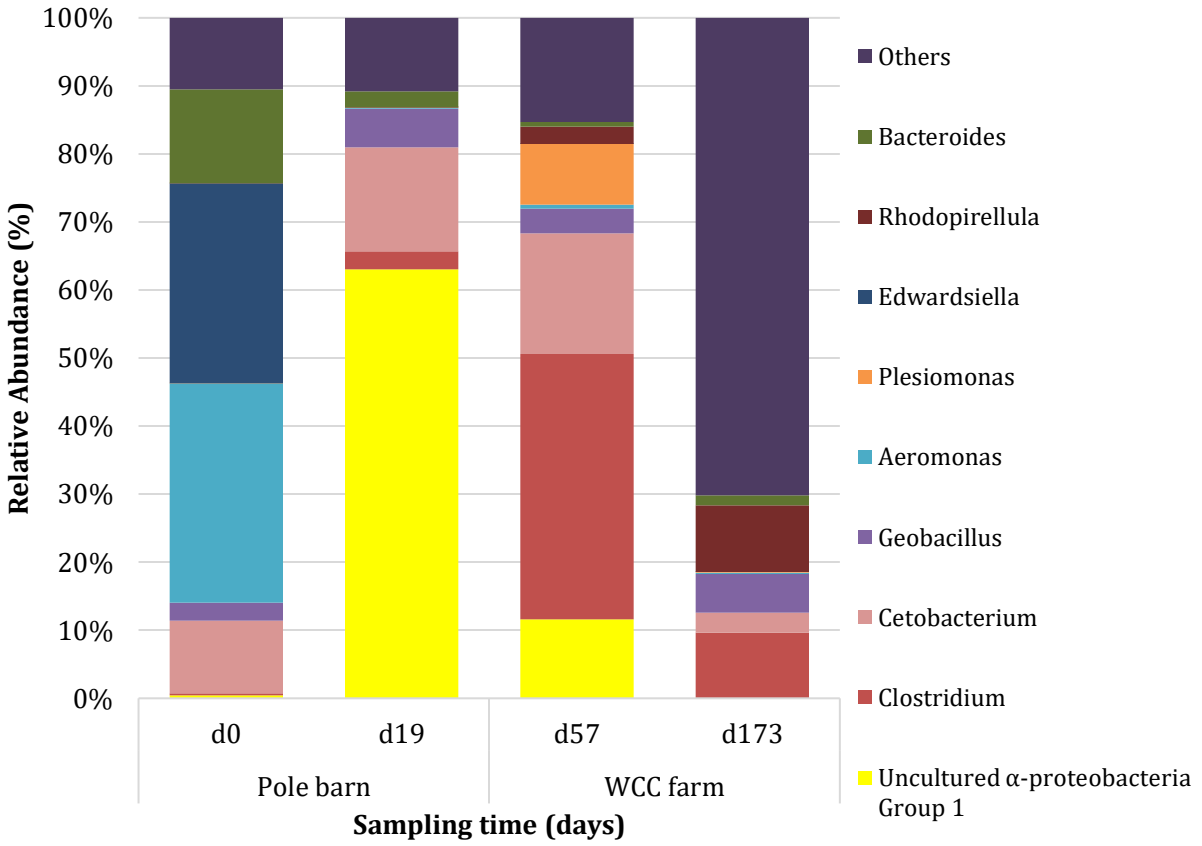

Figure 6-7. Average of Bacterial diversity at the genus level (bar graph) based on 16S rRNA gene sequencing across all gill samples. The bar graph shows the microbial community composition along the study, based in the microbial composition at specific period of time and location

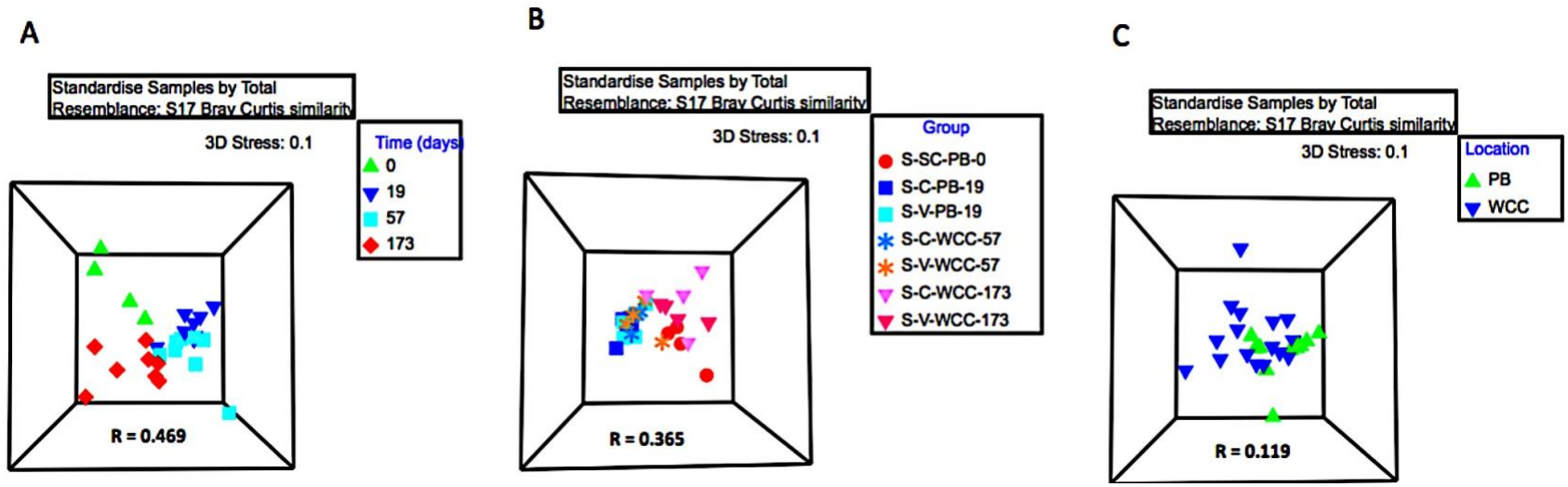

Figure 6-8. Multidimensional scaling (MDS) plot based on percent similarity in OTU abundance for skin samples based on A, sampling time; B, Group, and C, Location.

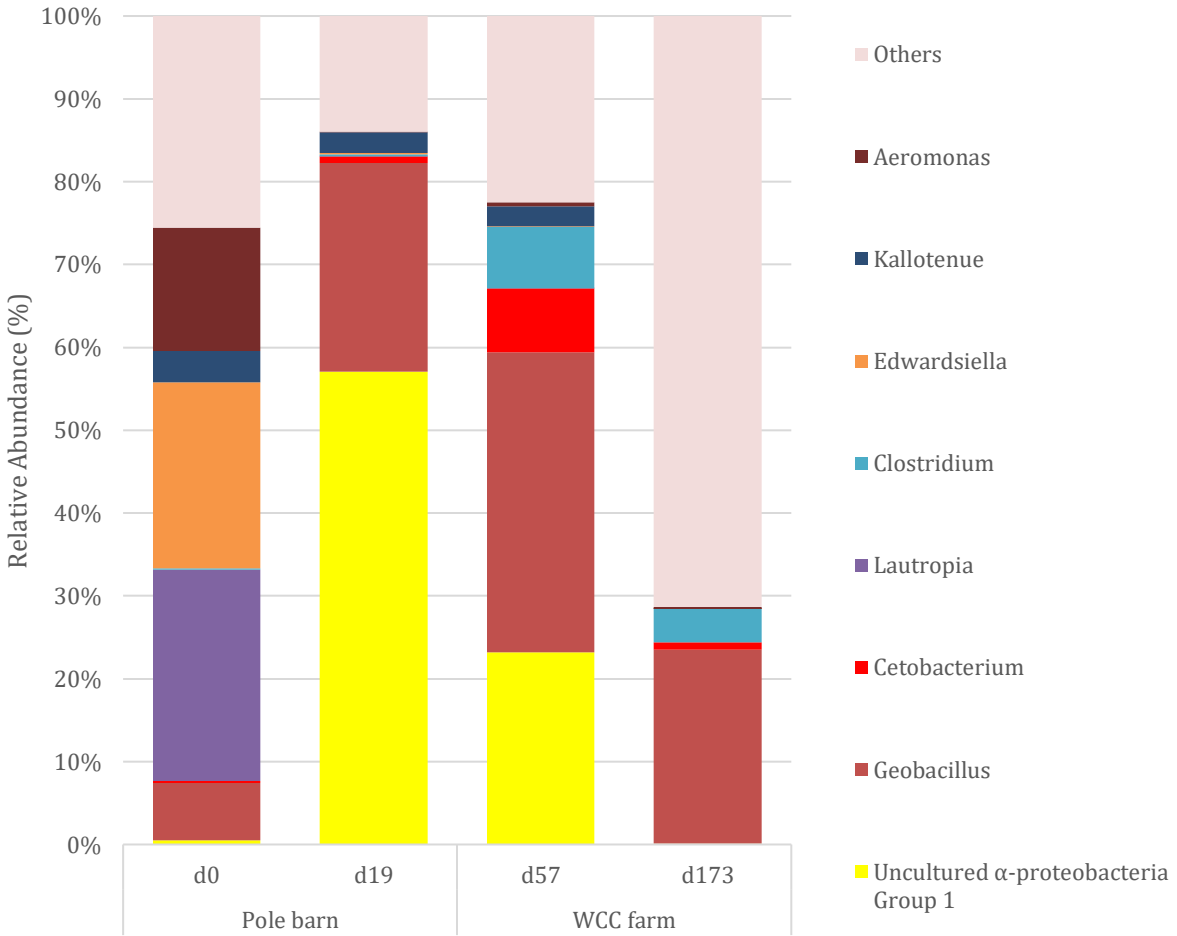

Figure 6-9. Average of Bacterial diversity at genus level (bar graph) based on 16S rRNA gene sequencing from all skin samples. The bar graph shows the microbial community composition along the study, based in the microbial composition at specific period of time and location.

CHAPTER 7. SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

In this dissertation, the phylum Proteobacteria dominated the microbiome of all the samples analyzed, specifically the classes Gammaproteobacteria and Alphaproteobacteria. Interestingly, Alphaproteobacteria seemed to be associated with the pond environment at the E. W. Shell Fisheries Station at Auburn University, specifically at the Pole Barn or S6 site. I identified a large number of OTUs from fish housed at that pond that exhibited very low sequence similarity with any known bacterial species and likely constitute a new genus or even a new family. Their uniqueness deserves further investigation.

The gut microbiome of channel catfish was influenced by the age and physiological maturity of the fish. During early stages of development, the gut microbiome was highly dynamic and reflected, for the most part, the microbial communities present in the water. However, after the first feed (swim-up fry) there was a drastic shift in the communities and feed exerted a large influence on the gut communities from that point on. After fish reached the fingerling stage, their microbiomes become more stable and resilient to changes in feed. I hypothetize that the best window to manipulate the gut microbiome in catfish (using pro- or pre-biotics) starts at the first feeding and continues till approximately 90 dph.

A balanced and well-established gut microbiome can be easily disrupted by a strong factor such as a broad-spectrum antibiotic. The post-antibiotic microbial composition-structure is characterized by an unbalanced composition with an increase of abundance of opportunistic and

pathogen bacteria. Contrary to my hypothesis, results from the zebrafish study suggested that dysbiosis did not induced inflammation in the gut yet fish were more susceptible to colonization by a common opportunistic pathogen. Further studies are needed to decipher the mechanisms that allowed *Aeromonas hydrophila* to cause disease in a dysbiotic gut.

A surprising result from this dissertation was the effect of mechanical injuries on the gut microbiome. The brain-gut axis has been well characterized in mammals and has just started to be investigated in teleosts. Mechanical injuries in high-stocking densities are common in fish farm and are typically a concern because they create a port of entry for opportunistic pathogens. The alteration of the gut microbiome as consequence of a wound can have a synergistic effect to the injury itself and further weaken the host in favor of opportunistic pathogens.

Noteworthy, *Cetobacterium* seemed to play an important functional role as member of the core gut microbiome in both zebrafish and channel catfish and its prevalence is reduced in dysbiotic gut. Therefore, *Cetobacterium* should be considered as potential candidate for use as a probiotic in freshwater fishes.

Each chapter revealed a serie of conclusions that are described below:

Chapter 2.

- The establishment of the gut microbiome starts at early stages in channel catfish as fertilized eggs and gets established at fingerling stage.
- The gut microbiome composition and structure is influenced by the age of the fish; characterized with a high bacterial richness with a decrease in diversity as fish mature.

- At early stages in channel catfish, the gut microbiome reflects their environment but once fish eat, diet leads the bacterial gut establishment. Once the animal matures the host lead the colonization.
- Particular genera were observed at specific ontogenic stage, such as *Vibrio* and *Flavobacterium* at fertilized eggs, *Opitutus* at sac-fry, *Opitutus*, *Phyllobacterium*, *Shewanella* and *Halospirulina* in swim-up, *Halospirulina*, and Candidatus *arthromitus* in pre-fingerling, and *Cetobacterium*, *Clostridium* and *Turicibacter* in fingerling stage.

Chapter 3.

- Florfenicol induced long last dysbiosis in the gut microbiome of zebrafish.
- Shift in the gut microbiome were observed in control fish, however those animals didn't show beign affected by the opportunistic pathogen like *Aeromonas hydrophila*.
- The relative abundance of bacterial sequences ascribed as *Mycoplasma* in the treated zebrafish were reduced to scarce level, suggesting that FFC had an effect on them and could be tested as chemotherapeutic alternative to treat *Mycoplasma* infections in tropical aquarium animals.
- *Cetobacterium* showed been susceptible to the Florfenicol but also to *Aeromonas hydrophila*.
- Dysbiosis observed not produce inflammation as was not overexpression of genes CCL20, IL1β, IL-8, TNF-α.

Chapter 4

- The gut microbial communities in fish treated with Florfenicol medicated feed didn't show significant differences compared with control.
- FFC medicated feed change the gut microbial evenness with the increased of *Cetobacterium* and *Pseudomonas*.
- The presence of Alphaproteobacteria (genus Uncultured- alphaproteobacteria Group 1) was higher in gut microbiome, and the bacterial sequences seems represent a new strain belongs to this class.

Chapter 5

- Mechanical injuries exerted a strong influence in the gut microbiome and could be related with GBA response.
- The presence of Gammaproteobcteria was higher in gut microbiome and Fusobacteria decreased.
- *Cetobacterium*, *Uncultured- alphaproteobacteria Group 1*, *Geobacillus* contributed with the most dissimilarities between treatments.

Chapter 6.

- The pilot multivalent vaccine (*Aeromonas hydrophila*, *Flavobacterium columnaris*, *Edwarsiella ictaluris*) didn't exert an effect in the microbial composition of the gut, gill and skin tissue studied under field conditions.

- The temporal seasonal shift influenced the microbiome changes in the tissues studied in the channel catfish, with a bacterial-tissue selection supported by the environmental condition in which the animals were kept during the study.
- The host plays an important role in the establishment of the new bacteria.
- The bacterial richness in the microbiome is benefit in a most natural environment.

In conclusion, there is an important relationship between a balanced microbial community and fish health. In a healthy animal with an established microbial composition, bacterial shifts can be observed as a response to common practices in aquaculture. However, not all the bacterial shift can turn into a pathobiome stage. External factors have to be strong or frequent enough to: a) unbalance the microbial composition and b) disrupt the host's symbiosis.