Studies on the Epidemiology, Vaccination, Susceptibility, and Treatment of Columnaris Disease in Fishes

by

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Abstract

In this dissertation, I explored the intraspecies genetic heterogeneity within *Flavobacterium columnare* isolates collected during particularly severe columnaris outbreaks observed at the E.W. Shell Fisheries Center, Aquatic Experiment Research Station at North Auburn, AL, USA. These outbreaks affected both farmed and sport fish species that were raised in ponds located under the same hydrologic unit from spring 2010 throughout summer 2012. All the recovered isolates were ascribed to genomovar II of *F. columnare* following restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene. Fingerprinting using amplified fragment length polymorphism (AFLP) revealed genetic diversity among the genomovar II isolates and persistence of certain clones throughout the watershed. Antimicrobial susceptibility testing of the isolates demonstrated resistance to some of the commonly used antimicrobial agents in aquaculture.

I also compared the vaccine efficacy of newly generated stable and safe rifampicin resistant genomovar II mutants to that of the attenuated mutant strain in the commercial vaccine (AQUAVAC-COL^{TM}, Merk), which is derived from a genomovar I (the less virulent genomovar) *F. columnare* strain. Based on relative percent survival rates in channel catfish, zebrafish, and Nile tilapia, both genomovar I and II mutants were equally protective against columnaris disease when a genomovar I strain of *F. columnare* was used to challenge the vaccinated fish. However, administration of the new genomovar II attenuated mutant as vaccine conferred greater

protection against columnaris disease caused by a genomovar II strain than that provided by the genomovar I mutant.

Because healthy microbiome on the skin and gills of fish was reported to benefit the hosts and prevent infections by opportunistic pathogens by hindering their invasion and/or stimulating the fish's immune system, I investigated the effect of disrupting the surface microbiome of channel catfish by short term exposure to one of the commonly used surface-acting disinfectants in aquaculture: potassium permanganate (PP) ($KMnO_4$). I tested the susceptibility of fish treated with PP to columnaris disease. Dysbiosis of the surface microbiome was analyzed by ribosomal intergenic spacer analysis (RISA) and pyrosequencing. Chemical treatment with PP altered the composition of the external microbiome and increased catfish mortality following experimental challenge with *F. columnare*.

Finally, I tested the *in vitro* and *in vivo* efficacy of *Nigella sativa*, a medicinal plant commonly used in folk medicine by many nations to treat a wide range of diseases, against columnaris disease. I found that *N. sativa* oil possess a potent antibacterial activity against all genomovars of *F. columnare* by disc diffusion method. Moreover, supplementation of fish diets with *N. sativa* seeds or oil were protective to zebrafish and channel catfish against columnaris disease in controlled laboratory challenges.

In summary, data obtained during my dissertation emphasized the importance of genomovar II isolates as causative agents of high mortality-columnaris disease in aquaculture and recreationally valued fish species. I tested two new promising methodologies to prevent and control columnaris disease and proved that caution should be used when treating external diseases in fish to avoid damaging the external microbiome.

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List of Abbreviations

RFLP	Restriction fragment length polymorphism
AFLP	Amplified fragment length polymorphism
PP	Potassium permanganate
PPD	Potassium permanganate demand
RISA	Ribosomal intergenic spacer analysis
ACE	Abundance-based coverage estimation
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
CFU	Colony forming unit
MDS	Multidimensional scaling
OTU	Operational taxonomic unit
SIMPER	Similarity percentages
UPGMA	Unweighted pair group method with arithmetic mean
EWSFC	E. W. Shell Fisheries Center
AML	Aquatic Microbiology Laboratory
OD	Optical density
RPS	Relative percent survival

CHAPTER 1. INTRODUCTION

Introduction

Flavobacterium columnare, the causative agent of columnaris disease, is a Gram negative bacterium, ubiquitous in most, if not all, aquatic environments of temperate and tropical latitudes and classically behaves as a secondary pathogen causing mortalities only when fish are stressed [1]. Although, some *F. columnare* strains can be a primary pathogen causing significant mortalities under normal culture conditions without the presence of predisposing stressors [2, 3]. *F. columnare* is a phenotypically homogeneous but genetically diverse species, therefore, the use of standard morphological and biochemical characterization are not useful for identification of *F. columnare* to the strain level. Based on DNA:DNA hybridization studies, three distinct genetic groups or genomovars (genomovars I, II and III) were described within the species [4]. Several other molecular studies have established the intraspecific genetic variability among *F. columnare* isolates by analyzing the 16S rRNA gene and 16S-23S rDNA intergenic spacer regions [4-8]. Different genetic groups of *F. columnare* have different virulence potential to fish [3, 9]. Although, all three genomovars had been reported from diseased fishes in the USA, several virulence and pathogenicity studies demonstrated that genomovar II are more virulent than genomovar I strains in channel catfish, *Ictalurus punctatus* [3, 9], in zebrafish, *Danio rerio* [10] and in rainbow trout, *Oncorhynchus mykiss* [11].

Annually, outbreaks of columnaris disease continues to increase in prevalence and severity in aquaculture to the extent that seriously threaten the fish farming industry in the USA and worldwide [11-13]. However, information regarding the epidemiology of columnaris disease

is still scarce [3]. Recently, several epizootics of columnaris occurred in a number of ponds at the E. W. Shell Fisheries Center at Auburn Experimental Station, Auburn, AL, USA. These outbreaks affected not only catfishes, but also other aquaculture and sport fish species [13]. Columnaris disease affecting recreationally valuable species has been observed by fisheries biologists across the Southeast but without published data, these observations are merely anecdotal.

Safe and efficient preventatives and curatives for columnaris disease are not yet available [14]. Eradication of columnaris from aquaculture settings is unlikely due to the cosmopolitan distribution of *F. columnare* in freshwater environments [15]. Preventive approaches such as vaccination are considered best measure to reduce the incidence of columnaris disease, although treatment options exist. Effective vaccines are ultimately the safest prophylactic approach to evade infectious diseases [16]. A modified live vaccine is currently available for commercial use to prevent columnaris disease in channel catfish under the licensed name AQUAVAC-COL™ (Intervet/Schering-Plough Animal Health, Boxmeer, Netherlands). The active ingredient in this vaccine is an avirulent rifampicin-resistant mutant of *F. columnare* genomovar I [5, 17]. Recently, our group has developed safe and permanently stable rifampicin-resistant mutants from genomovar II strains, the highly virulent group [18]. The genetic origin of the vaccine could affect the protective efficacy in fish. Since genomovar II strains are more virulent towards catfishes, I hypothesized that a genomovar II-based vaccine will increase the protective effect of vaccination against columnaris disease.

Columnaris disease appears in several forms depending on the host susceptibility, environmental conditions, and virulence of the strain [19]. Columnaris courses both as external and/or systemic infection [20, 21]. However, most cases of columnaris disease are restricted to

the external surfaces of fish [2, 22, 23] and skin and gills are believed to be the point of entry and the predilection sites of infection [23, 24]. Therefore, most conventional treatments proposed for columnaris disease involves surface-acting disinfection [25] and systemic antibiotic (to treat systemic columnaris) in the form of medicated feeds [26, 27]. Both chemicals and antibiotics have multiple drawbacks. Skin and gills of fish are normally colonized by diverse microbial communities forming the external microbiome [28-34]. A healthy intact microbiome is fundamental for maintaining health and excluding potential invading pathogens [35-37]. Previous studies have shown that survival and infectivity of *F. columnare* decline in presence of naturally occurring competitive bacteria species on the fish skin and gills [38-40]. Intensive production practices used in fish farms encompasses application of some harsh chemicals to control or treat specific pathogens. The effect of these treatments on the fish external microbiome is for the most part unknown.

The repetitive aggressive use of antibiotics in aquaculture has led to emergence of antibiotic resistance in many fish pathogens including *F. psychrophilum* [41] and *F. columnare* [42]. Moreover, aquaculture chemicals present a food safety hazard due to bioaccumulation, dangerous to farm workers who apply them to fish ponds, and can cause environmental pollution [43, 44]. Therefore, new efficacious and environment friendly alternatives are desperately required for sustainable treatment and prevention of diseases affecting aquaculture fishes. Recently, plant natural products are becoming a primary focus of research for discovery of novel pharmacologically active compounds [45].

Objectives

Thus, the overarching goal of my dissertation research was to study the epidemiology of columnaris disease in our latitude and to implement effective disease management strategies to help control columnaris outbreaks. To achieve that goal, I proposed the following objectives:

1. Investigate the intraspecific genetic diversity among *F. columnare* isolates collected during columnaris outbreaks
2. Test the antimicrobial susceptibility of the recovered *F. columnare* isolates to select the antimicrobial agent of choice for treatment.
3. Evaluate the vaccination efficacy of the new genomovar II rifampicin-resistant mutants and compare it to that of the genomovar I mutant of the commercial vaccine in commercially important fish species susceptible to columnaris disease.
4. Characterize and compare the composition of channel catfish external microbiome before and after exposure to chemical treatment (disinfectant) and challenge with *F. columnare* and its effect on susceptibility to columnaris disease.
5. Examine the antibacterial activity of *N. sativa* against *F. columnare* and the protective potential of dietary supplementation with *N. sativa* seeds or oil against experimental columnaris disease.

Dissertation Organization

This dissertation is organized into seven chapters. Chapter 1 includes a brief introduction, presents the research objectives, and clarifies dissertation organization. Chapter 2 provides a literature review of the current knowledge on columnaris disease and *F. columnare*. Chapter 3 gives the details of the frequencies and patterns of distribution of *F. columnare* strains isolated from sport and aquaculture fishes within the same drainage basin during recurrent

columnaris outbreaks. Chapter 4 discusses the efficacy of new rifampicin-resistant mutants of genomovar II *F. columnare* as vaccines against columnaris disease. Chapter 5 provides the results of disrupting the surface microbiome of channel catfish after pretreatment with PP and proves increased susceptibility to columnaris disease. Chapters 6 offers the efficacy results of using *N. sativa* seeds and oil extract against columnaris disease in zebrafish and channel catfish. Finally, Chapter 7 provides a summary of the research, presents conclusions, and also includes final recommendations.

References

[1] Durborow R., Thune R., Hawke J., Camus A., Columnaris disease: a bacterial infection caused by *Flavobacterium columnare*. Southern Regional Aquaculture Center, Publication No. 479. Stoneville, Mississippi, US Department of Agriculture, Stoneville, Mississippi. (1998).

[2] Plumb J.A., Health maintenance and principal microbial diseases of cultured fishes, Iowa State University Press, Ames, IA, 1999.

[3] Soto E., Mauel M., Karsi A., Lawrence M., Genetic and virulence characterization of *Flavobacterium columnare* from channel catfish (*Ictalurus punctatus*), Journal of Applied Microbiology. (2008) 104:1302-1310.

[4] Triyanto A., Wakabayashi H., Genotypic diversity of strains of *Flavobacterium columnare* from diseased fishes, Fish Pathology. (1999) 34:65–71.

[5] Arias C.R., Welker T.L., Shoemaker C.A., Abernathy J.W., Klesius P.H., Genetic fingerprinting of *Flavobacterium columnare* isolates from cultured fish, Journal of Applied Microbiology. (2004) 97:421-428.

[6] Thomas-Jinu S., Goodwin A., Morphological and genetic characteristics of *Flavobacterium columnare* isolates: correlations with virulence in fish, Journal of Fish Diseases. (2004) 27:29-35.

[7] Darwish A.M., Ismaiel A.A., Genetic diversity of *Flavobacterium columnare* examined by restriction fragment length polymorphism and sequencing of the 16S ribosomal RNA gene and the 16S–23S rDNA spacer, Molecular and Cellular Probes. (2005) 19:267-274.

[8] Olivares-Fuster O., Shoemaker C.A., Klesius P.H., Arias C.R., Molecular typing of isolates of the fish pathogen, *Flavobacterium columnare*, by single-strand conformation polymorphism analysis, Fems Microbiology Letters. (2007) 269:63-69.

[9] Shoemaker C.A., Olivares-Fuster O., Arias C.R., Klesius P.H., *Flavobacterium columnare* genomovar influences mortality in channel catfish (*Ictalurus punctatus*), Veterinary Microbiology. (2008) 127:353-359.

[10] Olivares-Fuster O., Bullard S.A., McElwain A., Llosa M.J., Arias C.R., Adhesion dynamics of *Flavobacterium columnare* to channel catfish *Ictalurus punctatus* and zebrafish *Danio rerio* after immersion challenge, Diseases of Aquatic Organisms. (2011) 96:221-227.

[11] LaFrentz B., LaPatra S., Shoemaker C., Klesius P., Reproducible challenge model to investigate the virulence of *Flavobacterium columnare* genomovars in rainbow trout *Oncorhynchus mykiss*, Diseases of Aquatic Organisms. (2012) 101:115-122.

[12] Suomalainen L.-R., *Flavobacterium columnare* in Finnish fish farming: characterisation and putative disease management strategies, PhD dissertation, University of Jyväskylä, 2005.

[13] Mohammed H., Arias C., Epidemiology of columnaris disease affecting fishes within the same watershed, Diseases of Aquatic Organisms. (2014) 109:201-211.

[14] Beck B., Barnett L., Farmer B., Peatman E., Carter D., Kaolinitic clay protects against *Flavobacterium columnare* infection in channel catfish *Ictalurus punctatus* (Rafinesque), Journal of Fish Diseases. (2014).

[15] Plumb J.A., Health maintainence and principal microbial diseases of cultured fish, Iowa State University Press, Ames, IA, 1999.

[16] Gudding R., Lillehaug A., Evensen O., Recent developments in fish vaccinology, Veterinary Immunology and Immunopathology. (1999) 72:203-212.

[17] Shoemaker C.A., Klesius P.H., Evans J.J., Modified live *Flavobacterium columnare* against columnaris disease in fish, Google Patents, 2005.

[18] Olivares-Fuster O., Arias C.R., Development and characterization of rifampicin-resistant mutants from high virulent strains of *Flavobacterium columnare*, Journal of Fish Diseases. (2011) 34:385-394.

[19] Amend D., Columnaris (*Flexibacter columnaris*) disease of freshwater fishes and a brief review of other flexibacterial diseases of fish, Antigens of fish pathogens. (1982):139-161.

[20] Pacha R., Ordal E., Myxobacterial diseases of salmonids, A symposium on diseases of fishes and shellfishes. The American Fisheries Society Special Publication 5, 1970.

[21] Becker C.D., Fujihara M., The bacterial pathogen *Flexibacter columnaris* and its epizootiology among Columbia River fish: A review and synthesis, Monograph-American Fisheries Society (USA). (1978) 2.

[22] Bernardet J.F., Immunization with bacterial antigens: *Flavobacterium* and *Flexibacter* infections, Dev Biol Stand. (1997) 90:179-188.

[23] Tripathi N.K., Latimer K.S., Gregory C.R., Ritchie B.W., Wooley R.E., Walker R.L., Development and evaluation of an experimental model of cutaneous columnaris disease in koi *Cyprinus carpio*, Journal of veterinary diagnostic investigation. (2005) 17:45-54.

[24] Esteban M.Á., An overview of the immunological defenses in fish skin, ISRN Immunology. (2012) 2012.

[25] Wakabayashi H., Effect of environmental conditions on the infectivity of *Flexibacter columnaris* to fish, Journal of Fish Diseases. (1991) 14:279-290.

[26] Thomas-Jinu S., Goodwin A., Acute columnaris infection in channel catfish, *Ictalurus punctatus* (Rafinesque): efficacy of practical treatments for warmwater aquaculture ponds, Journal of Fish Diseases. (2004) 27:23-28.

[27] Noga E.J., Fish disease: diagnosis and treatment, Ames: Iowa State University Press, 1996.

[28] Bullock A.M., Roberts R.J., The dermatology of marine teleost fish. I. The normal integument, Oceanogr. Mar. Biol. Annu. Rev. (1974) 13:383-411.

[29] Horsley R.W., A review of the bacterial flora of teleosts and elasmobranchs, including methods for its analysis, Journal of fish biology. (1977) 10:529-553.

[30] Hawkes J.W., The structure of fish skin, Cell and Tissue Research. (1974) 149:159-172.

[31] Austin B., The bacterial microflora of fish, revised, The Scientific World Journal. (2006) 6:931-945.

[32] Xu Z., Parra D., Gómez D., Salinas I., Zhang Y.-A., von Gersdorff Jørgensen L., Heinecke R.D., Buchmann K., LaPatra S., Sunyer J.O., Teleost skin, an ancient mucosal surface that elicits gut-like immune responses, Proceedings of the National Academy of Sciences. (2013) 110:13097-13102.

[33] Larsen A., Tao Z., Bullard S.A., Arias C.R., Diversity of the skin microbiota of fishes: evidence for host species specificity, FEMS microbiology ecology. (2013) 85:483-494.

[34] Boutin S., Sauvage C., Bernatchez L., Audet C., Derome N., Inter individual variations of the fish skin microbiota: host genetics basis of mutualism?, PloS one. (2014) 9:e102649.

[35] Balcázar J.L., Decamp O., Vendrell D., De Blas I., Ruiz-Zarzuela I., Health and nutritional properties of probiotics in fish and shellfish, Microbial ecology in health and disease. (2006) 18:65-70.

[36] Verschuere L., Rombaut G., Sorgeloos P., Verstraete W., Probiotic bacteria as biological control agents in aquaculture, Microbiology and Molecular Biology Reviews. (2000) 64:655-671.

[37] Dillon R., Charnley K., Mutualism between the desert locust *Schistocerca gregaria* and its gut microbiota, Research in Microbiology. (2002) 153:503-509.

[38] Chowdhury M.B., Wakabayashi H., A study on the mechanism of the bacterial competitive effects on *Flexibacter columnaris* infection: Effects of the time-lag between the exposures of fish to *F. columnaris* and its competitor, Fish Pathology. (1989) 24:105-110.

[39] Suomalainen L.R., Tiirola M.A., Valtonen E.T., Effect of Pseudomonas sp MT5 baths on Flavobacterium columnare infection of rainbow trout and on microbial diversity on fish skin and gills, Diseases of Aquatic Organisms. (2005) 63:61-68.

[40] Wakabayashi H., Egusa S., Preliminary experiments on environmental factors influencing the prevalence of columnaris disease, Fish Pathol. (1972) 7:58-63.

[41] Ekman E., Natural and experimental infections with *Flavobacterium psychrophilum* in salmonid fish, Doctoral thesis, Swedish University of Agricultural Sciences, Uppsala., 2003.

[42] Declercq A., Boyen F., den Broeck W., Bossier P., Karsi A., Haesebrouck F., Decostere A., Antimicrobial susceptibility pattern of *Flavobacterium columnare* isolates collected worldwide from 17 fish species, Journal of Fish Diseases. (2013) 36:45-55.

[43] Boyd C.E., Massaut L., Risks associated with the use of chemicals in pond aquaculture, Aquacultural Engineering. (1999) 20:113-132.

[44] Subasinghe R., Barg U., Phillips M., Bartley D., Tacon A., Aquatic animal health management: investment opportunities within developing countries, Journal of applied ichthyology. (1998) 14:123-129.

[45] Reverter M., Bontemps N., Lecchini D., Banaigs B., Sasal P., Use of plant extracts in fish aquaculture as an alternative to chemotherapy: current status and future perspectives, Aquaculture. (2014) 433:50-61.

CHAPTER 2. LITERATURE REVIEW

The Disease

Columnaris disease, caused by the fish pathogen *Flavobacterium columnare*, is one of the oldest known diseases affecting warm-water fishes, and was first described by Herbert Spencer Davis in 1922. It has been recognized as a globally-distributed acute to chronic bacterial infection of diverse freshwater fishes including most of the commercially important wild-caught, aquaculture-reared, and ornamental species [1-4]. Mainly, columnaris is an epithelial disease [5], however, it can occur as external and/or systemic infections that can greatly diminish the profitability of aquaculture operations by causing large-scale mortalities and tremendous economic losses every year [6-8]. Columnaris may occur as a primary or secondary infection and clinically may take the per-acute, acute, or chronic forms [9].

There appear to be little or no species resistance to columnaris disease. Anderson and Conroy (1969) listed 36 species of fish from which columnaris disease has been described worldwide. Columnaris disease has been reported to cause mortality in several cultured species including, but not limited to, the channel catfish (*Ictalurus punctatus* Rafinesque) and other catfishes; common carp (*Cyprinus carpio* Linnaeus) and other carps; rainbow trout (*Oncorhynchus mykiss* Walbaum) and other salmonids; eels (*Anguilla rostrata, A. japonica, A. anguilla*); and tilapia (*Oreochromis* spp.). The disease also assails important recreational species

such as the largemouth bass (*Micropterus salmoides*) and other basses, bluegill *(Lepomis macrochirus*) and other sunfishes; yellow perch (*Perca flavescens* Mitchill); crappies (*Pomoxis nigromaculatus* Lesueur, *P. annularis* Rafinesque); and numerous aquarium species as goldfish (*Carassius auratus* Linnaeus); platies (*Xiphophorus maculatus* Gunther); black mollies (*Poecilia latipinna* Lesueur, *P. sphenops*) or zebra fish (*Danio rerio* Hamilton) [1, 2, 10-16]. Ictalurids are often the most susceptible, however, all freshwater fishes are susceptible to columnaris under environmental conditions favorable to the bacterium and stressful to the fish [1, 17-20].

Historically, columnaris disease was considered the second leading cause of mortality in pond raised catfish in the southeastern United States next to enteric septicemia of catfish (ESC) caused by the bacterium *Edwardsiella ictaluri* [21, 22]. However, recent annual case reports from the Aquatic Research & Diagnostic Laboratory, Thad Cochran National Warmwater Aquaculture Center, Stoneville, MS, USA have indicated that columnaris disease is the most frequently diagnosed bacterial disease in commercially raised catfish in the USA [23]. Columnaris disease or mixed infections including columnaris are listed as the greatest cause of economic losses in catfish farms as reported by 70% of farmers from the four leading catfish producing states [24, 25]. Columnaris disease can occur as the primary disease in pond or tank raised channel catfish, with mortalities as high as 50% [1], however, the role of stressors is considered a key factor in outbreaks of the disease. The disease can be economically shattering for the cultured catfish industry with in-pond mortality rates among adults and fingerlings reaching up to 60 and 90%, respectively [1, 26]. In the US catfish industry, mortality ranges from 10 to 100% depending on the water temperature [27] and yearly losses are estimated at 30 million dollars [28]. Mortality rates as high as 34% have been documented in salmonids [29].

The Pathogen

Phenotypic description

Flavobacterium columnare is a long, slender, non-flagellated Gram-negative rod shaped bacterium, measuring 0.3 to 0.7 µm wide x 3 to 10 µm in length with some cells may reach up to 50 µm in length [30]. *F. columnare* grows on low nutrient media and cells exhibit flexing and gliding motility on solid surfaces. Colonies on agar plates are flat, yellow pigmented, tightly adherent, and rhizoid (spread across the surface forming irregular margins) with a convoluted center [31]. The optimum growth temperature is 20-25 ºC, however, *F. columnare* was reported to grow between 4 and 37 ºC [32]. The bacterium is non-halophilic and grows only under strict aerobic conditions [33, 34]. *F. columnare* is cytochrome oxidase and catalase positive; does not produce acid from carbohydrates; reduces nitrate to nitrite; produces hydrogen sulfide; does not hydrolyze cellulose, chitin, starch, esculin, and agar; hydrolyzes gelatin, casein, and tyrosine; does not decarboxylate arginine, lysine, or ornithine; and produces distinctive flexirubin type pigments [35, 36]. Reichenbach and Dworkin (1981) used potassium hydroxide method (20% solution) to demonstrate production of flexirubin pigments.

Some *F. columnare* isolates were reported to degrade hemoglobin, fibrinogen, and elastin as well [37]. *F. columnare* secretes Chondrotin AC lyase, an enzyme that degrades polysaccharides (chondroitin sulfates A and C), particularly those found in cartilaginous connective tissues and was suggested to contribute to the ability of the pathogen to establish and maintain infections in fish [38-41]. The bacterium is capsulated and the thickness of the capsule seems to be correlated with the degree of virulence. Transmission electron microscopy revealed a thick 120-130 nm capsule in high virulence strains, while low virulence strains showed a thinner

80-90 nm capsule [42]. The absorption of congo red dye into *F. columnare* colonies indicates production of extracellular galactosamine glycan [43, 44].

Table 2-1. Morphological and biochemical characteristics of F. columnare.

Characteristic	**Description**
Growth condition	Strictly aerobic
Colony color	Yellow pigmented
Colony morphology	Flat, rhizoid, and tightly adherent
Gram-staining	Gram-negative
Cell Morphology	Long, slender rods
Cell size (µm)	0.3-0.5 X 3-10
Motility	Gliding
Flexirubin pigment	+
Congo red absorption	+
H_2S production	+
Resistant to neomycin sulfate, polymixin B	+
Chondroitin lyases	+
Glucose as carbon source	-
Acid from carbohydrates	-
Gelatin degradation	+
Casein degradation	-
Catalase activity	+
Cytochrome oxidase activity	+
Tyrosine degradation	-
Starch degradation	-
Degradation of crystalline cellulose	-
Degradation of complex acidic polysaccharides of connective tissue	+
G + C content (mol%)	32-37

Griffin (1992) developed a simple, time-saving procedure consists of five characteristics that differentiates *F. columnare* from other morphologically similar yellow pigmented Gram negative aquatic bacteria [45]. These characteristics are (1) the ability to grow in the presence of neomycin sulfate and polymyxin B; (2) colonial morphology on agar plates typically has a rhizoid pattern and yellow pigmentation; (3) production of a diffusible, gelatin-degrading enzyme; (4) binding of aqueous congo red dye to the surface of the suspect colony; and (5)

production of a diffusible chondroitin sulfate A degrading enzyme. The bacteria form characteristic haystack-like columnar aggregates in cytological wet mounts of infected tissue [46]. The most important morphological and biochemical characteristics of *F. columnare* according to Plumb (1999) are summarized in (Table 2-1).

Taxonomy and nomenclature

Flavobacterium columnare is a member of the family *Flavobacteriaceae* [47-49] and was first observed (but unsuccessfully cultivated) by Davis in a major fish kill among warm-water fishes from the Mississippi River, USA as large numbers of slender, motile bacteria present in wet mounts of affected tissues arranged in columnar aggregates [46]. Hence, it was initially named *Bacillus columnaris* and the disease recognized as columnaris disease. The taxonomic classification and the nomenclature of the bacterium have been confusing and changed several times over the decades. In 1944, Ordal and Rucker were able to isolate the bacterium for the first time from a natural outbreak of columnaris disease among sockeye salmon (*Oncorhynchus nerka*) using diluted culture medium [50]. They identified columnaris bacterium as a *myxobacterium* based on cellular morphology and production of both fruiting bodies and microcysts, thus they named it *Chondrococcus columnaris* [50]. Garnjobst reported that neither fruiting bodies nor microcysts appear to be formed when a pathogenic bacterium resembling *Chondrococcus columnaris* morphologically was isolated from infected bullheads (*Ameiurus nebulosus*) and therefore excluded the bacterium from the Myxobacterales and allocated it under the family Cytophagaceae as *Cytophaga columnaris* [51]. Afterwards, the organism was placed in the genus *Flexibacter*, and assigned the name *Flexibacter columnaris* [31, 35]. In 1996, the bacterium received its final and current name, *Flavobacterium columnare*, based on protein and fatty acid profiles and DNA-rRNA hybridization studies [49].

Strain variability

Flavobacterium columnare shows wide intraspecies genetic and phenotypic variability as well as differences in virulence. Variation between isolates of *F. columnare* cultured from different hosts or different geographical regions have been demonstrated in several earlier studies. Serological studies by Anacker and Ordal (1959) divided the species into four main serotypes plus one miscellaneous group [52]. Pyle and Shotts (1980) were able to differentiate between *F. columnare* isolates from warmwater and coldwater based on results of biochemical tests [53]. However, Shamsudin and Plumb (1996) reported that four isolates of *F. columnare* recovered from four different fish hosts exhibited identical biochemical characteristics, but differed in their capacity to grow at 15 ºC on media with 0.5% NaCl or at a pH 6-10 [54]. Several authors have reported variations within *F. columnare* isolates based on their ability to grow at 15 and 37°C [55] and nitrate reduction [54]. *F. columnare* isolates recovered from tropical fish were reported to have temperature growth ranges different from those of temperate species [15].

Formation of different colony morphologies is also common among different *F. columnare* strains under laboratory conditions and changes in colony morphologies during subcultures of *F. columnare* strains has been detected previously [56-58]. Four different colony morphologies were formed among Finnish *F. columnare* strains on Shieh agar. Colony type 1 forms flat rhizoid colonies with yellow centers. Colony type 2 is hard, convex, dark orange in colour, non-rhizoid or only slightly rhizoid, and has irregular edges. Colony type 3 has round edges and a smooth, yellowish appearance. Colony type 4 is white or light yellow and smooth with an irregular shape [57].

F. columnare produces extracellular proteases with average molecular weights of 47, 40, 34, and 32 kD (one isolate from a diseased catfish in Louisiana, had a protease with average molecular weight of 44 kD rather than the 47 kD) [37]. Isolates of *F. columnare* have been separated into two groups based on the apparent molecular mass of the extracellular proteases produced with one group at 58 and 53.5 kD and a second group at 59.5, 48, and 44.5 kD [59]. Research comparing lipopolysacharide and protein profiles from different strains of *F. columnare* demonstrated a correlation with virulence among isolates exhibiting different profiles [60].

Moreover, there are significant differences in the virulence between different strains of *F. columnare* [16, 31]. Variation in the degree of virulence has been reported between isolates of *F. columnare* isolated from different species of fishes [15, 32, 61]. *F. columnare* strains can be divided into high virulence or low virulence depending on the site of infection, appearance of clinical signs on infected fish before death, mortality rate in the exposed fish, time to death after exposure to infection, and the temperature at which the bacterium produces disease. An association was found between high virulence stains of *F. columnare* and the ability to adhere to the gills tissue [16, 62, 63]. Wood (1974) described strains of high and low virulence; highly virulent forms attack gill tissue and the latter strains are primarily responsible for cutaneous infections [64]. Highly virulent strains of *F. columnare* have been reported to produce death without macroscopic evidence of tissue damage [65], and in young fish there is often negligible pathology before death [2]. On the other hand, low virulence strains produced necrotic lesions on the gills and/or body surface [66]. Although both categories (high and low virulence) of *F. columnare* are present in the aquatic environment and routinely isolated from diseased fish, the more virulent strains produce higher mortality rate (92-100% mortality) than that produced by

less virulent strains (0-46% mortality) in immersion challenge studies [67]. High virulence strains of *F. columnare* cause death within 24 to 48 hours post exposure while several days elapse before mortality results from infection by low virulence strains [34, 66]. Becker and Fujihara (1978) reported some highly virulent *F. columnare* strains causing mortality even at lower temperatures [8].

Several studies have shown that different *F. columnare* strains have different virulence potential to channel catfish, black mollies, common carp or golden shiners (*Notemigonus crysoleucas*) by intramuscular injection or immersion exposure [16, 17, 31, 66, 67]. Significant variation in the degree of virulence has also been reported between isolates of *F. columnare* recovered from salmonids [32, 66]. Although Thomas-Jinu and Goodwin (2004) suggested that virulence is a strain-specific characteristic rather than related to cell morphology or genetic group, differences in virulence were also correlated to the genomovar of the strain, with genomovar II being more virulent than genomovar I [67, 68].

Molecular techniques and genetic heterogeneity

Flavobacterium columnare is phenotypically and physiologically homogenous but genetically diverse group of organisms and, therefore, the use of standard biochemical tests or chemotaxonomic markers are not useful for characterization at the strain level. The term "genomovar" has been introduced to indicate phenotypically similar but genetically distinct groups of bacterial isolates within a species [69, 70]. The development of molecular techniques such as polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), single-strand conformation polymorphism (SSCP), end-label sequencing, DNA-DNA hybridization, random amplified polymorphic DNA (RAPD), amplified fragment length

polymorphism (AFLP), and pulsed filed gel electrophoresis (PFGE) have provided researchers with valuable tools to explore the genetic variability of *F. columnare*.

Molecular analyses of the *16S rDNA* gene or the intergenic spacer region (ISR) are the most frequently used techniques to study the genetic diversity of *F. columnare* [7, 17, 71-74]. DNA relatedness studies between *F. columnare* strains revealed homologies as low as 78% [35]. Toyama et al. (1996) showed an existing intra-species variation among strains of *F. columnare* based on 16S rDNA sequences [75]. Triyanto and Wakabayashi (1999) developed a 16S rRNA gene-based RFLP assay for genetic typing of *F. columnare* isolates that can be used to divide phenotypically identical strains of *F. columnare* into three distinct "genomovars" (genomovars I, II and III) and were also confirmed by DNA:DNA hybridization (DNA relatedness between strains belonging to the same genomovar was higher (ranging from 83 to 100% homology) than between strains from other genomovars (homologies lower than 69%)). Further research utilizing the same RFLP assay suggested the existence of subgroups within genomovars I and II, which were classified as genomovar I-B and genomovar II-B [76]. Afterwards, HaeIII-RFLP analysis of the 16S rDNA gene was frequently used as a marker for genomovar ascription in *F. columnare*. All three genomovars have been reported from fish in Asia and the USA, while only genomovar I has been isolated from an outbreak in Europe [15].

Interpretation of the restriction patterns was problematic due to the lack of a formal description of the expected number and sizes of the DNA fragments generated from each genomovar. Recently, LaFrentz et al. (2013) refined the protocol for typing isolates of *F. columnare* by RFLP of the 16S rRNA gene, provided a formal description of the expected restriction patterns for the previously described genomovars I, II, II-B and III [76], and described a new genomovar I/II [77]. The intraspecific genetic diversity between and among these groups

has been further explored using higher resolution fingerprinting methods and sequencing. Amplification of the more variable ISR of the bacteria has also allowed strain differentiation, and application of the SSCP technique has revealed a higher level of polymorphisms into these regions of the different *F. columnare* isolates [72, 76]. RAPD analysis was used to study the intraspecific heterogeneity among *F. columnare* isolates from warm water fish species [61], and AFLP, a more powerful and reproducible technique, revealed the polyclonal nature of this species as isolates from distant geographical locations showed surprisingly similar AFLP profiles [72]. PFGE was also used for genetic and virulence characterization of *F. columnare* isolates from channel catfish [78]. Tekedar et al. (2012) recently published the complete genome sequence of *F. columnare* strain ATCC 49512 [79].

Several pathogenicity studies using *F. columnare* isolates belonging to different genetic groups have proved genomovar II is the most virulent to channel catfish [67], rainbow trout [68], and other recreational fish species [18]. Moreover, a host-specific association between *F. columnare* genomovars and fish species in natural settings has been demonstrated, with genomovar II being mainly associated with channel catfish [17]. Genomovar II isolates of *F. columnare* showed a greater chemotactic response towards channel catfish skin mucus than that of genomovar I isolates [80]. Olivares-Fuster et al. (2011) demonstrated how cells of *F. columnare*, from both genomovars, adhere to channel catfish gills in high numbers within 1 h post-challenge. However, genomovar II cells persisted at higher levels and for a longer duration than cells of genomovar I [63]. All the above mentioned studies have given researches a better understanding of the intraspecies genetic diversity of *F. columnare* isolates cultured from a wide range of hosts from various geographical regions in an attempt to better understand the epidemiology of columnaris disease.

Epidemiology

Host range and susceptibility

Flavobacterium columnare has a broad host range, and attacks nearly all species of freshwater fishes and some amphibians [8, 81]. No wild or cultured freshwater fish are totally resistant to columnaris [5]. Most wild, cultured, and hatchery-reared species are highly susceptible under intensive culture conditions [15, 42, 47, 63, 82, 83]. The disease seems to be a problem among many freshwater tropical aquarium fishes, probably because of the high temperature of aquaria (25-30 ºC), which is favorable for many *F. columnare* strains [10, 21]. Catfish at any age, during all seasons, and under variety of water conditions are susceptible to *F. columnare* infection [84]. A similar filamentous organism, *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*), infects some marine fish causing tenacibaculosis (or flexibacteriosis) [85].

Predisposing factors

Typically, columnaris disease is not spontaneous and often thought of as an opportunistic infection with stress on the host being a prerequisite for infection. Although *F. columnare* can cause disease under normal culture conditions, healthy unstressed fish are generally not susceptible to columnaris disease [21]. Several environmental factors play an important role in the development of the disease, and water temperature is one of the main determinants. Columnaris disease has a pronounced seasonal occurrence, with both natural and hatchery epizootics are concentrated during the warm summer months [20, 86-88]. Most species of fish are susceptible to columnaris when water temperatures are in the preferred temperature range in spring, summer, and fall. Typically, outbreaks occur when the water temperature is in the range

of 18-22 ºC [89]. Durborow et al. (1998) found that columnaris disease commonly occurs in channel catfish when water temperatures are in the range of 25 to 32 ºC. Columnaris epidemics may occur in water temperatures below 25 ºC; even as low as 15 ºC, but mortalities and acuteness of disease are significantly less than in warmer temperatures [90]. Experiments by Holt et al. (1975) revealed that temperatures in excess of 12.2 ºC were required to induce mortality in fish infected with *F. columnare*. Pacha and Ordal (1970) suggested that temperatures below 12 ºC are not conductive to columnaris outbreaks, even with highly virulent strains. Moreover, *F. columnare* have higher adhesion capacity to gill epithelium at higher temperatures [62].

In addition to water temperature, other physical or environmental risk factors have been suggested to increase the susceptibility of fish to *F. columnare* infection. Factors related to water quality such as high mineral content (hardness), static water, low salinity, low dissolved oxygen, high ammonia, high nitrite, and organic pollution were shown to increase the severity of columnaris outbreaks [14, 20, 91, 92]. Chen et al. (1982) described the highest eel mortality levels to be associated with stagnant water, whereas the lowest losses occurred in running water. Interestingly, the total losses fell between these two extremes with aeration; the mortality rate was inversely correlated with the level of dissolved oxygen in the water. Moreover, even in presence of adequate dissolved oxygen, ammonia levels were directly correlated with the increased mortalities. Additional stress factors such as skin abrasions, netting injuries, rough handling, high rearing densities, feed deprivation, and transportation were shown to exacerbate the incidence of columnaris disease [5, 19, 86, 93, 94]. Columnaris occurs frequently in fish raised intensively in cages and in closed recirculating systems owing to overcrowding and cage abrasions [21]. Mechanically abraded fish are more vulnerable to infection by *F. columnare* [95],

and starving catfish for 7 days was reported to reduce their innate resistance to *F. columnare* [19].

Stress from parasites grazing on the skin has also been shown to increase the susceptibility of fish to *F. columnare* infection. The mortality amongst rainbow trout concomitantly challenged with *Argulus coregoni* and *F. columnare* was significantly higher and earlier in onset of disease when compared with fish challenged with *F. columnare* alone [96]. The interruption of the protective epithelial layer appears to create a portal of entry for *F. columnare*, and loss of the mucus or slime layer present on the epidermis, which normally serves as a barrier to bacterial infection [97], may predispose the fish for attachment of the bacteria. Adverse physiological stress also was suggested to increase susceptibility of fish to columnaris disease [4].

While stressful conditions can contribute to columnaris infections, the presence of columnaris may predispose to secondary infections by other pathogens as *Aeromonas* sp. or *Saprolegnia* sp. [5, 31, 90]. Winter kill (saprolegniasis) often is preceded by columnaris. In one case study, 80 percent of catfish ponds diagnosed with winter saprolegniasis had a columnaris infection in the preceding summer or fall [21].

Source of infection and mode of transmission

Flavobacterium columnare is ubiquitous in freshwater and probably occur in most, if not all, aquaculture environments [21]. The bacterium can be part of the normal bacterial community of freshwater fish, eggs and the fish rearing waters [98]. The natural reservoir for *F. columnare* is unknown and several studies have shown the ability of this pathogen to survive in water without nutrients for extended periods of time. Survival was shown to be determined by physical

and chemical properties of the water. In hard alkaline water with a high organic load, *F. columnare* was able to survive for up to 16 days at 25°C; while soft acidic water with low organic content did not provide a favorable conditions [99]. Chowdhury and Wakabayashi demonstrated the importance of calcium, magnesium, sodium and potassium ions for long-term survival of *F. columnare* in water. *F. columnare* persisted in water for up to 32 days when the hardness was 50 ppm or more, but a hardness of 10 ppm reduced the viability considerably [100].

Survival of *F. columnare* in static, sterile river water was found to be temperature-dependent, with a higher percent survival at lower temperatures [101]. When starved, *F. columnare* cells undergo specific morphological and ultrastructural alterations (coiled conformation) that make them able to tolerate such adverse conditions. Although long-term starvation appears to decrease cell fitness and resulted in loss of virulence, these coiled cell forms remained culturable over time and those changes were reversed upon encountering nutrients [102]. *F. columnare* kept in in lake water under laboratory conditions remain infective for at least five months [103]. Sterile river mud was also shown to contain adequate nutrients to maintain viability of *F. columnare* longer than river water [31]. Environmental waters were suggested as the actual source of *F. columnare* strains in fish farms and that stressful farm settings may select for virulent strains [103-106].

Arias et al. (2006) conducted a culture-independent study of the bacterial community in catfish ponds in Alabama and detected two main catfish pathogens, *F. columnare* and *Edwardsiella tarda*, in pond water in the absence of infective episodes (*F. columnare* represented up to 8% of the total clones sequenced). No correlation could be made between the

presence of the pathogens and the composition of the bacterial community or outbreak occurrences in those ponds [107], thus supporting the notion of their facultative pathogenicity.

Although *F. columnare* can survive in the aquatic environment and mud, fish seem to serve as the primary reservoir [5, 31, 108]. Infected fish with gill or cutaneous lesions serve as a source of infection within a population. During a columnaris outbreak, the bacterium invades the skin of the head region, mouth, lips, cheeks, fins, and gills. Afterwards, infected fish starts shedding many organisms into the surrounding environment, thus creating a self-perpetuating infection in the affected population [10]. It was suggested that fish may live over winter in a clinically healthy status (as a carrier) harboring the bacterium from a previous columnaris outbreak and act as a source of infection for other fish during the following summer months [8, 34, 109, 110]. Rainbow trout surviving a *F. columnare* infection can shed up to 5×10^3 colony forming units/mL/h of viable bacteria into the surrounding water and gills were shown to be the major site of release [109]. Dead fish were shown to be able to spread the disease at a higher rates compared to live fish [111]. In hatcheries supplied with open water, any species of infected fish may serve as a reservoir of infection for the disease [112]. Pacha and Ordal (1970) demonstrated that feral fish, such as catostomids, coregonids, and cyprinids may serve as reservoirs of infection. Research has shown that fish to fish contact was not needed for horizontal transmission of columnaris disease [108]. Transmission of *F. columnare* can be direct through contact with infected fish or indirect via the environment or by cohabitation with carrier fish which shed the bacterium into the water [6, 113].

Incubation period

The period between exposure to *F. columnare* and the appearance of visible clinical signs of disease varies, depending on the virulence of the bacterial strain, susceptibility of the fish

species, and the ambient water temperature. Strains of high virulence may induce acute columnaris disease characterized by an incubation period of less than 24 hours and the resulting mortalities peak two to three days post exposure [114], whereas less virulent forms may require 48 hours to several weeks. Holt et al. (1975) have shown experimentally the existing correlation between clinical disease and high water temperatures. Their studies also revealed that host species differ in the time from exposure to death.

Epizootiology

Epizootics of columnaris disease frequently occur in wild fish populations and have been devastating in fish farms, hatcheries, ponds, pens and cages particularly in fishes held under intensive culture conditions with poor water quality [112]. Morbidity under overcrowded poorly managed situations may reach 100% and mortality under the same conditions approaches 70%. In the wild, fish morbidity may vary from 1% to 30%, depending upon the stressful environmental parameters and the species at risk [4]. Although columnaris disease could occur throughout the whole year, most epizootics of columnaris occur between May and October in the northern hemisphere as warm-weather favors *F. columnare* infection [46].

Host specific association was suggested between wild fish species in the Mobile River (Alabama, USA) and specific *F. columnare* genomovars that may also contribute to the severity of the epizooty [17]. In this work, coexistence of *F. columnare* genomovars I and II in the natural environment was proved, as well as a significant association of the strains belonging to genomovar I with threadfin shad and the ones from genomovar II to catfishes, both channel and blue. However, in a recent epidemiological study, Mohammed and Arias (2014) found that genomovar II of *F. columnare* is the most predominant genomovar during columnaris outbreaks

affecting different fish species in farm and sport fishing ponds located in Auburn, Alabama, USA.

Another important factor to fully understand the epizootiology of this disease is the fact that columnaris is often diagnosed in a mixed infection associated with one or more other fish pathogens. Davis (1922) observed that besides *F. columnare*, large quantities of other bacteria were present in columnaris lesions. Hawke and Thune (1992) showed that out of 53 *F. columnare* infections, 46 involved *E. ictaluri* and/or *Aeromonas* spp.

Finally, because of the external nature of columnaris disease, *F. columnare* is known to encounter bacterial competition with other bacteria species in the environment or on the fish body and bacterial competition is considered one of the factors determining the degree of *F. columnare* infection [115]. The presence of competitive bacteria diminishes the ability of *F. columnare* to invade fish and, therefore, suppress the incidence of columnaris disease in fish farms [20, 116, 117]. Many competitive bacteria were reported to reduce the possibility of flavobacterial pathogens invasion to fish tissues in earlier studies [20, 118]. Moreover, survival and infectivity of *F. columnare* declined in presence of *Aeromonas hydrophila* or *Citrobacter freundii* [117]. In a competition study, *F. columnare* failed to invade fish in the presence of *C. freundii* at an initial number approximately 100 times that of *F. columnare* in water [117]. In a typical mixed columnaris infection, *F. columnare* can be masked by numerous saprophytic species, or its growth can be completely inhibited by antagonistic bacteria such as *Pseudomonas* spp. [119]. *Pseudomonas* sp. MSB1 was found to efficiently inhibit the growth of *F. columnare* and *F. psychrophilum* on solid growth media [120]. Moreover, *F. columnare* cells possess specific receptors for bacteriocins produced by some competitive bacterial strains and, consequently, *F. columnare* is sensitive to those strains [121]. However, Boutin et al. 2012

suggested that competitive exclusion is the mechanism involved in the antagonism of skin bacteria on growth and survival of *F. columnare* due to the absence of antimicrobials in the skin mucus of brook charr (*Salvelinus fontinalis*). On the other hand, different strains of *F. columnare* release specific, non-transmissible bactericidal substances, equivalent to colicins of *Escherichia coli* into the environment to reduce competition from other bacterial strains [121].

Virulence Factors and Pathogenesis

Different virulence factors have been described for *F. columnare*, but their virulence mechanisms are poorly defined and more research is needed to achieve a whole understanding of how this pathogenic organism elicits disease. The lack of robust methods for the genetic manipulation of *F. columnare* represents a substantial obstacle to understanding its virulence mechanisms [122, 123] , despite the recent reports on the establishment of genetic manipulation system for this important fish pathogen [124]. It has been suggested that plasmids, adhesion capabilities and enzyme activities could be related to virulence in flavobacterial fish pathogens [41, 125, 126]. The existence of plasmids in *F. columnare* isolates has not been investigated in depth; therefore, the possibility of plasmid-mediated virulence remain largely unidentified [127]. Li et al. (2012) identified the type I restriction-modification system (R-M system) in *F. columnare* to improve electroporation efficiency and suggested examination of the composition and diversity of R-M systems in strains of *F. columnare* in order to set up a suitable molecular manipulation system for the bacterium [122].

Although various studies have attempted to elucidate the pathogenesis of columnaris disease, the information currently available is scarce, making adoption of effective preventive measures to combat the infection difficult. Generally, pathogenicity of *F. columnare* can be

divided into two phases. First phase: in which the affinity and adhesion to fish play a major role in the virulence of the strain. In the second phase, adhered bacterial cells start to divide and produce lyases and proteases, which degrade fish connective tissue establishing visible signs of disease [128]. *F. columnare* was found to have preference for the skin and gill of channel catfish as entry sites and the mucus from the skin and gills of catfish promoted chemotaxis of *F. columnare* by using traditional capillary tube method [80]. At least three carbohydrate-binding receptors associated with the capsule of *F. columnare* (D-mannose, D-glucose and *N*-acetyl-D-glucosamine) might play a role in this chemotactic responses [129].Although the role of chemotaxis in the virulence of *F. columnare* is not well-defined, the authors suggested a correlation between chemotactic response and virulence of the strain.

Attachement is a necessary prerequisite for successful colonization of the fish tissue and bacteria seldom rely upon one single mechanism of adhesion, but both specific and nonspecific mechanisms are often involved [130]. In contrast to numerous other bacterial fish pathogens, experimental challenges with *F. columnare* are more effective by contact exposure (i.e., immersion) than by injection rout [34, 42]. As early as 1967, Pate and Ordal noted a capsular outer glycocalyx of mucopolysaccharide associated with *F. columnare* and correlates with adhesion [131]. A positive correlation was established between virulence of *F. columnare* and its ability to adhere to cells. The highly virulent *F. columnare* strains were found in close association with the gill tissue. This was not the case for the low virulence strains suggesting that adhesion of *F. columnare* to the gill tissue constitutes an important step in the pathogenesis of columnaris disease [42].

Olivares-Fuster et al. compared adhesion of *F. columnare* genomovar I and II strains to the gill tissue of channel catfish and zebrafish after immersion challenge. At 0.5 h post-

challenge, both genomovars adhered to the gill of channel catfish at comparable levels, but at later time points, genomovar II was able to persist longer than genomovar one [63]. Loss of adhesion was shown to severely reduce the virulence of *F. columnare* when Bader et al. adopted an adhesion defective mutant of *F. columnare* (developed by serial passage on ampicillin medium) in immersion challenges and found that the adhesion defective mutant did not cause disease after immersion exposure (the mortality was reduced by 75% and occurred 24 h later compared to the strains that still possessed the adhesion capacities), but produce columnaris infection after injection into fish [58]. Although injection is not a natural way to produce columnaris infection because all natural defense mechanisms are bypassed, this indicates that virulence factors other than adhesion are also important [7].

Additionally, the gliding motility of *F. columnare* [15, 47] may have a role in adhesion. Pate and Ordal described a spanning fibrillar structures present in the gap between the outer membrane and the mucopeptide layer of the *F. columnare* bacterial cells that might be responsible for the gliding motility [131]. Significant upregulation of the gliding motility gene *gld*H was found in *F. columnare* as soon as five minutes post-exposure to catfish mucus. However, no upregulation of gliding motility genes was observed when pretreated with D-mannose [129]. Decostere et al. found that following treatment of *F. columnare* cells with sodium metaperiodate or incubating them with D-glucose, *N*-acetyl-D-glucosamine, D-galactose and D-sucrose, the adhesion capabilities of the highly virulent strain to the gill were significantly reduced [42]. Therefore, they speculated that a lectin-like carbohydrate substance incorporated in the capsule might be partially responsible for lectin-mediated attachment to the gill tissue.

The first transcriptomic profiling of host responses to columnaris disease following an immersion challenge was carried out by using illumina-based RNA-sequencing expression

profiling [132]. A rhamnose-binding lectin (RBL) was the most upregulated gene observed in a differentially expressed set in the gill of fish infected with *F. columnare* in comparison to naive fish, with expression increasing 105-folds by four hours following infection. This upregulation dramatically decreased at the later verified timepoints (24 h and 48 h), suggesting the importance of this gene during early infection events [132].

In two distinct catfish families with differential susceptibilities to columnaris disease (one family was found to be completely resistant while the other was susceptible [133]), acute and robust upregulation in catfish RBL was observed in the susceptible family. Moreover, when catfish exposed to different doses of the putative RBL ligands L-rhamnose and D-galactose, these sugars were found to protect the fish against columnaris disease, likely through competition with *F. columnare* binding of host RBL. Additionally, RBL expression was found to be upregulated (>120-fold) in fish fasted for 7 d when compared to fish fed to satiation daily (expression levels returned to those of satiated fish within 4 h after re-feeding) [133]. Interestingly, Immunohistochemical staining with antisera against an RBL in rainbow trout revealed the presence of these RBLs in mucous cells of the gill and in various cells related to innate immunity [134]. Similarly, *F. columnare* aggregates were observed within and surrounding mucus pores of the skin and capping tissue of the gill filaments from common carp and catfish [62, 63], suggesting distinct, mucus-dependent areas for pathogen attachment.

Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria and plays crucial role in the structural integrity and protection of the bacteria from the host immune defenses [60]. LPS has been well-known as a virulence factor in many other bacterial species [135-137]. Earlier studies have shown that LPS along with other capsular polysaccharides play important role in columnaris pathogenesis (Bader et al. 2005; Zhang et al.

2006); however, no studies on *F. columnare* LPS composition or immunogenic role are yet available. Kunttu et al. determined LPS-profiles of different colony morphotypes of *F. columnare*. Colony morphology variants of the same strain produced a similar single LPS band. However, there were size differences between different strains [138]. The same authors also hypothesized that colony morphology of different *F. columnare* morphotypes affects the adhesion capacity of *F. columnare* to polystyrene in an *in vitro* model and suggested that rhizoid colony morphology is needed for virulence [138, 139].

Biofilm formation is considered an essential feature in the pathogenicity of *F. columnare*. Biofilm formation capacity was demonstrated *in vitro* for *F. columnare* when exposed to salmon surface mucus [140]. Cai et al. studied the dynamics of biofilm formation under static and flow conditions (in microfluidic chambers) by light microscopy, confocal laser scanning microscopy, and scanning electron microscopy. *F. columnare* cells were able to attach to and colonize inert surfaces producing biofilm. They also evaluated the effects of several physicochemical parameters including temperature, pH, salinity, hardness, and carbohydrates on biofilm formation by *F. columnare*. Salinity and hardness were the main factors modulating biofilm formation. The authors confirmed the virulence potential of biofilm by cutaneous inoculation of channel catfish fingerlings with mature biofilm [30].

As for enzymatic activities, pathogenic bacteria, when attaching onto host surfaces, release proteases to break down proteins of the host's extracellular matrix [21, 141], thus causing necrotic lesions [142]. The extensive necrosis associated with *F. columnare* infections suggests production of strong tissue-destroying enzymes that degrade proteins and connective tissue, and potentially contribute to its ability to cause disease. Newton et al. (1997) partially characterized 23 proteases produced by isolates of *F. columnare* derived from channel catfish raised in the

southeastern USA [59]. *In vitro* proteolytic activity of *F. columnare* suggests the importance of the extracellular proteases in the virulence of the bacterium [37, 38] as they have been found to contribute to direct tissue damage and/or invasiveness.

Chondroitin AC lyase, an enzyme produced by *F. columnare* [45], degrades polysaccharides, particularly those found in animal cartilaginous or connective tissue [39, 143]. Chondroitin sulphate (ChS) is a mucopolysaccahride found in animal connective tissues, and the production of ChS-degrading enzymes is associated with pathogenicity in bacteria [144]. Not all isolates of *F. columnare* exhibit the same level of chondroitin AC lyase activity. Chondroitin AC lyase activity was related to strain virulence, being significantly higher in the high virulence strains than in the low virulence strains at 25 ºC [40]. The enzymatic activity of the chondroitin AC lyase showed temperature dependence [39], with significantly higher activity at high temperatures than at lower temperatures [40, 41, 74].

There are at least four ways in which high temperature increases the severity of columnaris outbreaks. First, the growth rate of *F. columnare* increase by 30% when temperature rises from 17 to 25 ºC. Second, the adhesion capacity of *F. columnare* increases at higher temperatures [62]. Third, the chondroitinase activity is higher at 25 ºC than at 20 ºC. The fourth possible reason is related to the physiological response of the fish to warmer water temperature as higher water temperatures create stress, and therefore may render fish more susceptible to the infection [86].

Bacteria not only need to enter the host tissue, but also need to eliminate competitive bacteria. A pathogen like *F. columnare* with a poor ability to compete with other bacteria species can cause diseases and dominate the bacterial community for many reasons. First, *F. columnare* has a strong affinity to fish tissues. Second, bacterial competition on fish skin may differ from

that under culture conditions *in vitro* and *F. columnare* may benefit from some interactions [20, 116]. Finally, *F. columnare* is known to produce bactericidal substances, which can enhance its dominance over other bacteria in infected fish [121].

Host Defense

In contrast to other fish diseases, little is known about host immune responses to columnaris. The mucosal surfaces of fish such as the skin and gill are important sites of bacterial exposure and provide the first line of host defense against invading pathogens. When a healthy fish encounters *F. columnare*, surface mucus is the first physical-immunological barrier encountered by the bacterium during the initial steps of colonization and invasion of the fish [63]. Antibacterial properties of the fish mucus against *F. columnare* have been previously demonstrated and abrading the skin and/or removal of the mucus layer were successfully used in experimental challenges to increase fish susceptibility to columnaris disease [95, 145]. In a laboratory experiment, lower number of colonies was counted on incubated agar plates to which mucus was added after inoculation with *F. columnare*. Moreover, fluorescent microscopy of a stain-based bacterial viability assay also revealed a higher number of dead *F. columnare* cultured with mucus [145]. Fish mucus contains a variety of antimicrobial compounds such as antibacterial peptides, lysozyme, proteases, immunoglobulins, and antibodies that may protect underlying epidermal cells from bacterial colonization [146-148]. Conversely, salmon surface mucus was shown to promote the growth, induce biofilm formation and increase extracellular protease activity of a highly virulent *F. columnare* strain when cultured in media supplemented with juvenile Atlantic salmon (*Salmo salar* L.) skin mucus compared to the same media without

mucus [140]. These findings suggest differences between fish species in skin mucus response to *F. columnare* infection.

The ability of *F. columnare* to evade parts of the hot immune system was described in some studies. Ourth and Bachinski reported that the alternative complement pathway (ACP) of catfish was inhibited by sialic acid contained by many Gram-negative bacterial pathogens, including *F. columnare*. They suggested the importance of sialic acid for the pathogenicity of *F. columnare*, as they found very little or no bactericidal activity was produced against the bacterium by the catfish ACP, while removal of sialic acid with neuraminidase resulted in greater ACP activity [149]. Another distinguishing feature in *F. columnare* infections is the absence of an inflammatory response upon examining affected tissues microscopically. It was postulated that *F. columnare* triggers the endogenous programmed cell death machinery of immune cells to evade the immune system. Apoptosis of phagocytic cells has already proven to be a very powerful pathogenic strategy [150]. Sun et al. showed that one of the apoptotic factors G3BP1 (Rasputin), that plays a potential role in negative regulation of apoptosis, was highly down-regulated greater than 30 fold at all examined timepoints following *F. columnare* infection [132]. This could clearly explain the lack of inflammatory reaction in early infections with *F. columnare* [151]. Transferrin was also found to influence the virulence of *F. columnare*. When iron was injected prior to experimentally challenging fish with *F. columnare* by intraperitoneal injection with iron-free human transferrin, the survival time of fish was reduced [152].

Clinical Signs

Columnaris disease appears in several forms depending on the host, environment, and virulence of the bacterial strain [32]. It occurs both as external or systemic infections, however,

most cases of columnaris disease are restricted to the gills, the integumentary system (including fins), and the oropharynx [22, 153, 154]. Based on the site of infection and appearance of infected tissues, the disease has been commonly known as saddleback, fin rot, cigar mouth, or cotton wool disease [84]. The progression of the disease in fish is fast; fish may die within few hours of the first visible sign, in some acute cases fish may die before any of the signs are observed [145, 151]. Highly virulent strains of *F. columnare* were reported to produce death without macroscopic evidence of tissue damage [65].

The infection with *F. columnare* begins at fins, gills and the mouth as primary sites, but injuries elsewhere on the body may provide a primary infection site. Typically, skin lesions produced by columnaris initially are very shallow and may appear as an area that has lost its natural shiny appearance. The disease may be noted as an increase in thickening of the mucus on the head, opercula, fins and around injuries, which continues to become thicker until definite areas of skin involvement appears as circular areas of grayish opalescent growth having a brown to yellowish-brown tint. Fish with columnaris disease usually have sores in the area between the head and dorsal fin, with a mucus-like coating [155, 156]. Fins usually have necrotic lesions on the outer edges which progress down the rays of the fins [157]. In advanced cases, widespread erythemic spots appear, which may result in extensive necrosis, bacteraemia and ultimately death [4].

The most typical and characteristic lesion produced by columnaris is a pale white band along the dorsal midline, later extend to the dorsal fin and laterally down both sides of the abdomen/flanks encircling the body, often referred to as "saddleback" lesion [45] that may cover as much as 25% of the total surface area of the fish body. Acute saddle back lesions are often confined to the head and back, such lesions are white or yellow surrounded by a ring of inflamed

skin with a reddish zone of hyperaemia around the periphery and contain bacterial cells and necrotic tissue covering haemorrhagic ulcers [4]. As the infection progresses, an open ulcer, round or oval in shape, is frequently found in the center of the saddle [155]. Erosion and necrosis of the skin tissue around the dorsal fin may progress to expose the underlying muscle tissue or may sometimes reach the spine [64, 84]. Tripathi et al. (2005) suggested that the extensive damage of the skin leads to osmotic and electrolyte imbalances and eventually to death of the infected fish.

Ulceration of the oral mucosa also occurs; resulting in mouthrot. It is not unusual to find the mouth and inner walls of the oral cavity covered with a yellowish brown, mucus-like growth of *F. columnare*, the name “mouth fungus” is commonly used to describe this condition [47]. The yellowish coloration is due to massive presence of yellow pigmented bacteria and the brownish coloration is usually due to mud and detritus particles trapped in the slime layer [155]. The bacteria multiply rapidly in infected tissues and spread quickly to the surrounding areas, mandible and maxilla [93]. Mouth lesions are sometimes more lethal than are the cutaneous lesions, since the painful oropharynx render the fish anorexic, resulting in death from starvation [158]. Secondary infections with fungi or other bacteria may deteriorate the situation and can be seen together with the filamentous *F. columnare*. As the disease progresses, *F. columnare* invades the dermis and destroys fish’s connective tissues which is the most damaging stage of the infection [156].

Another common clinical sign of columnaris disease is the pronounced erosion and necrosis of the gills which is often a major site of damage [46]. The bacteria attach to the gill surface, grow in spreading patches, and eventually cover the entire gill filaments. Scattered hemorrhaging areas may be found [65].Gill lesions in the form of whitish spots begin at the tips

of the gill filaments and necrosis progresses toward the gill arch. The normal structure of the primary and secondary lamellae in the affected gill may disappear due to sloughing of the fringes of gill filaments by the advancing necrosis induced by the bacterial degrading enzymes resulting in actual loss of the respiratory tissue [65, 155, 157].

Although *Flavobacterium spp*., are capable of causing internal infections, such infections are rarely seen with *F. columnare*, and superficial lesions are more commonly observed indicating that systemic entry into the host is not required for the disease to occur [95]. However, in some cases, columnaris infection becomes systemic with little or no visible pathological signs [2, 31]. Once the integument is compromised, the bacterium is able to enter the blood stream through the external lesions, causing septicaemia, although this has only been reported in a limited number of cases [1, 159]. In a study conducted in Mississippi, *F. columnare* was isolated internally from 40 percent of the cases where it was found externally on the fish [21]. *F. columnare* have been previously isolated from the internal organs of fish, but no distinct clinical signs were characteristic of these internal infections and internal lesions are often lacking [22, 160]. Only Hawke and Thune (1992) have reported swelling of the posterior kidney in some cases of columnaris disease.

Histopathologically, acute ulcerative dermatitis and epidermal spongiosis that may proceed to severe necrosis and sloughing of the epidermis are common [65, 158]. Epidermal damage breaks down the osmotic barrier enabling water infiltration into the exposed tissues resulting in severe dermal edema, a process commonly known as “waterlogging” [158]. Haystack-like columnar aggregates of bacteria can be seen in the dermis between the collagen fibers upon examining hematoxylin and eosin (H&E) or Giemsa stained sections from affected tissue [158]. Pathological alterations in the gill structure are associated with cardiac changes. In

the first day after columnaris infection, the observed hyperplastic gill lesions were accompanied by bradycardia. In the following days, degeneration of the lamellae results in a compensatory tachycardia. The interaction between the impaired gill blood circulation and the cardiac changes was suggested to cause death of the fish [161]. Proliferation of the epithelial cells of the gill filaments can be accompanied by proliferation of mucus glands and chloride cells [162]. The proliferating tissue can occlude the space between adjacent gill lamellae [161]. Congestion of the branchial blood vessels occurs, followed by edema which causes lifting of the surface epithelium of gill lamellae from the underlying capillary bed [2, 65, 161]. Moreover, clusters of *F. columnare* can be found on the cell surface and/or in between necrotic sites. Complete fusion and clubbing of gill filaments can finally result in circulatory failure and extensive internal hemorrhage [161].

By using scanning electron microscopy (SEM), Bullard et al. were the first to describe the ultrastructural features of saddleback lesions associated with experimental columnaris disease in channel catfish and zebrafish. Channel catfish skin lesion showed margins typified by epidermal sloughing and lesion centers that exhibited a multitude of rod-shaped bacterial cells, approximately 3-10 µm long × 0.3-0.5 µm wide, intermingled with cellular debris across a surface characterized by denuded, strongly ridged, or folded dermal connective tissue. Zebrafish skin lesion samples displayed a multitude of rod-shaped bacterial cells and exhibited similar ultrastructural changes but some scales were missing [163]. SEM of affected gill arches revealed the presence of rod-shaped bacterial cells, approximately 0.3-0.5 µm wide and 3-10 µm long adhering on the gill surface and appeared to be aggregated rather than evenly distributed across the gill epithelium [63].

Diagnosis

Presumptive diagnosis of columnaris disease is obtained by observing the aforementioned clinical signs, and the presence of non-flagellated, long, slender, possibly filamentous (approximately 10 to 20 times longer than wide), rod-shaped, Gram-negative bacteria exhibiting the characteristic “haystacks” formation and “flexing/gliding” motility of *F. columnare* in scrapings or wet mount preparations from infected tissues magnified microscopically 100 to 400 times [5, 10]. Several minutes are required for the characteristic “haystacks” to form in the wet mount slide. Organisms are best seen on fresh tissue from live fish and are most numerous at the expanding lesion margins.

Although, the morphological (colonial and cellular) features of *F. columnare* are unique, a definitive identification is essential. Definitive diagnosis of the disease can be achieved through isolation and culture of the bacterium from body surface lesions or from infected tissues, followed by identification based on biochemical, serological or molecular methods [4, 90]. However, cultivation is a time-consuming approach to detect flavobacterial pathogens, especially *F. columnare* [119]. A list of the biochemical tests that can be done to confirm positive diagnosis of *F. columnare* is given in Table 1 [1]. The most common biochemical tests used for identification of this bacterium are Gram staining; catalase and oxidade tests [54]; flexirubin pigment [36]; congo red adsorption [43]; Tween-20 hydrolysis [164]; or the tests described in MacFaddin (2000) for starch, gelatin, casein, tributyrin, tyrosine and lecithin hydrolysis [165]. Accurate biochemical identification of *F. columnare* may take 1-2 weeks to completion [49], so faster alternative methods have been developed.

Some serological techniques, such as slide agglutination test, indirect ELISA, and immunofluorescent test, for the detection of specific humoral responses against *F. columnare*,

are useful tools for rapid diagnosis and to determine natural exposure to the organism [166-168]. Gas chromatography was used for the analysis of the whole-cell fatty acid profiles of the bacterium [169]. Moreover, several molecular methods have been developed for the genetic identification of *F. columnare*, most of them based on PCR using species-specific primers to identify and distinguish *F. columnare* from other related and common water bacteria [72, 73, 75, 108, 119]. Histopathology can be used to provide information regarding the severity of the infection [151] and Immunohistochemical staining can provide information on the location of the pathogen within the affected tissues [170].

Isolation and Culture

The primary isolation of most Flavobacteria is problematic, and has hindered investigations of the pathogenesis of *Flavobacterium* species in many cases [4, 143, 171]. Isolation of *F. columnare* can be accomplished from gill or cutaneous lesions or from the kidneys of chronically infected fish on specialized media [52]. Although in some cases *F. columnare* can be isolated from internal organs; skin and gills remain the tissues of choice for isolation [108, 172]. The bacterium was also isolated from skin mucus samples collected from moribund or diseased aquarium fish including black mollies, platies, guppies (*Poecilia reticulata*), tetras (*Cheirodon axelrod*) [15].

Columnaris disease is an epidermal disease and when isolation is made from the fish surface, contamination can rarely be avoided. Moreover, the slow growth rate of *F. columnare* under lab conditions gives the opportunity to other spreading or opportunistic bacteria growing on the agar plate to overgrow *F. columnare*, preventing the formation of distinct isolated

colonies [99, 119]. Tiirola et al. (2002) reported that the isolation of *Flavobacteria* was unsuccessful from a number of fish samples (44%) that contained filamentous Gram negative bacteria in microscopic examination. Columnaris typically occurs as a mixed infection with numerous opportunistic bacteria; therefore, a specialized media is often needed for the primary isolation of *F. columnare* to inhibit the growth of the secondary bacteria [45]. In 46.5% of *F. columnare* cases submitted to the Louisiana Aquatic Diagnostic Laboratory in 1992, the bacterium was present in mixed infections with other pathogens such as: *Aeromonas* spp., *Edwardsiella ictaluri*, and *E. tarda* [22].

Flavobacterium columnare does not grow on standard bacteriological media such as “blood agar”, “brain-heart infusion” and “tryptic soy agar”, or on standard Mueller Hinton agar. It requires specialized media for isolation, culture and antibiotic sensitivity testing. *F. columnare* grow on low nutrient, low agar and high moisture content media [50]. The medium should be prepared for same day use to ensure the correct moisture level. Media formulated for isolation and culture of the bacteria include Ordal’s [65], Hsu-Shotts [31], a modification of Fijan’s media [99] by Hawke and Thune (1992), and Shieh media [173] or some of its proposed modifications [56, 169].

To select for *F. columnare* during primary isolation, researchers took the advantage of the ability of *F. columnare* to grow in the presence of neomycin and polymyxin B, whereas most other fish pathogens and aquatic bacteria are inhibited. The minimal inhibitory concentration (MIC) of *F. columnare* was determining to be 1000 units/ml and 100 μg/ml for polymixin B and neomycin, respectively [99]. The MIC corresponds to the lowest concentration of a drug in a dilution series that inhibits growth of a bacterial strain [174]. Hence, Fijan recommended addition of five μg/ml neomyocin and five units/ml polymixin B to cytophaga agar to make the

medium selective for *F. columnare* and selective against other inhibiting bacteria. Hawke and Thune (1992) reported the selective media of Fijan did not inhibit many of the bacteria in mixed infections from diseased channel catfish. They improved the formulation to contain five µg/ml neomycin and 200 units/ml polymixin B, and this medium was effective in inhibiting all of the other species of bacteria tested except *Flavobacterium sp*. and *Streptococcus sp*. [22]. Shieh medium supplemented with tobramycin has also been reported to be selective for primary isolation of *F. columnare* from diseased fish [175].

Several days after the primary isolation of *F. columnare* from an infected fish, yellow pigmented, rhizoid and tightly adherent colonies start to appear. However, during several subcultivations in the laboratory, these colonial characteristics may change. Less rhizoid and atypical colonies are formed, eventually leading to a complete loss of the typical colony shape [55]. Pure cultures have to be subcultivated from primary isolates and it takes several days before new colonies can be seen (due to the slow growth rate of the bacterium) [128].

When working with *F. columnare*, culture maintenance is a problem as the viability of *F. columnare* typically does not extend beyond 48 hours on standard agar plates. By maintaining high moisture content (by adding one ml of sterile saline to a tube of slanted agar medium), cultures can survive past 30 days at which an agar plate culture normally loses viability [59]. The presence of salts in the media also increases the chance of survival of *F. columnare*, which is very sensitive to changes in osmotic pressure [56]. Moreover, *F. columnare* is a slow grower and cells often clump or auto-agglutinate in broth culture. This phenomenon results in difficulties with bacterial enumeration and in producing a uniform inoculum for various tests [114].

Farmer (2004) evaluated different culture media formulated for *F. columnare* and concluded that the low nutrient content of the selective Cytophaga agar (SCA) offered the best inhibition to other bacteria and improved the isolation of *F. columnare* in agar media. The best results for broth culture were obtained by *F. columnare* growing media (FCGM) which yielded faster growth, higher number of cells and seemed to prevent cell clumping. The best medium for maintenance of the cultures was tryptone yeast extract (TYA) agar with increased moisture. Other studies have shown the growth response of *F. columnare* to be better in Chase, Shieh, and Liewes media containing salts, with the best growth in Shieh medium at 24 hours [56].

Therapy

Although prevention is the best measure against columnaris disease, after epidemics begin, therapeutants are used excessively in aquaculture to reduce high mortalities associated with columnaris disease. Before determining the best treatment approach, economics must be considered. The cost of the treatment should be weighed against the value of the fish, and whether the number of fish dying (or likely to die) have a high enough value to justify the cost of the treatment [155]. The preferential ectopic pathogenesis of *F. columnare* makes it highly amenable to interventions with surface-acting compounds. However, ideal treatment of columnaris disease should include both external bath and medicated feed with antimicrobial agent to combat both cutaneous and systemic infections [89], as well as, treating the culture water with an approved chemical. Surface disinfection is effective only in early stages of the disease, when the infection is still superficial [31]. Bath treatments using chloramphenicol [176], nifurpirinol [89, 177], nifurprazine [178] and oxolinic acid [179] have been used successfully

before. However, in advanced or septicaemic stages of columnaris, administration of antimicrobials in the feed is necessary.

Three antibiotics (Romet, oxytetracycline and Aquaflor) are available for use as medicated feed in aquaculture. Thomas-Jinu and Goodwin recorded no mortalities when Romet (ormetoprim and sulfadimethoxine) and oxytetracycline where administered prior to bacterial challenge [180]. Oxytetracycline given orally for up to 10 days has been reported successful in early as well as advanced columnaris infections in Pacific salmon (*Salmo salar*) [64]. On the contrary, lack of success of orally administered oxytetracycline has also been reported in earlier studies [160]. Better results are usually obtained when the treatment starts soon after the disease onset, as sick fish tend to eat less or stop eating entirely. The withdrawal periods are 3 days in catfish or 42 days in salmonids for Romet and 21 for oxytetracycline in any food fish [181]. Nitrofuran can also be administered orally for 3 to 5 days [8, 31]. However, the use of these antibiotics is not approved for treatment of columnaris disease in catfish or other species and fall under the “extra label use”.

Gaunt et al. demonstrated the efficacy of florfenicol in the feed against columnaris disease in channel catfish [182]. Darwish et al. also showed the clear benefit of florfenicol against a mixed infection of *A. hydrophila* and *F. columnare* in Sunshine bass (*Morone chrysops* female ×*Morone saxatilis* male) [183]. The U.S. Food and Drug Administration (FDA) conditionally approved Aquaflor (florfenicol) as the first drug for treatment of columnaris disease in catfish [184]. Aquaflor is a Veterinary Feed Directive (VFD) drug, meaning that users must receive a signed VFD order from a licensed veterinarian before obtaining the drug through normal feed-distribution channels.

Because *F. columnare* primarily attacks the skin and gills, the most effective treatments for columnaris disease are surface-acting disinfectants [20]. Potassium permanganate (PP) is a commonly used therapy at a dose of 2 mg/l in ponds or at a higher concentration if the water's organic load is high. The amount of PP used to treat columnaris is based on the 15 minute demand test [185]. Darwish et al. suggested that copper sulfate has a clear therapeutic value against *F. columnare* infections in channel catfish [186]. Thomas-Jinu and Goodwin on the other hand reported inefficacy of copper sulfate against columnaris disease, which might be due to the advanced stage of the infection at the time of treatment [180]. Neither PP nor copper sulfate are approved by the FDA, and their effectiveness has been questioned [1, 155]. According to Plumb (1999), a combination of PP and oxytetracycline-medicated feed is the most effective way to treat columnaris outbreaks.

Another common treatment for columnaris is immersion in a salt bath, or increasing and maintaining the salt level at approximately 3 parts per thousand (ppt) [114]. Although *in vitro* salinity tolerance studies have shown growth inhibition of *F. columnare* at 10 ppt NaCl, but not at 5 ppt and growth was best at 3 ppt [55]. The adhesion of *F. columnare* was reduced at 3 ppt, which explains the lower mortality rates at higher salinities [14]. Suomalainen et al. have reported the use of high concentrations of salt and low pH as a treatment option since these conditions significantly reduced the numbers of viable *F. columnare* cells after *in vitro* exposures [110].

Other chemicals were also adopted for treatment of columnaris disease as well. Efficacy of hydrogen peroxide was studied and recommended to control the disease [187, 188]. Bath treatment by the herbicide Diquat was shown to significantly reduce channel catfish mortalities following challenge with *F. columnare* [180, 189] and has also proven to be effective treatment

of columnaris disease in salmonids [31]. Bath treatments of channel catfish with chloramine-T significantly decreased mortality following experimentally induced columnaris disease [180, 190]. Bowker et al. (2013) demonstrate the efficacy of both chloramine-T and hydrogen peroxide in controlling mortality associated with external columnaris in Florida Largemouth Bass *Micropterus salmoides floridanus* and Bluegill *Lepomis macrochirus* [191].

Nevertheless, the excessive use of antibiotics in aquaculture is problematic and has several drawbacks [28]. Some of these negative attributes are: (1) Antibiotics are costly and diseased fish usually loose appetite reducing the antibiotic intake, (2) Using antibiotic treatments necessitates withdrawal periods before the fish can be marketed as a food item which adds to the production cost, (3) The repetitive application of antibiotics is accompanied by the risk of emergence of antibiotic resistant strains which is a major public health concern (Antibiotic resistance has already been detected in *F. columnare* strains [18, 192]), (4) The discharge of antibiotic residues into the environment downstream from fish farms can possibly lead to emergence of drug-resistance in environmental bacterial communities due to the transfer of resistant traits between bacteria species [193-195], and (5) possible allergic reactions elicited in the consumers after food contact [194].

Furthermore, aquaculture chemicals can be bioaccumulative, present a food safety hazard, pose risks to fish farm personnel, and can cause environmental pollution [196]. Besides, some chemical treatments can induce dysbiosis to the fish's healthy microbiome, reducing the numbers of beneficial microorganisms competing with pathogens and increasing susceptibility to *F. columnare* infection (Mohammed and Arias, unpublished data). Consumer concerns over the presence of antibiotic residues in foods as well as the evolution of antibiotic resistant microbes

has sparked interest in development of ecologically safer and economic alternatives for treatment and prevention of columnaris disease.

Recently, Mohammed and Arias showed the potent inhibitory effect of *Nigella sativa* oil towards *F. columnare* isolates of different genomovars and the protective potential of dietary supplementation with *N. sativa* seeds or oil extract against columnaris disease (unpublished data). Phage treatment of *F. columnare* was described to be successful in combating columnaris disease too [197, 198]. Kaolinitic clay, an inert clay which has been principally used in medicine to adsorb pathogenic bacteria, was also found to protect against *F. columnare* infection in channel catfish [199]. Beck et al. (2014) showed that Kaolin can improve survival, reduce gill pathologies and reduce bacterial attachment to channel catfish tissues by binding to *F. columnare*.

Prevention and Control Measures

Up until now, no effective preventive measures against columnaris disease are available. Control of columnaris disease is a combination of good management practices, appropriate use of the available chemotherapeutics or antibiotics, and vaccination when feasible. Good management practices are crucial in any aquaculture operation and are the foundation of all disease prevention programs. Ideally, the incidence of columnaris could be reduced by alleviating stress on the cultured fish population. Aquaculture, however, involves stocking and feeding fish at rates that will ensure production efficiency and profitability. These necessary management practices create stressful conditions and increase the likelihood of illness in the farmed fish. Since aquaculture practices are inherently stressful and because columnaris is a

stress-triggered type of infection, efforts to minimize the impacts of stress through maintaining proper husbandry, water quality, feeding practices, and stocking densities must be made [1].

Eradication of columnaris disease from aquaculture settings is unrealistic since the bacterium is ubiquitous in freshwater environments [1]. However, avoidance of exposure to the disease is a primary method of prevention. This can be achieved by the use of disease-free water or by U. V. disinfection of water supplies when appropriate. Elimination of wild fish in an open water supply that may be carriers could be helpful. When water temperature manipulation is feasible, temperatures above 12.8 °C (55 °F) should be avoided since they favor development of the disease. If the fish must be handled or crowded, certain prophylactic treatments (summarized below) should be administered [112]. During high water temperatures, reduction of fish density could be used to prevent columnaris disease [86]. Conrad et al. reported that ozone treatment of water significantly reduced the numbers of *F. columnare*, which could be a practical method of prevention [200]. In the absence of natural food, fish should be fed at least once every other day to apparent satiation in order to maintain normal physiological functions and to improve resistance to *F. columnare*, since starving juvenile channel catfish reduced their innate resistance to columnaris disease [19].

Besides implementing good management practices, chemical agents can also be applied as a preventive approach. Davis determined that treating fish for 20 min in a copper sulfate ($CuSO_4$) baths at 37 mg/L or by adding copper sulfate to pond water at 0.5 mg/L could be preventive to epizootic episodes of columnaris [46]. Addition of potassium permanganate (PP, $KMnO_4$) to the water at 2 mg/L for indefinite periods was suggested to have a prophylactic value [81, 201, 202]. Copper sulfate should be used with care since it is highly toxic to fish in soft waters. Similarly, PP should be used with caution since it can be toxic to certain species,

particularly in soft waters with low levels of organic matter. Prophylactic treatment of channel catfish with 15 mg/L chloramine-T reduced fish mortality from a *F. columnare* infection from 84-100% to 6-14% [190]. Prophylactically given oxytetracycline and romet in feed prior to columnaris challenge with four virulent strains of *F. columnare* were effective in reducing channel catfish mortality [180]. Additionally, if the fish can be adapted to high salt levels (at least 1 ppt) in the culture systems, this could be used as a possible preventive measure against columnaris disease [14].

Fish vaccination is a potential approach for prevention and control of columnaris disease; hence, continuous efforts have been devoted over the last few decades to develop safe and effective columnaris vaccines. Although vaccination trials have not always been successful, fish vaccination has proved to be a successful approach to prevent columnaris disease in aquaculture [28, 203]. Earlier studies report that fish surviving columnaris outbreak were immune upon re-exposure and therefore carrier fish are not likely to get the disease again [109]. However, this is rarely seen in practice in fish farms because farmers usually start treatments shortly after the first signs of the disease allowing no time for protective immunity against the disease to develop [204].

Early vaccination attempts against columnaris disease by oral immunization with heat-killed *F. columnare* cells incorporated into fish feed resulted in protection in juvenile coho salmon [109]. Prolonged feeding of formalin-killed preparations for periods more than three months proved to provide high levels of protection in salmonids [205]. Liewes et al. (1982) found that bath immunization with a *F. columnare* bacterin protects carp against experimental columnaris, but no agglutinins could be detected in immunized fish sera [206]. Annual immersion of channel catfish in a *F. columnare* bacterin was shown to induce a significant

decrease in mortality compared to unvaccinated fish [207]. Mano et al. (1996) administered formalized cells of *F. columnare* to eel by immersion and injection, that resulted in an immune response two weeks later [208]. However, vaccination using formalin killed bacterins delivered with or without adjuvant resulted in limited efficacy [204, 207], because formalin treatment destroys important bacterial antigenic structures [204].

Intraperitoneal injection of tilapia with formalin-killed sonicated cells in Freund's complete adjuvant resulted in a significant systemic humoral response within two weeks and antibody levels almost tripled following a poster immunization. The antibodies were detected in the cutaneous mucus of these tilapia at six and eight weeks postimmunization [209]. Passive immunization studies in channel catfish using antiserum generated against *F. columnare* showed partial protection [210]. However, in a field trial, Bebak et al. tested a commercial oral vaccine in largemouth bass fry and found that vaccinated fish had a 43% lower risk of death by *F. columnare* [13].

Among the several types of vaccines available, live attenuated vaccines are those in which the pathogen has been modified and is no longer virulent to the host [211]. The use of attenuated vaccines holds tremendous potential because it mimics natural infection and elicits strong humoral and cell-mediated protection [212]. In catfish aquaculture, where fish handling is cost-prohibitive and deleterious to fish health, attenuated vaccines have the additional advantage of ease of delivery through immersion. Rifampicin resistance (by successive passage of virulent wild-type isolates onto a medium containing increasing concentrations of the antibiotic rifampicin) is one of the most successful strategies of generating attenuated mutants in Gram-negative bacteria. Resistance to antibiotics consequently results in attenuation of virulence as a fitness cost (as the resistance to an antibiotic increases, some virulence-associated genes are

downregulated) [213]. This strategy has been successfully used to develop modified live attenuated bacterial vaccines for commercial use in mammals and in aquaculture [214-216].

Currently, a modified live *F. columnare* vaccine developed by Shoemaker et al. [217] is available for commercial use to prevent columnaris disease in catfish under the licensed name AQUAVAC-COL™ (Merk). The active ingredient in this vaccine is an avirulent rifampicin-resistant mutant of *F. columnare*, strain FCRR, which is derived from a parent belonging to the less virulent genomovar I of *F. columnare* [218]. The mutant showed non virulence and protective capabilities when administrated to channel catfish. Immersion vaccination of catfish fry between 10 to 48 days post hatch using this mutant achieved a relative percent survival between 57 and 94% following *F. columnare* challenge [28]. However, the efficacy of this vaccine under farm settings has not been reported, and its effectiveness has been publically questioned [219].

Recently; in our lab, we have developed novel rifampicin-resistant mutants from genomovar II strains, the highly virulent group [203]. Out of four selected genetically different genomovar II strains, we were able to obtain 13 rifampicin-resistant mutants. Extensive characterization of these mutants showed marked genetic diversity, indicating a random mutation which means that not all mutations were introduced into the same loci in all mutants [203, 220]. Mohammed and Arias conducted several vaccination trials in commercially valuable fish species and demonstrated that the new genomovar II mutants outperformed FCRR post-exposure to virulent strains of *F. columnare* [221]. The authors suggest that administration of the genomovar II mutants as potential modified-live vaccines is safe and elicits greater protection against columnaris disease than the use of genomovar I mutants.

Finally, fish could benefit from manipulation of their microbial communities and manipulating the fish microbiome composition was suggested as a more ecological and natural approach to prevent columnaris disease [128]. Bacteria associated with fish mucosal surfaces play an important role in fish health as they can compete with pathogens for space and nutrients. Competitive bacteria are known to inhibit the growth of *F. columnare* [20, 116]. Moreover, when *F. columnare* is present in low numbers, it may not be able to compete with other naturally occurring microbiota on the fish skin and gills [222]. Several bacteria strains were isolated from the resident microbiota on the skin of fish and showed very strong antagonistic activity against *F. columnare* in agar diffusion assays [119, 120, 223].

References

[1] Plumb J.A., Health maintenance and principal microbial diseases of cultured fishes, Iowa State University Press, Ames, IA, 1999.

[2] Austin B., Austin D.A., Bacterial fish pathogens: disease of farmed and wild fish, Springer Verlag, 1999.

[3] Shamsudin M.N., Pathogenesis of *Flexibacter columnaris* and immunity in channel catfish, 1994.

[4] Shotts E., Starliper C., Flavobacterial diseases: columnaris disease, cold-water disease and bacterial gill disease. (1999).

[5] Noga E.J., Fish disease: diagnosis and treatment, John Wiley & Sons, 2010.

[6] Groff J.M., Lapatra S.E., Infectious diseases impacting the commercial culture of salmonids, Journal of Applied Aquaculture. (2000) 10:17-90.

[7] Figueiredo H., Klesius P., Arias C., Evans J., Shoemaker C., Pereira Jr D., Peixoto M., Isolation and characterization of strains of *Flavobacterium columnare* from Brazil, Journal of Fish Diseases. (2005) 28:199-204.

[8] Becker C.D., Fujihara M., The bacterial pathogen *Flexibacter columnaris* and its epizootiology among Columbia River fish: A review and synthesis, Monograph-American Fisheries Society (USA). (1978) 2.

[9] Roberts R.J., Fish pathology, John Wiley & Sons, 2012.

[10] Post G., Textbook of fish health. Revised and expanded edition Ltd, TFH Publications, Inc., Neptune City, New Jersey. P, 1987.

[11] Anderson J., Conroy D., (Symposium on Myxobacteria and Flavobacteria: Paper VII). The Pathogenic Myxobacteria with Special Reference to Fish Diseases, Journal of Applied Bacteriology. (1969) 32:30-39.

[12] Davis H., *Cytophaga columnaris* as a cause of fish epidemics, Transactions of the American Fisheries Society. (1949) 77:102-104.

[13] Bebak J., Matthews M., Shoemaker C., Survival of vaccinated, feed-trained largemouth bass fry (*Micropterus salmoides floridanus*) during natural exposure to *Flavobacterium columnare*, Vaccine. (2009) 27:4297-4301.

[14] Altinok I., Grizzle J., Effects of low salinities on *Flavobacterium columnare* infection of euryhaline and freshwater stenohaline fish, Journal of Fish Diseases. (2001) 24:361-367.

[15] Decostere A., Haesebrouck F., Devriese L.A., Characterization of four *Flavobacterium columnare* (*Flexibacter columnaris*) strains isolated from tropical fish, Veterinary Microbiology. (1998) 62:35-45.

[16] Decostere A., Haesebrouck F., Charlier G., Ducatelle R., The association of *Flavobacterium columnare* strains of high and low virulence with gill tissue of black mollies (*Poecilia sphenops*), Veterinary Microbiology. (1999) 67:287-298.

[17] Olivares-Fuster O., Baker J.L., Terhune J.S., Shoemaker C.A., Klesius P.H., Arias C.R., Host-specific association between *Flavobacterium columnare* genomovars and fish species, Systematic and Applied Microbiology. (2007) 30:624-633.

[18] Mohammed H., Arias C., Epidemiology of columnaris disease affecting fishes within the same watershed, Diseases of Aquatic Organisms. (2014) 109:201-211.

[19] Shoemaker C.A., Klesius P.H., Lim C., Yildirim M., Feed deprivation of channel catfish, *Ictalurus punctatus* (Rafinesque), influences organosomatic indices, chemical composition and susceptibility to *Flavobacterium columnare*, Journal of Fish Diseases. (2003) 26:553-561.

[20] Wakabayashi H., Effect of environmental conditions on the infectivity of *Flexibacter columnaris* to fish, Journal of Fish Diseases. (1991) 14:279-290.

[21] Durborow R., Columnaris Disease: A Bacterial Infection Caused by *Flavobacterium Columnare*, South Regional Aquaculture Centre SRAC, Texas A & M University, Publication. (1998).

[22] Hawke J.P., Thune R.L., Systemic isolation and antimicrobial susceptibility of *Cytophaga columnaris* from commercially reared channel catfish, Journal of Aquatic Animal Health. (1992) 4:109-113.

[23] MSU, 2012 annual case summary report of the Aquatic Research and Diagnostic Laboratory. Mississippi State University, College of Veterinary Medicine, Thad Cochran National Warmwater Aquaculture Center available at (http://tcnwac.msstate.edu/cvm2012.pdf). (2012).

[24] USDA, United States Department of Agriculture Reference of 2002 U. S. catfish health and production practices, Center for Epidemiology and Animal Health. (2003) Fort Collins, Colorado:64-67.

[25] Khoo L., Annual fish diagnostic laboratory report for 2000, NWAC News. (2001) 4:6-7.

[26] Wagner B.A., Wise D.J., Khoo L.H., Terhune J.S., The epidemiology of bacterial diseases in food-size channel catfish, Journal of Aquatic Animal Health. (2002) 14:263-272.

[27] Holt R., Sanders J., Zinn J., Fryer J., Pilcher K., Relation of water temperature to *Flexibacter columnaris* infection in steelhead trout (*Salmo gairdneri*), coho (*Oncorhynchus kisutch*) and chinook (*O. tshawytscha*) salmon, Journal of the Fisheries Board of Canada. (1975) 32:1553-1559.

[28] Shoemaker C.A., Klesius P.H., Drennan J.D., Evans J.J., Efficacy of a modified live *Flavobacterium columnare* vaccine in fish, Fish & Shellfish Immunology. (2011) 30:304-308.

[29] Williams I., Investigations of the prespawning mortality of sockeye in Horsefly River and McKinney Creek in 1969, International Pacific Salmon Fisheries Commission. (1973):1-42.

[30] Cai W., De La Fuente L., Arias C.R., Biofilm Formation by the Fish Pathogen *Flavobacterium columnare*: Development and Parameters Affecting Surface Attachment, Applied and Environmental Microbiology. (2013) 79:5633-5642.

[31] Bullock G., Hsu T.C., Shotts Jr E., Columnaris disease of fishes, USFWS Fish Disease Leaflet. (1986) 1986:1-9.

[32] Amend D., Columnaris (*Flexibacter columnaris*) disease of freshwater fishes and a brief review of other flexibacterial diseases of fish, Antigens of fish pathogens. (1982):139-161.

[33] Pacha R., Porter S., Characteristics of myxobacteria isolated from the surface of freshwater fish, Applied microbiology. (1968) 16:1901-1906.

[34] Pacha R., Ordal E., Myxobacterial diseases of salmonids, A symposium on diseases of fishes and shellfishes. The American Fisheries Society Special Publication 5, 1970.

[35] Bernardet J.-F., Grimont P.A., Deoxyribonucleic acid relatedness and phenotypic characterization of *Flexibacter columnaris* sp. nov., nom. rev., *Flexibacter psychrophilus* sp. nov., nom. rev., and *Flexibacter maritimus* Wakabayashi, Hikida, and Masumura 1986, International journal of Systematic and Evolutionary Microbiology. (1989) 39:346-354.

[36] Reichenbach H., Dworkin M., Introduction to the gliding bacteria, The prokaryotes, Springer, 1981, pp. 315-327.

[37] Betolini J., Rohovec J., Electrophoretic detection of proteases from different *Flexibacter columnaris* strains and assessment of their variability, Diseases of Aquatic Organisms. (1992) 12:121-128.

[38] Griffin B., Characteristics of a chondroitin AC lyase produced by *Cytophaga columnaris*, Transactions of the American Fisheries Society. (1991) 120:391-395.

[39] Teska J.D., Assay to Evaluate the Reaction Kinetics of Chondroitin AC Lyase Produced by *Cytophaga columnaris*, Journal of Aquatic Animal Health. (1993) 5:259 - 264.

[40] Suomalainen L.R., Tiirola M., Valtonen E.T., Chondroitin AC lyase activity is related to virulence of fish pathogenic *Flavobacterium columnare*, Journal of Fish Diseases. (2006) 29:757-763.

[41] Stringer-Roth K.M., Yunghans W., Caslake L.F., Differences in chondroitin AC lyase activity of *Flavobacterium columnare* isolates, Journal of Fish Diseases. (2002) 25:687-691.

[42] Decostere A., Haesebrouck F., Van Driessche E., Charlier G., Ducatelle R., Characterization of the adhesion of *Flavobacterium columnare* (*Flexibacter columnaris*) to gill tissue, Journal of Fish Diseases. (1999) 22:465-474.

[43] Johnson J.L., Chilton W., Galactosamine glycan of *Chondrococcus columnaris*, Science. (1966) 152:1247-1248.

[44] McCurdy H.D., Studies on the taxonomy of the Myxobacterales, Canadian journal of microbiology. (1969) 15:1453-1461.

[45] Griffin B., A simple procedure for identification of *Cytophaga columnaris*, Journal of Aquatic Animal Health. (1992) 4:63-66.

[46] Davis H.S., A new bacterial disease of fresh-water fishes: By HS Davis, Government Printing Office., 1922.

[47] Bernardet J.F., Bowman J.P., The genus *Flavobacterium*, Prokaryotes. (2006) 7:481-531.

[48] Bernardet J.-F., Nakagawa Y., Holmes B., Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family, International journal of systematic and evolutionary microbiology. (2002) 52:1049-1070.

[49] Bernardet J.-F., Segers P., Vancanneyt M., Berthe F., Kersters K., Vandamme P., Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov.(basonym, *Cytophaga aquatilis* Strohl and Tait 1978), International journal of systematic bacteriology. (1996) 46:128-148.

[50] Ordal E.J., Rucker R.R., Pathogenic myxobacteria, Experimental Biology and Medicine. (1944) 56:15-18.

[51] Garnjobst L., *Cytophaga columnaris* (Davis) in pure culture: a myxobacterium pathogenic to fish, Journal of Bacteriology. (1945) 49:113.

[52] Anacker R.L., Ordal E.J., Studies on the myxobacterium *Chondrococcus columnaris*: I. Serological typing, Journal of Bacteriology. (1959) 78:25-32.

[53] Pyle S.W., Shotts Jr E.B., A New Approach for Differentiating Flexibacteria Isolated from Cold-water and Warmwater Fish, Canadian Journal of Fisheries and Aquatic Sciences. (1980) 37:1040-1042.

[54] Shamsudin M.N., Plumb J.A., Morphological, biochemical, and physiological characterization of *Flexibacter columnaris* isolates from four species of fish, Journal of Aquatic Animal Health. (1996) 8:335-339.

[55] Bernardet J., '*Flexibacter columnaris*': first description in France and comparison with bacterial strains from other origins, Diseases of Aquatic Organisms. (1989) 6:37-44.

[56] Song Y., Fryer J., Rohovec J., Comparison of six media for the cultivation of *Flexibacter columnaris*, Fish Pathology. (1988) 23.

[57] Kunttu H., Characterizing the bacterial fish pathogen *Flavobacterium columnare*, and some factors affecting its pathogenicity. (2010).

[58] Bader J.A., Shoemaker C.A., Klesius P.H., Production, characterization and evaluation of virulence of an adhesion defective mutant of *Flavobacterium columnare* produced by beta-lactam selection, Letters in Applied Microbiology. (2005) 40:123-127.

[59] Newton J.C., Wood T.M., Hartley M.M., Isolation and partial characterization of extracellular proteases produced by isolates of *Flavobacterium columnare* derived from channel catfish, Journal of Aquatic Animal Health. (1997) 9:75-85.

[60] Zhang Y., Arias C.R., Shoemaker C.A., Klesius P.H., Comparison of lipopolysaccharide and protein profiles between *Flavobacterium columnare* strains from different genomovars, Journal of Fish Diseases. (2006) 29:657-663.

[61] Thomas-Jinu S., Goodwin A., Morphological and genetic characteristics of *Flavobacterium columnare* isolates: correlations with virulence in fish, Journal of Fish Diseases. (2004) 27:29-35.

[62] Decostere A., Haesebrouck F., Turnbull J., Charlier G., Influence of water quality and temperature on adhesion of high and low virulence *Flavobacterium columnare* strains to isolated gill arches, Journal of Fish Diseases. (1999) 22:1-11.

[63] Olivares-Fuster O., Bullard S.A., McElwain A., Llosa M.J., Arias C.R., Adhesion dynamics of *Flavobacterium columnare* to channel catfish *Ictalurus punctatus* and zebrafish *Danio rerio* after immersion challenge, Diseases of Aquatic Organisms. (2011) 96:221-227.

[64] Wood J.W., Diseases of Pacific salmon, their prevention and treatment, State of Washington, Department of Fisheries, Hatchery Division, 1974.

[65] Pacha R., Ordal E., Histopathology of experimental columnaris disease in young salmon, Journal of comparative pathology. (1967) 77:419-423.

[66] Rucker R.R., Earp B.J., Ordal E.J., Infectious diseases of Pacific salmon, Transactions of the American Fisheries Society. (1954) 83:297-312.

[67] Shoemaker C.A., Olivares-Fuster O., Arias C.R., Klesius P.H., *Flavobacterium columnare* genomovar influences mortality in channel catfish (*Ictalurus punctatus*), Veterinary Microbiology. (2008) 127:353-359.

[68] LaFrentz B., LaPatra S., Shoemaker C., Klesius P., Reproducible challenge model to investigate the virulence of *Flavobacterium columnare* genomovars in rainbow trout *Oncorhynchus mykiss*, Diseases of Aquatic Organisms. (2012) 101:115-122.

[69] Rosselló R., García-Valdés E., Lalucat J., Ursing J., Genotypic and phenotypic diversity of *Pseudomonas stutzeri*, Systematic and Applied Microbiology. (1991) 14:150-157.

[70] Ursing J., Rossellö-Mora R., Garcia-Valdes E., Lalucat J., Taxonomic note: a pragmatic approach to the nomenclature of phenotypically similar genomic groups, International journal of systematic bacteriology. (1995) 45:604-604.

[71] Triyanto A., Wakabayashi H., Genotypic diversity of strains of *Flavobacterium columnare* from diseased fishes, Fish Pathology. (1999) 34:65–71.

[72] Arias C.R., Welker T.L., Shoemaker C.A., Abernathy J.W., Klesius P.H., Genetic fingerprinting of *Flavobacterium columnare* isolates from cultured fish, Journal of Applied Microbiology. (2004) 97:421-428.

[73] Darwish A.M., Ismaiel A.A., Genetic diversity of *Flavobacterium columnare* examined by restriction fragment length polymorphism and sequencing of the 16S ribosomal RNA gene and the 16S–23S rDNA spacer, Molecular and Cellular Probes. (2005) 19:267-274.

[74] Schneck J.L., Caslake L.F., Genetic diversity of *Flavobacterium columnare* isolated from fish collected from warm and cold water, Journal of Fish Diseases. (2006) 29:245-248.

[75] Toyama T., Kita-Tsukamoto K., Wakabayashi H., Identification of *Flexibacter maritimus*, *Flavobacterium branchiophilum* and *Cytophaga columnaris* by PCR targeted 16S ribosomal DNA, Gyobyo Kenkyu= Fish Pathology. (1996) 31:25-31.

[76] Olivares-Fuster O., Shoemaker C.A., Klesius P.H., Arias C.R., Molecular typing of isolates of the fish pathogen, *Flavobacterium columnare*, by single-strand conformation polymorphism analysis, Fems Microbiology Letters. (2007) 269:63-69.

[77] LaFrentz B.R., Waldbieser G.C., Welch T.J., Shoemaker C.A., Intragenomic heterogeneity in the 16S rRNA genes of *Flavobacterium columnare* and standard protocol for genomovar assignment, Journal of Fish Diseases. (2014) 37:657-669.

[78] Soto E., Mauel M., Karsi A., Lawrence M., Genetic and virulence characterization of *Flavobacterium columnare* from channel catfish (*Ictalurus punctatus*), Journal of Applied Microbiology. (2008) 104:1302-1310.

[79] Tekedar H.C., Karsi A., Gillaspy A.F., Dyer D.W., Benton N.R., Zaitshik J., Vamenta S., Banes M.M., Gulsoy N., Aboko-Cole M., Waldbieser G.C., Lawrence M.L., Genome Sequence of the Fish Pathogen *Flavobacterium columnare* ATCC 49512, Journal of Bacteriology. (2012) 194:2763-2764.

[80] Klesius P.H., Shoemaker C.A., Evans J.J., *Flavobacterium columnare* chemotaxis to channel catfish mucus, Fems Microbiology Letters. (2008) 288:216-220.

[81] Snieszko S.F., Bullock G., Columnaris disease of fishes, US Fish and Wildlife Service, Fish Disease Leaflet. (1976) 45:10 Pp.

[82] Suomalainen L.R., Bandilla M., Valtonen E.T., Immunostimulants in prevention of columnaris disease of rainbow trout, *Oncorhynchus mykiss* (Walbaum), Journal of Fish Diseases. (2009) 32:723-726.

[83] Morley N.J., Lewis J.W., Consequences of an outbreak of columnaris disease (*Flavobacterium columnare*) to the helminth fauna of perch (*Perca fluviatilis*) in the Queen Mary reservoir, south-east England, Journal of Helminthology. (2010) 84:186-192.

[84] Griffin B., Columnaris disease: recent advances in research, Aquaculture. (1987) 13:48-50.

[85] Wakabayashi H., Hikida M., Masumura K., *Flexibacter maritimus* sp. nov., a pathogen of marine fishes, International journal of systematic bacteriology. (1986) 36:396-398.

[86] Suomalainen L., Tiirola M., Valtonen E., Influence of rearing conditions on *Flavobacterium columnare* infection of rainbow trout, *Oncorhynchus mykiss* (Walbaum), Journal of Fish Diseases. (2005) 28:271-277.

[87] Karvonen A., Rintamäki P., Jokela J., Valtonen E.T., Increasing water temperature and disease risks in aquatic systems: Climate change increases the risk of some, but not all, diseases, International Journal for Parasitology. (2010) 40:1483-1488.

[88] Fujihara M.P., Olson P.A., Nakatani R.E., Some Factors in Susceptibility of Juvenile Rainbow Trout and Chinook Salmon to *Chondrococcus columnaris*, Journal of the Fisheries Research Board of Canada. (1971) 28:1739-1743.

[89] Amend D.F., Ross A.J., Experimental control of columnaris disease with a new nitrofuran drug, P-7138, The Progressive Fish-Culturist. (1970) 32:19-25.

[90] Stoskopf M.K., Fish medicine, WB Saunders Company, 1993.

[91] Chen C., Chung Y., Kuo G., Studies on the pathogenicity of *Flexibacter columnaris*. I, Effect of dissolved oxygen and ammonia on the pathogenicity of *Flexibacter columnaris* to eel (*Anguilla japonica*). CAPD Fisheries Series. (1982):57-61.

[92] Hanson L.A., Grizzle J.M., Nitrite-induced predisposition of channel catfish to bacterial diseases, The Progressive Fish-Culturist. (1985) 47:98-101.

[93] Kumar D., Suresh K., Dey R.K., Mishra B.K., Stress-mediated columnaris disease in rohu, *Labeo rohita* (Hamilton), Journal of Fish Diseases. (1986) 9:87-89.

[94] Bader J.A., Moore S.A., Nusbaum K.E., The effect of cutaneous injury on a reproducible immersion challenge model for *Flavobacterium columnare* infection in channel catfish (*Ictalurus punctatus*), Aquaculture. (2006) 253:1-9.

[95] Bader J., Nusbaum K., Shoemaker C., Comparative challenge model of *Flavobacterium columnare* using abraded and unabraded channel catfish, *Ictalurus punctatus* (Rafinesque), Journal of Fish Diseases. (2003) 26:461-467.

[96] Bandilla M., Valtonen E.T., Suomalainen L.R., Aphalo P.J., Hakalahti T., A link between ectoparasite infection and susceptibility to bacterial disease in rainbow trout, International Journal for Parasitology. (2006) 36:987-991.

[97] Ellis A.E., Innate host defense mechanisms of fish against viruses and bacteria, Developmental & Comparative Immunology. (2001) 25:827-839.

[98] Barker G.A., Smith S.N., Bromage N.R., Effect of oxolinic acid on bacterial flora and hatching success rate of rainbow trout, *Oncorhynchus mykiss*, eggs, Aquaculture. (1990) 91:205-222.

[99] Fijan N.N., Antibiotic additives for the isolation of *Chondrococcus columnaris* from fish, Applied microbiology. (1969) 17:333–334.

[100] Chowdhury M., Wakabayashi H., Survival of four major bacterial fish pathogens in different types of experimental water, Bangladesh Journal of Microbiology. (1990) 7:47-54.

[101] Ross A., Smith C.A., Effect of temperature on survival of *Aeromonas liquefaciens*, *Aeromonas salmonicida*, *Chondrococcus columnaris*, and *Pseudomonas fluorescens*, The Progressive Fish-Culturist. (1974) 36:51-52.

[102] Arias C.R., LaFrentz S., Cai W., Olivares-Fuster O., Adaptive response to starvation in the fish pathogen *Flavobacterium columnare*: cell viability and ultrastructural changes, BMC microbiology. (2012) 12:266.

[103] Kunttu H.M., Sundberg L.R., Pulkkinen K., Valtonen E.T., Environment may be the source of *Flavobacterium columnare* outbreaks at fish farms, Environmental microbiology reports. (2012) 4:398-402.

[104] Pulkkinen K., Suomalainen L.R., Read A., Ebert D., Rintamäki P., Valtonen E., Intensive fish farming and the evolution of pathogen virulence: the case of columnaris disease in Finland, Proceedings of the Royal Society B: Biological Sciences. (2010) 277:593-600.

[105] Rickard A., McBain A., Ledder R., Handley P., Gilbert P., Coaggregation between freshwater bacteria within biofilm and planktonic communities, Fems Microbiology Letters. (2003) 220:133-140.

[106] Revetta R., Rodgers M., Kinkle B., Isolation and identification of freshwater bacteria antagonistic to *Giardia intestinalis* cysts, J Water Health. (2005) 3:83-88.

[107] Arias C.R., Abernathy J.W., Liu Z., Combined use of 16S ribosomal DNA and automated ribosomal intergenic spacer analysis to study the bacterial community in catfish ponds, Letters in Applied Microbiology. (2006) 43:287-292.

[108] Welker T.L., Shoemaker C.A., Arias C.R., Klesius P.H., Transmission and detection of *Flavobacterium columnare* in channel catfish *Ictalurus punctatus*, Diseases of Aquatic Organisms. (2005) 63:129-138.

[109] Fujihara M., Nakatani R., Antibody production and immune responses of rainbow trout and coho salmon to *Chondrococcus columnaris*, Journal of the Fisheries Board of Canada. (1971) 28:1253-1258.

[110] Suomalainen L., Tiirola M., Valtonen E., Treatment of columnaris disease of rainbow trout: low pH and salt as possible tools?, Diseases of Aquatic Organisms. (2005) 65:115-120.

[111] Kunttu H.M.T., Valtonen E.T., Jokinen E.I., Suomalainen L.-R., Saprophytism of a fish pathogen as a transmission strategy, Epidemics. (2009) 1:96-100.

[112] Schachte J.H., Columnaris disease, A guide to integrated fish health management in the Great Lakes basin. Great Lakes Fishery Commission, Special Publication. (1983):199-203.

[113] Wakabayashi H., Columnaris disease. In: Inglis V, Roberts RJ, Bromage NR, (eds), Bacterial diseases of fish. (1993) Blackwell Scientific Publications, Oxford:23-39.

[114] Farmer B., Improved methods for the isolation and characterization of *Flavobacterium columnare*, 2004.

[115] Wakabayashi H., Egusa S., Preliminary experiments on environmental factors influencing the prevalence of columnaris disease, Fish Pathol. (1972) 7:58-63.

[116] Chowdhury M.B., Wakabayashi H., Effects of competitive bacteria on the survival and infectivity of *Flexibacter columnaris*, Fish Pathology. (1989) 24:9-15.

[117] Chowdhury M.B., Wakabayashi H., A study on the mechanism of the bacterial competitive effects on *Flexibacter columnaris* infection: Effects of the time-lag between the exposures of fish to *F. columnaris* and its competitor, Fish Pathology. (1989) 24:105-110.

[118] Chowdhury M., Wakabayashi H., A study on *Flexibacter columnaris* infection in loach, *Misgurnus anguillicaudatus* (Bleeker, Günther), Journal of Fish Diseases. (1991) 14:389-394.

[119] Tiirola M., Valtonen E.T., Rintamäki-Kinnunen P., Kulomaa M.S., Diagnosis of flavobacteriosis by direct amplification of rRNA genes, Diseases of Aquatic Organisms. (2002) 51:93-100.

[120] Ström-Bestor M., Wiklund T., Inhibitory activity of *Pseudomonas* sp. on *Flavobacterium psychrophilum*, *in vitro*, Journal of Fish Diseases. (2011) 34:255-264.

[121] Anacker R.L., Ordal E.J., Studies on the Myxobacterium *chondrococcus columnaris*: II. Bacteriocins, Journal of Bacteriology. (1959) 78:33.

[122] Li N., Zhang L., Zhang J., Liu Z., Huang B., Zhang S., Nie P., Type I restriction-modification system and its resistance in electroporation efficiency in *Flavobacterium columnare*, Veterinary Microbiology. (2012).

[123] Staroscik A.M., Hunnicutt D.W., Archibald K.E., Nelson D.R., Development of methods for the genetic manipulation of *Flavobacterium columnare*, BMC microbiology. (2008) 8:115.

[124] Zhang J., Zou H., Wang L., Huang B., Li N., Wang G., Nie P., Construction of two selectable markers for integrative/conjugative plasmids in *Flavobacterium columnare*, Chinese Journal of Oceanology and Limnology. (2012) 30:269-278.

[125] Dalsgaard I., Madsen L., Bacterial pathogens in rainbow trout, *Oncorhynchus mykiss* (Walbaum), reared at Danish freshwater farms, Journal of Fish Diseases. (2000) 23:199-209.

[126] Nematollahi A., Decostere A., Pasmans F., Ducatelle R., Haesebrouck F., Adhesion of high and low virulence *Flavobacterium psychrophilum* strains to isolated gill arches of rainbow trout *Oncorhynchus mykiss*, Diseases of Aquatic Organisms. (2003) 55:101-107.

[127] Suomalainen L., Kunttu H., Valtonen E., Hirvelä-Koski V., Tiirola M., Molecular diversity and growth features of *Flavobacterium columnare* strains isolated in Finland, Diseases of Aquatic Organisms. (2006) 70:55-61.

[128] Suomalainen L.-R., *Flavobacterium columnare* in Finnish fish farming: characterisation and putative disease management strategies, PhD dissertation, University of Jyväskylä, 2005.

[129] Klesius P.H., Pridgeon J.W., Aksoy M., Chemotactic factors of Flavobacterium columnare to skin mucus of healthy channel catfish (Ictalurus punctatus), Fems Microbiology Letters. (2010) 310:145-151.

[130] Ofek I., Doyle R.J., Bacterial adhesion to cells and tissues, Springer, 1994.

[131] Pate J.L., Ordal E.J., The fine structure of *Chondrococcus columnaris* III. The surface layers of Chondrococcus columnaris, The Journal of cell biology. (1967) 35:37-51.

[132] Sun F., Peatman E., Li C., Liu S., Jiang Y., Zhou Z., Liu Z., Transcriptomic signatures of attachment, NF-κB suppression and IFN stimulation in the catfish gill following columnaris bacterial infection, Developmental & Comparative Immunology. (2012).

[133] Beck B.H., Farmer B.D., Straus D.L., Li C., Peatman E., Putative roles for a rhamnose binding lectin in *Flavobacterium columnare* pathogenesis in channel catfish *Ictalurus punctatus*, Fish & Shellfish Immunology. (2012).

[134] Tateno H., Yamaguchi T., Ogawa T., Muramoto K., Watanabe T., Kamiya H., Saneyoshi M., Immunohistochemical localization of rhamnose-binding lectins in the steelhead trout (*Oncorhynchus mykiss*), Developmental & Comparative Immunology. (2002) 26:543-550.

[135] Mäkelä P.H., Valtonen V., Valtonen M., Role of O-antigen (lipopolysaccharide) factors in the virulence of *Salmonella*, Journal of Infectious Diseases. (1973) 128:S81-S85.

[136] Arias C., Shoemaker C., Evans J., Klesius P., A comparative study of *Edwardsiella ictaluri* parent (EILO) and *E. ictaluri* rifampicin mutant (RE-33) isolates using lipopolysaccharides, outer membrane proteins, fatty acids, Biolog, API 20E and genomic analyses, Journal of Fish Diseases. (2003) 26:415-421.

[137] MacLean L.L., Vinogradov E., Crump E.M., Perry M.B., Kay W.W., The structure of the lipopolysaccharide O-antigen produced by *Flavobacterium psychrophilum* (259-93), European Journal of Biochemistry. (2001) 268:2710-2716.

[138] Kunttu H.M.T., Jokinen E.I., Valtonen E.T., Sundberg L.R., Virulent and nonvirulent *Flavobacterium columnare* colony morphologies: characterization of chondroitin AC lyase activity and adhesion to polystyrene, Journal of Applied Microbiology. (2011) 111:1319-1326.

[139] Kunttu H.M.T., Suomalainen L.R., Jokinen E.I., Valtonen E.T., *Flavobacterium columnare* colony types: Connection to adhesion and virulence?, Microbial Pathogenesis. (2009) 46:21-27.

[140] Staroscik A.M., Nelson D.R., The influence of salmon surface mucus on the growth of *Flavobacterium columnare*, Journal of Fish Diseases. (2008) 31:59-69.

[141] Aumailley M., Gayraud B., Structure and biological activity of the extracellular matrix, Journal of Molecular Medicine. (1998) 76:253-265.

[142] Miyoshi S.-i., Shinoda S., Microbial metalloproteases and pathogenesis, Microbes and infection. (2000) 2:91-98.

[143] Dalsgaard I., Virulence mechanisms in *Cytophaga psychrophila* and other *Cytophaga*-like bacteria pathogenic for fish, Annual Review of Fish Diseases. (1993) 3:127-144.

[144] Kitamikado M., Lee Y.-Z., Chondroitinase-producing bacteria in natural habitats, Applied microbiology. (1975) 29:414-421.

[145] Tripathi N.K., Latimer K.S., Gregory C.R., Ritchie B.W., Wooley R.E., Walker R.L., Development and evaluation of an experimental model of cutaneous columnaris disease in koi *Cyprinus carpio*, Journal of veterinary diagnostic investigation. (2005) 17:45-54.

[146] Kitani Y., Kikuchi N., Zhang G., Ishizaki S., Shimakura K., Shiomi K., Nagashima Y., Antibacterial action of L-amino acid oxidase from the skin mucus of rockfish *Sebastes schlegelii*, Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. (2008) 149:394-400.

[147] Alexander J.B., Ingram G.A., Noncellular nonspecific defence mechanisms of fish, Annual Review of Fish Diseases. (1992) 2:249-279.

[148] Ellis A.E., Immunity to bacteria in fish, Fish & Shellfish Immunology. (1999) 9:291-308.

[149] Ourth D.D., Bachinski L.M., Bacterial sialic acid modulates activation of the alternative complement pathway of channel catfish (*Ictaluruspunctatus*), Developmental & Comparative Immunology. (1987) 11:551-564.

[150] Do Vale A., Marques F., Silva M.T., Apoptosis of sea bass (*Dicentrarchus labrax* L.) neutrophils and macrophages induced by experimental infection with *Photobacterium damselae* subsp. *piscicida*, Fish & Shellfish Immunology. (2003) 15:129-144.

[151] Morrison C., Cornick J., Shum G., Zwicker B., Microbiology and histopathology of ‘saddleback’ disease of underyearling Atlantic salmon, *Salmo salar* L, Journal of Fish Diseases. (1981) 4:243-258.

[152] Kuo S.-C., Chung H.-Y., Kou G.-H., Studies on artificial infection of the gliding bacteria in cultured fishes, Fish Pathology. (1981) 15:309-314.

[153] Amend D., Myxobacterial infections of salmonids: prevention and treatment, A symposium on diseases of fish and shellfishes. Am. Fish. Soc. Spec. Pubi, 1970.

[154] Austin B., Austin D., Bacterial fish pathogens: Diseases in farmed and Wild Fish, Ellis Horwood Limited, Chichester, England. 304pp, 1987.

[155] Durborow R., Thune R., Hawke J., Camus A., Columnaris disease: a bacterial infection caused by *Flavobacterium columnare*. Southern Regional Aquaculture Center, Publication No. 479. Stoneville, Mississippi, US Department of Agriculture, Stoneville, Mississippi. (1998).

[156] Davis H.S., Culture and diseases of game fishes, University of California Press, 1953.

[157] Post G.W., Textbook of fish health, TFH Publications Inc., Neptune City, New Jersey, 1983.

[158] Ferguson H., Systemic pathology of fish: A text and atlas of normal tissue in teleosts and their responses in disease, Scotian Press London:, 2006.

[159] Wolke R., Ribelin W., Migaki G., Pathology of bacterial and fungal diseases affecting fish, The pathology of fishes. (1975):33-116.

[160] Koski P., Hirvela Koski V., Bernardet J., *Flexibacter columnaris* infection in Artic char (*Salvelinus alpinus* (L.)) first isolation in Finland, Bulletin of the European Association of Fish Pathologists. (1993) 13:66-69.

[161] Foscarini R., Induction and development of bacterial gill disease in the eel (*Anguilla japonica*) experimentally infected with *Flexibacter columnaris*: pathological changes in the gill vascular structure and in cardiac performance, Aquaculture. (1989) 78:1-20.

[162] Roberts R.J., Sommerville C., Diseases of tilapias, Manila, 1982.

[163] Bullard S.A., McElwain A., Arias C.R., Scanning Electron Microscopy of "Saddleback" Lesions Associated with Experimental Infections of *Flavobacterium columnare* in Channel Catfish, *Ictalurus punctatus* (Siluriformes: Ictaluridae), and Zebrafish, *Danio rerio* (Cypriniformes: Cyprinidae), Journal of the World Aquaculture Society. (2011) 42:906-913.

[164] Sierra G., A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates, Antonie van Leeuwenhoek. (1957) 23:15-22.

[165] Mac Faddin J.F., Biochemical tests for identification of medical bacteria, Williams & Wilkins. Baltimore, MD. (2000).

[166] Shoemaker C.A., Shelby R.A., Klesius P.H., Development of an indirect ELISA to detect humoral response to *Flavobacterium columnare* infection of channel catfish, *Ictalurus punctatus*, Journal of Applied Aquaculture. (2003) 14:43-52.

[167] Panangalal V., Shelby R.A., Shoemaker C.A., Klesius P.H., Mitra A., Morrison E.E., Immunofluorescent test for simultaneous detection of *Edwardsiella ictaluri* and *Flavobacterium columnare*, Diseases of Aquatic Organisms. (2006) 68:197-207.

[168] Lorenzen E., Karas N., Detection of *Flexibacter psychrophilus* by immunofluorescence in fish suffering from fry mortality syndrome: a rapid diagnostic method, Diseases of Aquatic Organisms. (1992) 13:231-234.

[169] Shoemaker C.A., Arias C.R., Klesius P.H., Welker T.L., Technique for identifying *Flavobacterium columnare* using whole-cell fatty acid profiles, Journal of Aquatic Animal Health. (2005) 17:267-274.

[170] Madetoja J., Nyman P., Wiklund T., *Flavobacterium psychrophilum*, invasion into and shedding by rainbow trout *Oncorhynchus mykiss*, Diseases of Aquatic Organisms. (2000) 43:27-38.

[171] Anderson I., Norton J., Diseases of barramundi in aquaculture, Australian Aquaculture. (1991) 5:21-24.

[172] Bader J.A., Shoemaker C.A., Klesius P.H., Rapid detection of columnaris disease in channel catfish (*Ictalurus punctatus*) with a new species-specific 16-S rRNA gene-based PCR primer for *Flavobacterium columnare*, Journal of Microbiological Methods. (2003) 52:209-220.

[173] Shieh H., Studies on the nutrition of a fish pathogen, *Flexibacter columnaris*, Microbios Letters. (1980) 13:129-133.

[174] Andrews J.M., Determination of minimum inhibitory concentrations, Journal of Antimicrobial Chemotherapy. (2001) 48:5-16.

[175] Decostere A., Haesebrouck F., Devriese L.A., Shieh medium supplemented with tobramycin for selective isolation of *Flavobacterium columnare* (*Flexibacter columnaris*) from diseased fish, Journal of clinical microbiology. (1997) 35:322-324.

[176] Snieszko S., Therapy of bacterial fish diseases, Transactions of the American Fisheries Society. (1954) 83:313-330.

[177] Ross A., In vitro studies with nifurpirinol (P-7138) and bacterial fish pathogens, The Progressive Fish-Culturist. (1972) 34:18-20.

[178] Shiraki K., Miyamoto F., Sato T., Sonezaki I., Yano K., Studies on a new chemotherapeutic agent nifurprazine (HB-115) against fish infectious diseases, Fish Pathol. (1970) 4:130-137.

[179] Soltani M., Shanker S., Munday B., Chemotherapy of *Cytophaga/Flexibacter*-like bacteria (CFLB) infections in fish: studies validating clinical efficacies of selected antimicrobials, Journal of Fish Diseases. (1995) 18:555-565.

[180] Thomas-Jinu S., Goodwin A., Acute columnaris infection in channel catfish, *Ictalurus punctatus* (Rafinesque): efficacy of practical treatments for warmwater aquaculture ponds, Journal of Fish Diseases. (2004) 27:23-28.

[181] Benbrook C.M., Antibiotic drug use in US aquaculture, Institute for Agriculture and Trade Policy Report. (2002) 2.

[182] Gaunt P.S., Gao D.N., Sun F.S., Endris R., Efficacy of Florfenicol for Control of Mortality Caused by *Flavobacterium columnare* Infection in Channel Catfish, Journal of Aquatic Animal Health. (2010) 22:115-122.

[183] Darwish A.M., Bebak J.A., Schrader K.K., Assessment of Aquaflor®, copper sulphate and potassium permanganate for control of *Aeromonas hydrophila* and *Flavobacterium columnare* infection in sunshine bass, *Morone chrysops* female × *Morone saxatilis* male, Journal of Fish Diseases. (2012) 35:637-647.

[184] US Food and Drug Administration, First FDA Conditionally Approved New Animal Drug for Columnaris Disease in Catfish., US Food and Drug Administration, Rockville, MD, USA. Available at http://www.fda.gov/ (accessed August 2009). (2009).

[185] Boyd C.E., Water quality in warmwater fish ponds, Auburn University, Agricultural Experiment Station Auburn, Alabama, 1979.

[186] Darwish A.M., Mitchell A., Straus D.L., Evaluation of a 4-h static copper sulphate treatment against experimental infection of *Flavobacterium columnare* in channel catfish (*Ictalurus punctatus*), Aquaculture Research. (2012) 43:688-695.

[187] Speare D.J., Arsenault G.J., Effects of intermittent hydrogen peroxide exposure on growth and columnaris disease prevention of juvenile rainbow trout (*Oncorhynchus mykiss*), Canadian Journal of Fisheries and Aquatic Sciences. (1997) 54:2653-2658.

[188] Rach J.J., Schleis S.M., Gaikowski M., Johnson A., Efficacy of hydrogen peroxide in controlling mortality associated with external columnaris on walleye and channel catfish fingerlings, North American Journal of Aquaculture. (2003) 65:300-305.

[189] Darwish A., Mitchell A., Evaluation of diquat against an acute experimental infection of *Flavobacterium columnare* in channel catfish, *Ictalurus punctatus* (Rafinesque), Journal of Fish Diseases. (2009) 32:401-408.

[190] Riley T., Treatment of *Flavobacterium columnare* and toxicity of chloramine-T in channel catfish, *Ictalurus punctatus*, MS thesis: Auburn University, Auburn, Alabama, 2000.

[191] Bowker J.D., Carty D., Trushenski J.T., Bowman M.P., Wandelear N., Matthews M., Controlling mortality caused by external columnaris in Largemouth Bass and Bluegill with Chloramine-T or Hydrogen Peroxide, North American Journal of Aquaculture. (2013) 75:342-351.

[192] Declercq A.M., Haesebrouck F., Van den Broeck W., Bossier P., Decostere A., Columnaris disease in fish: a review with emphasis on bacterium-host interactions, Veterinary research. (2013) 44:27.

[193] Schmidt A.S., Bruun M.S., Dalsgaard I., Pedersen K., Larsen J.L., Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms, Applied and Environmental Microbiology. (2000) 66:4908-4915.

[194] Serrano P.H., Responsible use of antibiotics in aquaculture, Food & Agriculture Org FAO., Rome, 2005.

[195] Miranda C.D., Rojas R., Occurrence of florfenicol resistance in bacteria associated with two Chilean salmon farms with different history of antibacterial usage, Aquaculture. (2007) 266:39-46.

[196] Boyd C.E., Massaut L., Risks associated with the use of chemicals in pond aquaculture, Aquacultural Engineering. (1999) 20:113-132.

[197] Laanto E., Sundberg L.R., Bamford J.K.H., Phage Specificity of the Freshwater Fish Pathogen *Flavobacterium columnare*, Applied and Environmental Microbiology. (2011) 77:7868-7872.

[198] Prasad Y., Arpana, Kumar D., Sharma A.K., Lytic bacteriophages specific to *Flavobacterium columnare* rescue catfish, *Clarias batrachus* (Linn.) from columnaris disease, Journal of Environmental Biology. (2011) 32:161-168.

[199] Beck B., Barnett L., Farmer B., Peatman E., Carter D., Kaolinitic clay protects against *Flavobacterium columnare* infection in channel catfish *Ictalurus punctatus* (Rafinesque), Journal of Fish Diseases. (2014).

[200] Conrad J., Holt R., Kreps T., Ozone disinfection of flowing water, The Progressive Fish-Culturist. (1975) 37:134-136.

[201] Rogers W.A., Principal diseases of catfish: how to identify and fight them, Watt Publishing Company, 1971.

[202] Darwish A., Mitchell A., Straus D., Evaluation of potassium permanganate against an experimental subacute infection of *Flavobacterium columnare* in channel catfish, *Ictalurus punctatus* (Rafinesque), Journal of Fish Diseases. (2009) 32:193-199.

[203] Olivares-Fuster O., Development, Characterization and Early Evaluation of New Modified Live Vaccines Against Columnaris Disease, 2010.

[204] Bernardet J., Immunization with bacterial antigens: *Flavobacterium* and *Flexibacter* infections, Developments in biological standardization. (1996) 90:179-188.

[205] Ransom D.P., Immune responses of salmonids: a) Oral immunization against *Flexibacter columnaris*: b) Effects of combining antigens in parenterally administered polyvalent vaccines, 1975.

[206] Liewes E., Van Dam R., Procedures and application of the in vitro fish leukocyte stimulation assay, Dev Comp Immunol. (1982) 2:223-232.

[207] Moore A.A., Eimers M.E., Cardella M.A., Attempts to control *Flexibacter columnaris* epizootics in pond-reared channel catfish by vaccination, Journal of Aquatic Animal Health. (1990) 2:109-111.

[208] Mano N., Inui T., Arai D., Hirose H., Deguchi Y., Immune response in the skin of eel against *Cytophaga columnaris*, Fish Pathology. (1996) 31:65-70.

[209] Grabowski L.D., LaPatra S.E., Cain K.D., Systemic and mucosal antibody response in tilapia, Oreochromis niloticus (L.), following immunization with Flavobacterium columnare, Journal of Fish Diseases. (2004) 27:573-581.

[210] Shelby R., Shoemaker C., Klesius P., Passive immunization of channel catfish *Ictalurus punctatus* with anti-*Flavobacterium columnare* sera, Diseases of Aquatic Organisms. (2007) 77:143-147.

[211] Shoemaker C.A., Klesius P.H., Evans J.J., Arias C.R., Use of modified live vacccine in aquaculture, Journal of the World Aquaculture Society. (2009) 40:573-585.

[212] Woodrow K.A., Bennett K.M., Lo D.D., Mucosal vaccine design and delivery, Annual Review of Biomedical Engineering. (2012) 14:17-46.

[213] McCallum N., Karauzum H., Getzmann R., Bischoff M., Majcherczyk P., Berger-Bächi B., Landmann R., In vivo survival of teicoplanin-resistant *Staphylococcus aureus* and fitness cost of teicoplanin resistance, Antimicrobial agents and chemotherapy. (2006) 50:2352-2360.

[214] Klesius P.H., Shoemaker C.A., Development and use of modified live *Edwardsiella ictaluri* vaccine against enteric septicemia of catfish, Advanced Veterinay Medicine. (1999) 41:523-537.

[215] Schurig G.G., Roop R.M., Bagchi T., Boyle S., Buhrman D., Sriranganathan N., Biological properties of RB51; a stable rough strain of *Brucella abortus*, Veterinary Microbiology. (1991) 28:171-188.

[216] Shoemaker C.A., Klesius P.H., Evans J.J., Immunization of eyed channel catfish, *Ictalurus punctatus*, eggs with monovalent *Flavobacterium columnare* vaccine and bivalent *F. columnare* and *Edwardsiella ictaluri* vaccine, Vaccine. (2007) 25:1126-1131.

[217] Shoemaker C.A., Klesius P.H., Evans J.J., Modified live *Flavobacterium columnare* against columnaris disease in fish, Google Patents, 2005.

[218] Zhang Y., Arias C.R., Shoemaker C.A., Klesius P.H., Comparison of lipopolysaccharide and protein profiles between *Flavobacterium columnare* strains from different genomovars, Journal of Fish Diseases. (2006) 29:657-663.

[219] Kirkland S., Evaluation of the Live-Attenuated Vaccine AquaVac-COL® on Hybrid Channel x Blue Catfish Fingerlings in Earthen Ponds, 2010.

[220] Olivares-Fuster O., Arias C.R., Development and characterization of rifampicin-resistant mutants from high virulent strains of *Flavobacterium columnare*, Journal of Fish Diseases. (2011) 34:385-394.

[221] Mohammed H., Olivares-Fuster O., LaFrentz S., Arias C.R., New attenuated vaccine against columnaris disease in fish: Choosing the right parental strain is critical for vaccine efficacy, Vaccine. (2013) 31:5276-5280.

[222] Suomalainen L.R., Tiirola M.A., Valtonen E.T., Effect of *Pseudomonas* sp MT5 baths on *Flavobacterium columnare* infection of rainbow trout and on microbial diversity on fish skin and gills, Diseases of Aquatic Organisms. (2005) 63:61-68.

[223] Boutin S., Bernatchez L., Audet C., Derôme N., Antagonistic effect of indigenous skin bacteria of brook charr (*Salvelinus fontinalis*) against *Flavobacterium columnare* and *F. psychrophilum*, Veterinary Microbiology. (2012) 155:355-361.

CHAPTER 3. EPIDEMIOLOGY OF COLUMNARIS DISEASE AFFECTING FISHES WITHIN THE SAME WATERSHED

Abstract

In the Southeastern United States, columnaris disease (caused by *Flavobacterium columnare*) typically affects catfish raised in earthen ponds from early spring until late summer. Recently, unusually severe outbreaks of columnaris disease occurred at the E. W. Shell Fisheries Center (EWSFC) located in Auburn, AL, USA. During these outbreaks, catfish and other aquaculture and sport fish species that were in ponds located within the same watershed were affected. My objective was to investigate the genetic diversity among *F. columnare* isolates recovered from different sites, sources and dates in order to clarify the origin of these outbreaks and, ultimately, to better understand the epidemiology of columnaris disease. A total of 102 *F. columnare* isolates were recovered from catfishes (channel catfish *Ictalurus puntactus,* blue catfish *I. furcatus*, and their hybrid), bluegill (*Lepomis microchirus*), Nile tilapia (*Oreochromis niloticus*), largemouth bass (*Micropterus salmoides*), egg masses, and water during columnaris outbreaks (from spring 2010 to summer 2012). Putative *F. columnare* colonies were identified following standard protocols. All isolates were ascribed to genomovar II following restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene. Genetic variability among the isolates was revealed by amplified fragment length polymorphism (AFLP). Date of

isolation explained most of the variability among the isolates, while host was the least influential parameter denoting a lack of host-specificity within genomovar II isolates. The susceptibility of each of the isolates against commonly used antibiotics was tested by antibiogram. The data showed that 19.6 and 12.7 % of the isolates were resistant to oxytetracycline and kanamycin, respectively.

Introduction

Columnaris disease is an acute to chronic bacterial infection that affects a variety of freshwater fishes, including commercially important species worldwide [1-4]. *Flavobacterium columnare*, the causal agent of columnaris disease, is a Gram negative bacterium, ubiquitous in aquatic environments. In the Southeastern US, columnaris disease is the second most prevalent bacterial infection in channel catfish (*Ictalurus punctatus*) after enteric septicemia of catfish (ESC) caused by the bacterium *Edwardsiella ictaluri*. Recently, a summary of cases compiled from 2001 to 2012 by the Aquatic Research and Diagnostic Laboratory, Mississippi State University [5, 6] reported *F. columnare* as the most frequently diagnosed pathogen in catfish farms.

Several factors have been suggested to influence the susceptibility of fish to *F. columnare* infection although, limited information is available from field studies. Some of these predisposing factors have been used to induce acute columnaris disease under experimental conditions including skin abrasions [7, 8], feed deprivation [9], static challenge water [10], high stocking densities [11-13], and temperature stress [11, 12, 14, 15]. However, columnaris disease outbreaks can occur without any stressor [1].

Besides environmental factors, the severity of columnaris disease is greatly influenced by the genetic type of the strain causing the infection [16]. *Flavobacterium columnare* is a phenotypically homogeneous species but harbors a large degree of genetic diversity. Three different genetic groups or genomovars have been described within the species, and all of them have been isolated from diseased fish in the US [14, 17-20]. Recently, LaFrentz et al. (2013) refined the protocol for typing *F. columnare* isolates using Restriction Fragment Length Polymorphism (RFLP) of the 16S rRNA gene and formally described the restriction patterns for the previously described genomovars I, II, II-B and III [20] and described a new genomovar, I/II. Many virulence studies have shown that genomovar II strains are more virulent than genomovar I strains in channel catfish [3, 16], blue catfish and their hybrid [21], zebrafish (*Danio rerio*) [22], and rainbow trout (*Oncorhynchus mykiss*) [23]. Ascription to genomovar using 16S-RFLP is easy to perform and is a good indicator of strain virulence. However, for molecular epidemiology studies, a higher resolution method is needed in order to unveil the intraspecies genetic diversity. Among all the typing methods used to describe the genetic diversity in *F. columnare*, AFLP has been proven to have one of the highest indices of discriminatory power (D=0.949) [20]. AFLP analyzes nucleotide positions distributed over the whole genome and thus is likely to find more polymorphisms than techniques screening for just a few loci. AFLP can detect polymorphisms more efficiently than other DNA technologies, such as RFLP, random amplified polymorphic DNA (RAPD), and microsatellite or simple sequence repeat polymorphism (SSR) analysis [24].

Currently, preventive measures against columnaris disease are limited. Proper husbandry and health management practices are crucial to prevent *F. columnare* infections [25-27]. However, after disease onset, the use of therapeutants, primarily antibiotics, is necessary to

reduce high mortalities associated with the disease. The repetitive application of antibiotics to any animal production system is linked with a higher incidence of antibiotic resistant bacteria. This has already been detected in both fish pathogens, *F. psychrophilum* [28] and *F. columnare* [29]. Moreover, the discharge of even small quantities of antibiotics into the environment can lead to the emergence of drug-resistant strains in environmental bacterial communities resulting from lateral transfer of these resistant traits between bacteria species [30-32].

Despite best management practices, the incidence and severity of columnaris disease outbreaks in aquaculture have not decreased worldwide. On the contrary, columnaris disease prevalence in commercial rainbow trout farming in Idaho, US (S. E. LaPatra, personal communication) has increased in recent years [23]. Similarly, in Finland, columnaris outbreaks were first reported in the early 1990s and have increased annually to the point of seriously threatening their fish farming industry [33]. The Southeastern US concentrates the vast majority of US aquaculture production (focused on channel catfish) where columnaris disease is considered endemic. Despite columnaris disease impacts on the industry, few studies have analyzed in depth the epidemiology of this disease [3]. Recently, severe epizootics of columnaris disease occurred at the E. W. Shell Fisheries Center (EWSFC) at Auburn, AL, US. These outbreaks affected both aquaculture and sport fish species in ponds located within the same hydrologic unit from spring 2010 till summer 2012. The objective of this study was to investigate the intraspecific genetic diversity among *F. columnare* isolates collected during these outbreaks to better understand columnaris disease epidemiology in open aquaculture systems. In addition, I wanted to investigate the antibiotic susceptibility patterns of these isolates to determine the antimicrobial agent of choice for treatment during columnaris outbreaks.

Material and Methods

Sampling sites. The EWSFC is located in Auburn, AL, USA (32°40’43.3” N, 85°30’15.4” W) and comprises approximately 6.5 km^2 out of which 1 km^2 is surface water distributed in ponds that range in size from 400 m^2 to 105,500 m^2. Water is collected from rainfall on watersheds surrounding the ponds. Water flows by gravity North to South, and most of the ponds, as well as the research buildings, are connected by permanent or intermittent water flows (Figure 3-1). In addition, heavy rainfall events, birds, vehicles, and seining equipment facilitate the transfer of pathogens between ponds. For the purpose of this study, I considered all ponds and buildings at the EWSFC to be part of the same watershed. Two additional sampling locations situated on main campus (10 km south of EWSFC) were included in the study but were not hydrologically linked with the EWSFC.

Samples. Unusually high mortalities due to columnaris disease occurred at the EWSFC in 2010, 2011, and 2012. Moribund fish showing signs of columnaris disease were sampled using standard protocols for isolation of *F. columnare* [34]. In 2010, columnaris disease affected channel catfish fingerlings and tilapia stocked in ponds located at the south end of the EWSFC. These samples were labeled as LS (lower station). In 2011, columnaris disease affected channel catfish fry at the hatchery. Samples were taken from fry, water, and egg masses from spawning tanks. All samples collected at the hatchery were labeled as HA. Some egg masses were transported to the School of Veterinary Medicine, Auburn University, located on main campus where they were disinfected upon arrival using 100 ppm iodine solution for 10 min. After hatching, most of the fry were lost due to columnaris diseases. Samples were collected from moribund fry and recorded as VS (Vet School). In 2012, columnaris disease affected large ponds at the upper station. High mortalities in sport fishing ponds (S1, S3 and S6 stocked with bluegill

and largemouth bass) occurred from April till August (the percentage of fish that succumbed to the infection could not be estimated; it was the highest mortality observed in those ponds within the last 15 years, Dr. Russell Wright, Auburn University, personal communication). Typical columnaris disease signs (i.e. saddle back lesions, skin discoloration, gill necrosis) were observed in dead and moribund bluegill and largemouth bass. Channel, blue and hybrid catfish reared in raceways using water from the affected S1 pond also succumbed to columnaris along with hybrid and channel catfish stocked in ponds S10 and S11. During the summer of 2012, columnaris disease widely spread throughout the EWSFC and affected the genetics unit (GL), research buildings (RB), and ponds nearby the hatchery (F-ponds). In addition, fish maintained in the wet lab located on main campus (WL) that were brought in from East Alabama (not produced at the EWSFC) suffered from columnaris disease and were incorporated into the study. Water samples were taken from recirculating tanks with active columnaris infections.

Bacterial isolation. One hundred two isolates of *F. columnare* (Table 3-1) were recovered from four different fish species, egg masses and water samples during high mortality columnaris outbreaks. Bacteria were recovered from external surfaces (i.e. skin, gills) on selective modified Shieh (MS) agar supplemented with 1 μg/ml tobramycin [35] incubated at 28 °C for 48 hrs. Putative *F. columnare* colonies were selected based on their typical rhizoid morphology and further confirmed by species-specific PCR (see below). All bacteria isolates were stored at −80 °C in 20% glycerol.

DNA extraction and isolate identification. Genomic DNA of isolated bacteria was extracted using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Maryland, USA) following manufacturer's instructions for Gram-negative bacteria. Total DNA was quantified using a Nanodrop (*ND*-1000) spectrophotometer (Thermo Scientific; Nanodrop Technologies,

Wilmington, DE, USA) and properly diluted at 20 ng/µl. Isolates were confirmed as *F. columnare* by amplifying a specific *F. columnare* locus using primer pair FCISRFL (5'-TGCGGCTGGATCACCTCCTTTCTA-GAGACA-3') and FCISRR1 (5'-TAATYRCTAAAGATGTTCTTTCTACTTGTTTG-3') that recognize the 16S–23S rDNA intergenic spacer region (ISR) of the *F. columnare* ribosomal RNA operon. Protocols have been previously described by [36].

RFLP analysis of 16S rRNA gene and AFLP analysis. The 16S rRNA gene was amplified by PCR using universal primers 20F (5'-AGAGTTTGATC(AC)TGGCTCAG-3') and 1500R (5'- CGATCCTACTTGCGTAG-3'), and amplicons were digested as described previously by [17, 20]. Two previously characterized *F. columnare* strains, genomovar I strain ARS-1 and a genomovar II strain ALG-00-530, were used as genomovar controls for the RFLP analysis. AFLP fingerprinting was carried out according to Arias et al. (1997, 2004). Briefly, 100 ng of DNA was digested with *Taq*I and *Hin*dIII (Promega, Madison, WI, USA). Following digestions, corresponding adapters (Arias et al., 1998) were added and ligated to the restriction fragments using T4 DNA ligase (Promega) . Two specific primers were used in these AFLP reactions to amplify the digested fragments, the oligonucleotide T000 (5′-CGATGAGTCCTGACCGAA-3′) matching the *Taq*I-ends, and H00A matching the *Hin*dIII-ends (5′-GACTGCGTACCAGCTT<u>A</u>-3′, the selective base at the 3′-end is underlined). PCR and electrophoresis conditions have been previously described [18]. Ten isolates were AFLP-type in triplicate to ensure the repeatability of the method (Arias et al., 1998). Gel images were exported to Bionumerics software (version 7.0; Applied Maths, Inc., Sint-Martens-Latem, Belgium) for analysis. After conversion, normalization, and background subtraction, the levels of similarity between resulting fingerprints were calculated using the Pearson product moment correlation coefficient. Cluster analysis was

computed by the unweighted pair-group method using average linkages (UPGMA) to identify groups of similar genotypes among the collected strains.

Antibiogram. The disc diffusion technique [37] was used for testing the antimicrobial susceptibility of all the isolates against the 5 selected antimicrobial agents: erythromycin 15 µg, oxytetracycline 30 µg, ampicillin 10 µg, kanamycin 30 µg and florfenicol 30 µg. All antimicrobials were obtained as commercial Sensi-Disc/50 discs Cartridge from (Becton Dickinson (BD), Franklin Lakes, NJ, USA), except for florfenicol, which was obtained from (Mast Diagnostics, Liverpool, UK). Strains were incubated for 18 h at 28 °C in MS broth under continuous shaking. Growing conditions (18 h cultures and vigorous shaking) facilitated the formation of uniform lawns as *F. columnare* rizhoid morphology could interfere with lawn formation. The optical density (OD) of the cultures was adjusted to 0.7 at 600 nm using an Eppendorf Biophotometer (UV spectrophotometer, Eppendorf, Hamburg, Germany). An OD of 0.7 was equivalent to ca. 10^7 colony forming units (CFU)/ml determined by plate counts on MS agar plates. Subsequently, 0.1 ml of each bacterial culture was uniformly distributed onto a MS agar plate. Plates were allowed to dry for 15 min at room temperature to eliminate excess moisture. Antibiotic discs were discharged from their cartridges and distributed evenly in a manner in which they were 15 mm away from the edge of the Petri-dish, and the distance between the centers of 2 disks was not less than 24 mm (5 discs on each plate). Discs were pressed gently against the agar surface to ensure contact and subsequent antibiotic diffusion. The diameters in millimeters (mm) of the inhibition zones were measured and recorded after incubation at 28 °C for 48 h. The strains were separated into 3 categories (susceptible (S), intermediate (I), or resistant (R)) based on the diameter of the zones according to manufacturer's

instructions as follows: erythromycin >21, 16–20, <13; oxytetracyclin >19, 15–18, <14; ampicillin >17, 14–16, <13; kanamycin >18, 14–17, <13; florfenicol >19, 15–18, <14.

Statistical analysis. An analysis of similarities (ANOSIM) was carried out [38] to measure the similarity between the clusters identified by AFLP. Significant differences between sites, sources and dates of isolation were determined using ANOSIM, testing the hypothesis that isolates from the same site, source, or date of isolation are more similar to each other than to those from different origins.

Results

Identification of bacterial isolates and genomovar ascription. Out of 110 putative *F. columnare* isolates identified based on colony morphology on MS medium, 102 isolates (Table 3-1) were confirmed as *F. columnare* by species-specific PCR. All tested isolates yielded the same RFLP pattern, identical to that of genomovar II strain ALG-00-530. Accordingly, all 102 *F. columnare* isolates were ascribed to genomovar II.

Genetic diversity of F. columnare genomovar II. Figure 3-2 shows the results of AFLP fingerprint cluster analysis. AFLP produced isolate-specific patterns consisting of 45 to 65 distinct bands ranging from 50 bp to 700 bp. Out of the 102 AFLP profiles generated from the recovered isolates, I defined 17 different profiles at 90% similarity (cut-off value for defining unique AFLP profiles was previously defined at 97% [39]; a more conservative 90% cut-off value was used to ensure the instraspecies diversity of *F. columnare* was not overestimated). Clusters 1-3 comprised 2011 isolates recovered from egg masses at the HA and from fry derived from those egg masses but hatched at the VS after disinfection. Clusters 4-5 contained all isolates recovered from S10 pond, which had been stocked with hybrid catfish fingerlings in 2012.

Cluster 6 was the largest cluster with 27 isolates (24 from 2012 and 3 from 2011) from bluegill and catfish species from different locations. Similarly, clusters 7-9 grouped 2012 isolates from bluegill, largemouth bass, and catfish species from ponds, the genetics unit (GL) and the research building (RB). Profiles 10 and 11 were represented by only one isolate each, one bluegill (profile 10) and one channel catfish broodstock (profile 11) with a shared similarity of 89%; both isolates were recovered from close proximity but different environments (open pond, profile 11 and close recirculating system, profile 10). Profile 12 was shared by two bluegill isolates from pond S6. Profiles 13, 14 and 16 grouped all 2012 isolates recovered from channel catfish fingerlings transferred from East Alabama that were housed in the on-campus facility. These clusters, along with cluster 17 which grouped all 2010 isolates from F-ponds, were notably different from profiles 1-12 with a shared similarity of 74%. Finally, cluster 17 was the most dissimilar group and included all 2012 isolates recovered from channel catfish in pond S11. Cluster/profile 15 grouped all the isolates from 2010, from the lower station (LS) and they were from tilapia and catfish.

ANOSIM was used to directly compare groups based on site, source and date of isolation. The isolates clustered significantly (p =0.001) by the three factors considered (site, source and date). However, the least influential factor was source (fish species, egg masses, water samples) with an R value of 0.166 and with 14 out of 50 pairwise comparisons not being significant. The R values for site and date were 0.671 and 0.424, respectively. By site, out of 72 pairwise comparisons, 66 were significant while by date, all the pairwise tests were significant. These R values indicate that groups defined by AFLP could be significantly correlated with all three factors, but site played the main determinant role. Figure 3-3 shows a multidimensional scaling analysis (MDS) of the similarities obtained by AFLP analysis. Three distinct groups were

evident based on date of isolation and confirmed the common origin of isolates in each year outbreaks.

Antibiogram. Table 3-2 shows the results from the antibiogram study. Among all tested isolates (n=102), none were resistant to florfenicol, erythromycin and ampicillin. Resistance to oxytetracycline was detected in 19.6% of the isolates and 12.7% of the isolates were resistant to kanamycin. Interestingly, two strains (one from skin and one from gill) from the same fish showed different susceptibilities towards oxytetracycline indicating columnaris disease was caused by more than one strain. Also noteworthy, isolates recovered from eggs and from catfish fry immediately after hatching, were all resistant to oxytetracycline. However, isolates from water samples collected from the tanks in which the eggs were maintained, showed a combination of resistance/susceptibility pattern to oxytetracycline (some strains were resistant but others were sensitive).

Discussion

This study confirmed *F. columnare* genomovar II strains are responsible for severe columnaris disease outbreaks that can become recurrent within the same watershed. In addition, the data showed genomovar II is capable of causing very high mortalities in other fish species rarely affected by this pathogen. Previous studies showed that, in Alabama, both genomovars I and II coexisted in the natural environment [40]. However, in that study, the authors found an interesting host-genomovar association between genomovar II and catfishes, while genomovar I was predominantly recovered from threadfin shad. In salmonids, genomovar I seems to be the predominant causative agent of columnaris disease; however, experimental infections revealed genomovar II is indeed more virulent for rainbow trout than genomovar I [17, 23, 41-43].

Mydata supports the hypothesis that posits genomovar II as the most virulent type of columnaris disease regardless of the host.

Although all *F. columnare* isolates were genomovar II, AFLP fingerprinting provided strain-specific profiles, since the technique screens the whole genome and depicts chromosomal polymorphisms among the isolates [44]. In many instances (eg. clusters 6 and 9), isolates recovered from different fish species, whether reared together or at different facilities, shared the same AFLP profile suggesting some genomovar II types were widely spread throughout the station. This lack of host-specificity at the strain level has been previously reported in genomovar II [18]. Not surprisingly, source of isolation was the least significant variable in the study while site was the most significant one, suggesting that some strains were punctually introduced at different facilities. Isolates recovered in 2010 and 2011 clustered apart from those recovered in 2012, with just a few exceptions. This indicates that related strains were responsible for annual outbreaks, which also suggesting a successive introduction of strains into the watershed. Several factors can contribute to the introduction of *F. columnare* at different sites within the EWSFC. The exchange of fish fry or fingerlings between ponds including routine stocking of fish produced by commercial hatcheries outside the EWSFC, use of seining or fishing equipment, water flows that connect many ponds, and presence of wildlife and birds that feed on the fish could contribute to the spread of strains between different ponds at the experiment station.

Until recently, not much was known about the antimicrobial susceptibility patterns of *F. columnare* strains. In the present study, all isolates were sensitive to erythromycin, ampicillin and florfenicol. Not surprisingly, 20 isolates were found to be resistant to oxytetracycline as this antibiotic is one of the most commonly used tetracyclines for treatment of fish bacterial diseases

globally [45]. Several studies have revealed tetracycline resistance in other fish pathogens such as *Aeromonas* spp. and *Vibrio* spp. [46-48]. However, earlier studies showed no resistance of *F. columnare* strains to oxytetracycline [10, 14, 43], and not until recently, resistance of *F. columnare* towards oxytetracycline was reported [29]. Eight Finnish *F. columnare* strains were susceptible to ampicillin, erythromycin, and florfenicol [43]. This study also reported resistance of thirteen *F. columnare* isolates towards kanamycin. Formerly, the bacterium was reported to have 0 mm disk diffusion zones around kanamycin (30 μg) [49].

Nowadays, florfenicol is widely used for treatment of fish diseases in aquaculture [50, 51] and is currently approved for aquaculture purposes in 25 countries [52]. AQUAFLOR® (Merck) contains florfenicol as an active ingredient and is approved for the treatment of coldwater disease and furunculosis in salmonids as well as enteric septicaemia and columnaris disease in catfishes [53]. In this study, none of the *F. columnare* isolates displayed resistance towards florfenicol, likewise susceptibility of *F. columnare* to this antibiotic has been reported elsewhere [43, 52]. During my study, only catfish raised in F-ponds, LS ponds and raceways received AQUAFLOR®, but medicated feed is routinely used at the EWSFC during disease outbreaks.

The existence of several genotypes in disease outbreaks has been previously documented in *F. columnare* [43, 54] and *F. psychrophilum* [55]. However, co-infection by more than one strain in the same fish is rarely documented. In this study, I isolated two *F. columnare* strains that although shared the same AFLP profile did differ in their antibiotic susceptibility pattern from the same infected fish. The coexistence of more than one strain in the fish may be due to the ubiquitous distribution of *F. columnare* in aquatic environments and the lack of host-specificity observed within genomovar II strains. In addition, I observed a trend associated with

oxytetracycline resistance in the isolates from the egg masses, catfish fry and water samples. *F. columnare* isolates from the egg masses and from the catfish fry that hatched from these egg masses were resistant to oxytetracycline. However, water samples from the same tanks contained both oxytetracycline resistant and oxytetracycline sensitive isolates. It is plausible that the resistance to oxytetracycline changes the adhesion properties of the *F. columnare* cell membranes thus facilitating attachment to eggs. This hypothesis is supported by the close genetic relationship observed among egg and fry isolates (AFLP types 1-3). The possible effect of iodine disinfection in strain selection warrants further investigation.

In conclusion, genomovar II strains of *F. columnare* were responsible for the severe columnaris outbreaks affecting the EWSFC from 2010-2012. I confirmed that genomovar II of *F. columnare* is the prevalent genomovar in aquaculture and sport fishing ponds during columnaris outbreaks in the experiment station. Genomovar II strains showed a marked genetic diversity among themselves, but strain relatedness was primarily influenced by date and site of isolation suggesting periodic introductions of new strains into the watershed and punctual introductions at different facilities within the watershed. According to the results of the present study, resistance to florfenicol has not yet developed in our facilities and florfenicol remains the drug of choice for treatment of fish during columnaris outbreaks.

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References

[1] Plumb J.A., Health maintainence and principal microbial diseases of cultured fishes, Iowa State University Press, Ames, IA, 1999.

[2] Austin B., Austin D.A., Bacterial fish pathogens: disease of farmed and wild fish, Springer Verlag, 1999.

[3] Soto E., Mauel M., Karsi A., Lawrence M., Genetic and virulence characterization of *Flavobacterium columnare* from channel catfish (*Ictalurus punctatus*), Journal of Applied Microbiology. (2008) 104:1302-1310.

[4] Suomalainen L., Bandilla M., Valtonen E., Immunostimulants in prevention of columnaris disease of rainbow trout, *Oncorhynchus mykiss* (Walbaum), Journal of Fish Diseases. (2009) 32:723-726.

[5] MSU, 2010 Annual Case Summary Report, Aquatic Research and Diagnostic Laboratory, Mississippi State University, Stoneville, MS. <http://tcnwac.msstate.edu//cvm2010.pdf>. (2010): (accessed 19.14.12).

[6] MSU, 2012 ANNUAL CASE SUMMARY REPORT of the AQUATIC RESEARCH & DIAGNOSTIC LABORATORY, Mississippi State University, College of Veterinary Medicine, Thad Cochran National Warmwater Aquaculture Center (http://tcnwac.msstate.edu/cvm2012.pdf). (2012).

[7] Bader J., Nusbaum K., Shoemaker C., Comparative challenge model of *Flavobacterium columnare* using abraded and unabraded channel catfish, *Ictalurus punctatus* (Rafinesque), Journal of Fish Diseases. (2003) 26:461-467.

[8] Bader J.A., Moore S.A., Nusbaum K.E., The effect of cutaneous injury on a reproducible immersion challenge model for *Flavobacterium columnare* infection in channel catfish (*Ictalurus punctatus*), Aquaculture. (2006) 253:1-9.

[9] Shoemaker C.A., Klesius P.H., Lim C., Yildirim M., Feed deprivation of channel catfish, *Ictalurus punctatus* (Rafinesque), influences organosomatic indices, chemical composition and susceptibility to *Flavobacterium columnare*, Journal of Fish Diseases. (2003) 26:553-561.

[10] Thomas-Jinu S., Goodwin A., Acute columnaris infection in channel catfish, *Ictalurus punctatus* (Rafinesque): efficacy of practical treatments for warmwater aquaculture ponds, Journal of Fish Diseases. (2004) 27:23-28.

[11] Wakabayashi H., Effect of environmental conditions on the infectivity of *Flexibacter columnaris* to fish, Journal of Fish Diseases. (1991) 14:279-290.

[12] Suomalainen L., Tiirola M., Valtonen E., Influence of rearing conditions on *Flavobacterium columnare* infection of rainbow trout, *Oncorhynchus mykiss* (Walbaum), Journal of Fish Diseases. (2005) 28:271-277.

[13] Iguchi K.i., Ogawa K., Nagae M., Ito F., The influence of rearing density on stress response and disease susceptibility of ayu (*Plecoglossus altivelis*), Aquaculture. (2003) 220:515-523.

[14] Thomas-Jinu S., Goodwin A., Morphological and genetic characteristics of *Flavobacterium columnare* isolates: correlations with virulence in fish, Journal of Fish Diseases. (2004) 27:29-35.

[15] Decostere A., Haesebrouck F., Turnbull J., Charlier G., Influence of water quality and temperature on adhesion of high and low virulence *Flavobacterium columnare* strains to isolated gill arches, Journal of Fish Diseases. (1999) 22:1-11.

[16] Shoemaker C.A., Olivares-Fuster O., Arias C.R., Klesius P.H., *Flavobacterium columnare* genomovar influences mortality in channel catfish (*Ictalurus punctatus*), Veterinary Microbiology. (2008) 127:353-359.

[17] Triyanto A., Wakabayashi H., Genotypic diversity of strains of *Flavobacterium columnare* from diseased fishes, Fish Pathology. (1999) 34:65–71.

[18] Arias C.R., Welker T.L., Shoemaker C.A., Abernathy J.W., Klesius P.H., Genetic fingerprinting of *Flavobacterium columnare* isolates from cultured fish, Journal of Applied Microbiology. (2004) 97:421-428.

[19] Darwish A.M., Ismaiel A.A., Genetic diversity of *Flavobacterium columnare* examined by restriction fragment length polymorphism and sequencing of the 16S ribosomal RNA gene and the 16S–23S rDNA spacer, Molecular and Cellular Probes. (2005) 19:267-274.

[20] Olivares-Fuster O., Shoemaker C.A., Klesius P.H., Arias C.R., Molecular typing of isolates of the fish pathogen, *Flavobacterium columnare*, by single-strand conformation polymorphism analysis, Fems Microbiology Letters. (2007) 269:63-69.

[21] Arias C.R., Cai W., Peatman E., Bullard S.A., Catfish hybrid *Ictalurus punctatus*× *I. furcatus* exhibits higher resistance to columnaris disease than the parental species, Diseases of Aquatic Organisms. (2012) 100:77.

[22] Olivares-Fuster O., Bullard S.A., McElwain A., Llosa M.J., Arias C.R., Adhesion dynamics of *Flavobacterium columnare* to channel catfish *Ictalurus punctatus* and zebrafish *Danio rerio* after immersion challenge, Diseases of Aquatic Organisms. (2011) 96:221.

[23] LaFrentz B., LaPatra S., Shoemaker C., Klesius P., Reproducible challenge model to investigate the virulence of *Flavobacterium columnare* genomovars in rainbow trout *Oncorhynchus mykiss*, Diseases of Aquatic Organisms. (2012) 101:115.

[24] Miyashita N.T., Kawabe A., Innan H., DNA variation in the wild plant *Arabidopsis thaliana* revealed by amplified fragment length polymorphism analysis, Genetics. (1999) 152:1723-1731.

[25] Declercq A.M., Haesebrouck F., Van den Broeck W., Bossier P., Decostere A., Columnaris disease in fish: a review with emphasis on bacterium-host interactions, Veterinary research. (2013) 44:27.

[26] Farmer B., Improved methods for the isolation and characterization of *Flavobacterium columnare*, 2004.

[27] Decostere A., *Flavobacterium columnare* infections in fish: the agent and its adhesion to the gill tissue, Verhandelingen-Koninklijke Academie voor Geneeskunde van België. (2002) 64:421.

[28] Ekman E., Natural and experimental infections with *Flavobacterium psychrophilum* in salmonid fish, Doctoral thesis, Swedish University of Agricultural Sciences, Uppsala., 2003.

[29] Declercq A., Boyen F., den Broeck W., Bossier P., Karsi A., Haesebrouck F., Decostere A., Antimicrobial susceptibility pattern of *Flavobacterium columnare* isolates collected worldwide from 17 fish species, Journal of Fish Diseases. (2013) 36:45-55.

[30] Serrano P.H., Responsible use of antibiotics in aquaculture, Food & Agriculture Org, 2005.

[31] Miranda C.D., Rojas R., Occurrence of florfenicol resistance in bacteria associated with two Chilean salmon farms with different history of antibacterial usage, Aquaculture. (2007) 266:39-46.

[32] Schmidt A.S., Bruun M.S., Dalsgaard I., Pedersen K., Larsen J.L., Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms, Applied and Environmental Microbiology. (2000) 66:4908-4915.

[33] Suomalainen L.-R., *Flavobacterium columnare* in Finnish fish farming: characterisation and putative disease management strategies, University of Jyväskylä, 2005.

[34] Thoesen J.C., Suggested procedures for the detection and identification of certain finfish and shellfish pathogens, Vol. American Fisheries Society-Fish Health Section, Bethesda, ML, American Fisheries Society, 2004.

[35] Decostere A., Haesebrouck F., Devriese L.A., Shieh medium supplemented with tobramycin for selective isolation of *Flavobacterium columnare* (*Flexibacter columnaris*) from diseased fish, Journal of clinical microbiology. (1997) 35:322-324.

[36] Welker T.L., Shoemaker C.A., Arias C.R., Klesius P.H., Transmission and detection of *Flavobacterium columnare* in channel catfish *Ictalurus punctatus*, Diseases of Aquatic Organisms. (2005) 63:129-138.

[37] Bauer A., Kirby W., Sherris J.C., turck, Turck M., Antibiotic susceptibility testing by a standardized single disk method, American journal of clinical pathology. (1966) 45:493.

[38] Clarke K.R., Non-parametric multivariate analyses of changes in community structure, Australian Journal of Ecology. (1993) 18:117-143.

[39] Arias C.R., Pujalte M.J., Garay E., Aznar R., Genetic relatedness among environmental, clinical, and diseased-eel Vibrio vulnificus isolates from different geographic regions by ribotyping and randomly amplified polymorphic DNA PCR, Applied and Environmental Microbiology. (1998) 64:3403-3410.

[40] Olivares-Fuster O., Baker J.L., Terhune J.S., Shoemaker C.A., Klesius P.H., Arias C.R., Host-specific association between *Flavobacterium columnare* genomovars and fish species, Systematic and Applied Microbiology. (2007) 30:624-633.

[41] Avendano-Herrera R., Gherardelli V., Olmos P., Godoy M.G., Heisinger A., Fernandez J., *Flavobacterium columnare* associated with mortality of salmonids farmed in Chile: a case report of two outbreaks, Bulletin of the European Association of Fish Pathologists. (2011) 31:36-44.

[42] Schneck J.L., Caslake L.F., Genetic diversity of *Flavobacterium columnare* isolated from fish collected from warm and cold water, Journal of Fish Diseases. (2006) 29:245-248.

[43] Suomalainen L., Kunttu H., Valtonen E., Hirvelä-Koski V., Tiirola M., Molecular diversity and growth features of *Flavobacterium columnare* strains isolated in Finland, Diseases of Aquatic Organisms. (2006) 70:55-61.

[44] Kokotovic B., Bölske G., Ahrens P., Johansson K.E., Genomic variations of *Mycoplasma capricolum* subsp. *capripneumoniae* detected by amplified fragment length polymorphism (AFLP) analysis, Fems Microbiology Letters. (2000) 184:63-68.

[45] Rigos G., Troisi G., Antibacterial agents in Mediterranean finfish farming: a synopsis of drug pharmacokinetics in important euryhaline fish species and possible environmental implications, Reviews in Fish Biology and Fisheries. (2005) 15:53-73.

[46] Čížek A., Dolejská M., Sochorová R., Strachotová K., Piačková V., Veselý T., Antimicrobial resistance and its genetic determinants in aeromonads isolated in ornamental (koi) carp (*Cyprinus carpio koi*) and common carp (*Cyprinus carpio*), Veterinary Microbiology. (2010) 142:435-439.

[47] Weir M., Rajic A., Dutil L., Cernicchiaro N., Uhland F., Mercier B., Tusevljak N., Zoonotic bacteria, antimicrobial use and antimicrobial resistance in ornamental fish: a systematic review of the existing research and survey of aquaculture-allied professionals, Epidemiology and infection. (2012) 140:192-206.

[48] Verner-Jeffreys D.W., Welch T.J., Schwarz T., Pond M.J., Woodward M.J., Haig S.J., Rimmer G.S., Roberts E., Morrison V., Baker-Austin C., High prevalence of multidrug-tolerant bacteria and associated antimicrobial resistance genes isolated from ornamental fish and their carriage water, PloS one. (2009) 4:e8388.

[49] Bernardet J.-F., Grimont P.A., Deoxyribonucleic acid relatedness and phenotypic characterization of *Flexibacter columnaris* sp. nov., nom. rev., *Flexibacter psychrophilus* sp. nov., nom. rev., and *Flexibacter maritimus* Wakabayashi, Hikida, and Masumura 1986, International journal of Systematic and Evolutionary Microbiology. (1989) 39:346-354.

[50] Aoki T., Transferable drug resistance plasmids in fish-pathogenic bacteria, Use of Chemicals in Aquaculture in Asia: Proceedings of the Meeting on the Use of Chemicals in Aquaculture in Asia 20-22 May 1996, Tigbauan, Iloilo, Philippines, 2000.

[51] Sapkota A., Sapkota A.R., Kucharski M., Burke J., McKenzie S., Walker P., Lawrence R., Aquaculture practices and potential human health risks: current knowledge and future priorities, Environment international. (2008) 34:1215-1226.

[52] Gaunt P.S., Gao D.N., Sun F.S., Endris R., Efficacy of Florfenicol for Control of Mortality Caused by *Flavobacterium columnare* Infection in Channel Catfish, Journal of Aquatic Animal Health. (2010) 22:115-122.

[53] FDA, Available at: http://www.fda.gov/AnimalVeterinary/DevelopmentApprovalProcess/Aquaculture/ucm132954.htm. (2011).

[54] Triyanto, Kumamaru A., Wakabayashi H., The use of PCR targeted 16S rDNA for identification of genomovars of *Flavobacterium columnare*, Fish Pathology. (1999) 34:217–218.

[55] Madetoja J., Hänninen M.L., Hirvelä–Koski V., Dalsgaard I., Wiklund T., Phenotypic and genotypic characterization of *Flavobacterium psychrophilum* from Finnish fish farms, Journal of Fish Diseases. (2001) 24:469-479.

Table 3-1. List of all 102 *F. columnare* isolates included in the study with isolate names, fish host, site of isolation, date of isolation.

Isolate name[a]	No. of Isolates	Source	Site[b]	Date
CC-S 25	1	Channel catfish	LS	2010
CC-G	1	Channel catfish	LS	2010
TL-S 1-3	3	Tilapia	LS	2010
TL-G	1	Tilapia	LS	2010
CC-E 2-6,9	6	Egg masses	HA	2011
CC-W 1-6	6	Water	HA	2011
CC-F 2,4-7	5	Channel catfish	VS	2011
BG-S 1-2, 15-22	10	Bluegill	S6	2012
BG-S 3-10	8	Bluegill	S3	2012
LB-S 1-2	2	Largemouth bass	S1	2012
BG-S 11-14	4	Bluegill	S1	2012
LB-S 3-4	2	Largemouth bass	S6	2012
BC-S 1-3	3	Blue catfish	GL	2012
HC-S 1-2	2	Hybrid catfish	GL	2012
CC-S 1-3	3	Channel catfish	GL	2012
HC-S 3-6	4	Hybrid catfish	S10	2012
SpCC-S 2-4	3	Channel catfish	RB	2012
SpCC-G	1	Channel catfish	RB	2012
SpCC-W 1-6	6	Water	RB	2012
CC-S 4-9	6	Channel catfish	RW-S1	2012
CC-S 10-14	5	Channel catfish	WL	2012
BG-S 23-27	5	Bluegill	WL	2012
CC-S 15-19	5	Channel catfish	S11	2012
CC-S 20-24	5	Channel catfish	RW-S1	2012
BG-S 28-32	5	Bluegill	F-ponds	2012

[a], CC, channel catfish; BC, blue catfish; HC, hybrid catfish; TL, tilapia; LB, largemouth bass; BG, bluegill; SpCC, spawning channel catfish; S, skin; G, gill; E, eggs; W, water

[b], LS, lower station; HA, hatchery; VS, veterinary school; GL, genetics laboratory; S, upper station ponds; RB, research buildings at the EWSFC; RW, raceways; WL; on campus-wet laboratory; F-ponds, lower station ponds

Table 3-2. Antimicrobial agents used in the study: compound concentrations, interpretation of inhibition zones, and percent of each class of susceptibility (Sensitive, Intermediate and Resistant). Number of isolates n= 102.

Interpretation[a]	**Erythromycin 15 µg**	**Oxytetracycline 30 µg**	**Ampicillin 10 µg**	**Kanamycin 30 µg**	**Florfenicol 30 µg**
S	99%	79.4	100%	1%	100%
I	1%	1%	0%	86.30%	0%
R	0%	19.6	0%	12.70%	0%
Ranges of zone diameter (mm)	R= <13, I= 16-20, S= >21	R= <14, I= 15-18, S= >19	R= <13, I= 14-16, S= >17	R= <13, I= 14-17, S= >18	R= <14, I= 15-18, S= >19

[a], S, sensitive or susceptible; I, intermediate; R, resistant

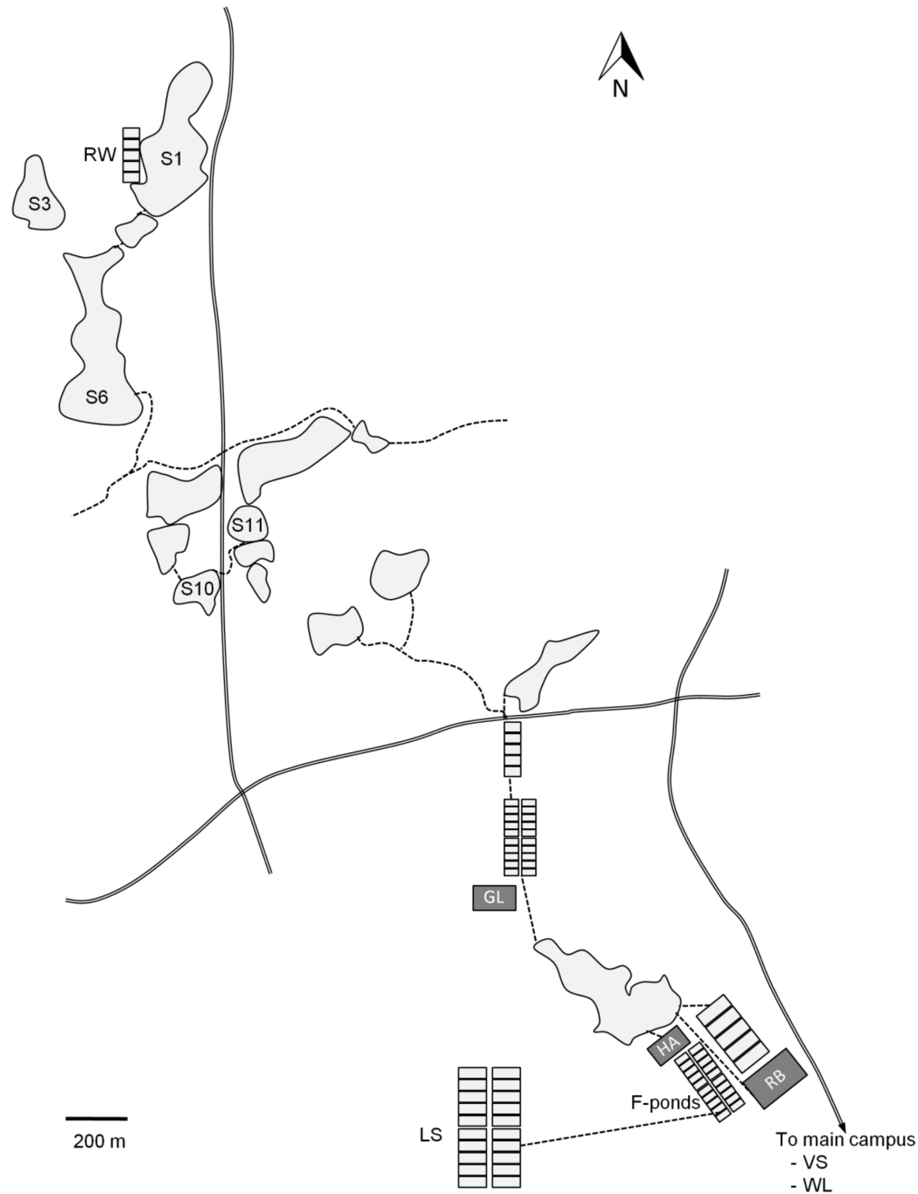
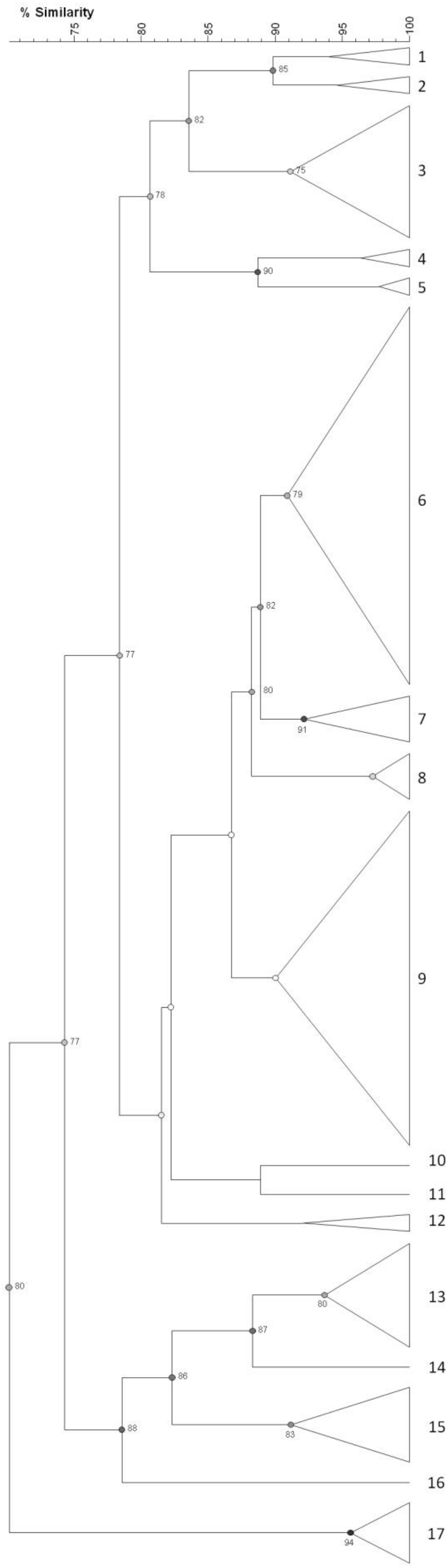


Figure 3-1. Map of the E. W. Shell Fisheries Center including the upper ponds (S1-S11), genetics unit (GL), and lower station (HA, hatchery; F-ponds; RB, research building; other lower station ponds, LS). Dotted lines represent permanent or intermitent water flows. Double lanes represent paved roads. All ponds and buildings are communicated by unpaved roads. Enclosed buildings are represented by grey squares. Main campus facilities (School of Veterinary Medicine, VS, and our on campus facility, WL) are located approximately 10 km south of the lower station.


% Similarity
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2011 VS CC-F 4
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2011 VS CC-F 5
2011 HA CC-W 6
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2011 HA CC-W 2
2011 VS CC-F 2
2011 VS CC-F 7
2011 HA CC-E 2
2011 HA CC-E 6
2011 HA CC-E 5
2011 HA CC-E 4
2011 HA CC-E 9
2011 HA CC-W 5
2012 S10 HC-S 3
2012 S10 HC-S 5
2012 S10 HC-S 4
2012 S10 HC-S 6
2012 S1 BG-S 12
2012 S1 BG-S 14
2012 RW-S1 CC-S 4
2012 S1 BG-S 13
2012 RW-S1 CC-S 7
2012 S3 BG-S 5
2012 S1 BG-S 11
2012 RW-S1 CC-S 5
2012 RW-S1 CC-S 6
2012 S3 BG-S 7
2012 S3 BG-S 9
2012 S3 BG-S 8
2012 S3 BG-S 10
2012 RW-S1 CC-S 8
2012 S3 BG-S 3
2012 S3 BG-S 6
2012 S6 BG-S 2
2012 RB SpCC-W 4
2012 RB SpCC-W 3
2012 RB SpCC-S 3
2012 RB SpCC-S 4
2012 RB SpCC-S 2
2012 RB SpCC-W 1
2012 RB SpCC-G
2011 HA CC-W 1
2011 HA CC-W 4
2011 HA CC-W 3
2012 F-Ponds BG-S 29
2012 F-Ponds BG-S 31
2012 RW-S1 CC-S 21
2012 F-Ponds BG-S 32
2012 S1 LB-S 1
2012 S1 LB-S 2
2012 S6 BG-S 1
2012 S3 BG-S 4
2012 RW-S1 CC-S 20
2012 RW-S1 CC-S 22
2012 RW-S1 CC-S 23
2012 RW-S1 CC-S 24
2012 F-Ponds BG-S 30
2012 GL CC-S 2
2012 GL CC-S 3
2012 RW-S1 CC-S 9
2012 S6 BG-S 22
2012 GL BC-S 1
2012 S6 LB-S 3
2012 GL BC-S 2
2012 GL HC-S 2
2012 GL BC-S 3
2012 GL HC-S 1
2012 RB SpCC-W 5
2012 RB SpCC-W 6
2012 S6 BG-S 16
2012 S6 BG-S 20
2012 S6 LB-S 4
2012 S6 BG-S 21
2012 GL CC-S 1
2012 S6 BG-S 17
2012 S6 BG-S 18
2012 RB SpCC-W 2
2012 F-Ponds BG-S 28
2012 S6 BG-S 15
2012 S6 BG-S 19
2012 WL CC-S 14
2012 WL BG-S 24
2012 WL CC-S 11
2012 WL CC-S 13
2012 WL BG-S 27
2012 WL CC-S 12
2012 WL BG-S 23
2012 WL CC-S 10
2012 WL BG-S 26
2010 LS TL-G
2010 LS TL-S 3
2010 LS TL-S 1
2010 LS TL-S 2
2010 LS CC-S 25
2010 LS CC-G
2012 WL BG-S 25
2012 S11 CC-S 15
2012 S11 CC-S 17
2012 S11 CC-S 16
2012 S11 CC-S 18
2012 S11 CC-S 19

Figure 3-2. Dendrogram was derived by UPGMA cluster analysis of the AFLP profiles of 102 *Flavobacterium columnare* isolates. Linkage levels are expressed as percentage similarity based on the Pearson correlation coefficient. Unique AFLP clusters were defined at 90% similarity.

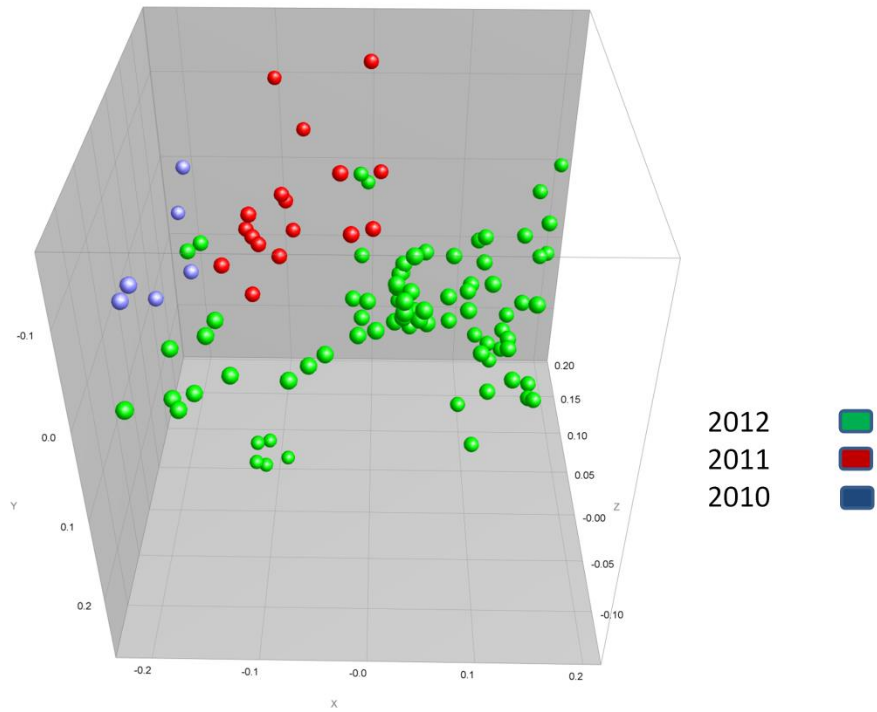

Figure 3-3. Multidimensional scaling (MDS) plot of the similarity matrix obtained by comparing AFLP fingerprints of all the recovered *F. columnare* isolates from 2010, 2011 and 2012. Distance between entries represents graphical dissimilarities obtained from the similarity matrix.

CHAPTER 4. NEW ATTENUATED VACCINE AGAINST COLUMNARIS DISEASE IN FISH: CHOOSING THE RIGHT PARENTAL STRAIN IS CRITICAL FOR VACCINE EFFICACY

Abstract

Flavobacterium columnare, the causative agent of columnaris disease, is a highly diverse species comprised by three genetic groups or genomovars. Genomovar II strains present a higher degree of virulence towards catfishes than genomovar I isolates. The objective of this study was to compare the vaccine efficacy of avirulent mutants derived from genomovars I and II using a rifampicin-resistance strategy. First, we compared the efficacy of 13 genomovar II mutants in channel catfish (*Ictalurus punctatus*) fingerlings and we identified mutant 17-23 as the best vaccine candidate based on their relative percent survival (RPS) against a highly virulent genomovar II strain (BGFS-27). In the second experiment, I vaccinated zebrafish (*Danio rerio*) with two genomovar II mutants (17-23 and 16-534) and FCRR (genomovar I mutant) followed by exposure to BGFS-27 strain. RPS values were 28.4, 20.3 and 8.1% for 17-23, 16-534, and FCRR, respectively. For experiments 3 and 4, I tested both 17-23 and FCRR in channel catfish fry and Nile tilapia (*Oreochromis niloticus*). In both experiments, vaccinated fish were divided in two groups and each challenged with either a genomovar I (ARS-1) or a II (BGFS-27) strain. Channel catfish fry vaccinated with 17-23 and FCRR followed by challenge with BGFS-27 resulted in RPS values of 37.0% and 4.4%. When fish were challenged with ARS-1, RPS value

were 90.9% and 72.7% for fish vaccinated with 17-23 and FCRR, respectively. Nile tilapia vaccinated with 17-23 and FCRR followed by challenged with BGFS-27 had RPS values of 82.1% and 16.1%, respectively. When fish were challenged with strain ARS-1, RPS values were 86.9% and 75.5%. Overall, the results demonstrated that vaccination with genomovar II mutant 17-23 confers better protection in channel catfish and Nile tilapia than FCRR against columnaris disease caused by genomovar II. Both mutants were equally protective against columnaris caused by genomovar I showing that 17-23 mutant cross-protected against both genomovars.

Introduction

Flavobacterium columnare is the causative agent of columnaris disease that affects wild, cultured and ornamental fish populations worldwide [1, 2]. In the United States, columnaris disease is one of the leading causes of mortality in catfish farms, with in-pond mortality rates among adults and fingerlings reaching up to 60 and 90%, respectively [3]. Eradication of columnaris disease from culture settings is unlikely since this bacterium is ubiquitous in freshwater environments [4]. Sustainable aquaculture needs to maximize disease prevention and vaccination has become one of the best tools to achieve that goal. Effective vaccines are ultimately the safest prophylactic approach to mitigate the effect of infectious diseases [5]. Among the several types of vaccines available, live attenuated vaccines are those in which the pathogen has been modified and is no longer virulent to the host [6]. The use of attenuated vaccines holds tremendous potential because they present multiple immunogens while building in adjuvanticity that elicits strong humoral and cell-mediated protection [7]. In catfish aquaculture, where individual vaccination is cost-prohibitive, attenuated vaccines have the additional advantage of ease of delivery through feed or by immersion [8, 9].

One of the most successful strategies used to obtain attenuated mutants in Gram-negative bacteria is by passing virulent isolates onto media containing increasing concentrations of the antibiotic rifampicin. This strategy has been successfully used to develop modified live attenuated bacterial vaccines for commercial use in cattle and fish [10-12]. Currently, a modified live *F. columnare* vaccine is available for commercial use to prevent columnaris disease under the licensed name AQUAVAC-COL^{TM} (Merk & Co., Inc.). The active ingredient in this vaccine is an avirulent rifampicin-resistant mutant of *F. columnare*, strain FCRR, derived from a genomovar I strain [13].

Recently, we have developed safe and permanently stable rifampicin-resistant mutants from highly virulent genomovar II strains [14]. Since genomovar II strains are more virulent towards catfishes, I hypothesized that a genomovar II-based vaccine will increase the protective effect of vaccination against columnaris disease. Therefore, the purpose of this study was to compare the efficacy of the genomovar I and II rifampicin-resistant mutants in fish species susceptible to columnaris disease. Channel catfish (*Ictalurus punctatus*), and Nile tilapia (*Oreochromis niloticus*) were chosen based on their economic relevance as food aquaculture species while zebrafish (*Danio rerio*) is cultured worldwide for both ornamental and experimental purposes.

Materials and Methods

Fish Husbandry. Channel catfish fingerlings (n = 540, mean weight = 5.5 ± 0.8 g) and 21 day post hatch channel catfish fry (n = 480; mean weight = 0.05 ± 0.003 g) were supplied by the School of Veterinary Medicine at Auburn University and a commercial hatchery in Mississippi, respectively. Fish were transferred in aerated containers to the Aquatic Microbiology Laboratory

(AML) at Auburn University. Non-sexed adult zebrafish (*Danio rerio*) (n = 360, mean weight = 0.45 ± 0.04 g) were purchased from Aquatica Tropicals (Plant City, FL, USA), and express shipped to AML. All male Nile tilapia juveniles (n = 600, mean weight = 9.4 ± 0.5 g) were obtained from E. W. Shell Fisheries Center at North Auburn Fisheries experiment Station and transported to AML in aerated containers. Upon arrival to AML, fish were stocked into 37 L glass aquaria/tanks (stocking rates are mentioned under each experiment). Fish were acclimated for at least 5 days before vaccination, and fed daily to apparent satiation with AQUAMAX Grower 100 (channel catfish fry and zebrafish) or 400 (channel catfish fingerlings and tilapia) (Purina Mills, Inc., St. Louis, MO, USA). Ten randomly-selected individuals of each fish species were examined and proved culture negative for *F. columnare* prior to stocking in the tanks following standard protocols [15]. Each tank had an individual biofilter and an air stone. Water was prepared with 340 g of Marine Salt (Seachem, Madison, Georgia) diluted in 10 L of deionized water to make the primary salt stock. For tank use, 0.97 g of $CaCO_3$, 2.26 g of $NaHCO_3$, and 110 mL of the stock were mixed overnight in 55 L of deionized water. Water quality was checked daily to maintain established parameters (80 ppm alkalinity, 40 ppm hardness, 0.1 ppt salinity, 27±1 °C, pH 7.8±0.2 [mean±standard error], ammonia and nitrites were non-detectable). At the time of vaccination, aquaria were assigned blindly to each treatment group. All animal protocols, including vaccination experiments, were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC number 2009-1609).

Bacterial strains and growth conditions. Previously generated and characterized rifampicin-resistant mutants of *F. columnare* genomovar I [13] and II [14] were used in the following experiments. Briefly, mutants were generated by successive passes on culture medium with increasing concentrations of rifampin (from 50 µg/mL to 300 µg/mL). Only one mutant

from genomovar I was available (FCRR) while 8 genomovar II mutants were included in the study. All *F. columnare* strains used in this study were stored at -80 °C as glycerol stocks and were cultured on modified Shieh (MS) agar [14] or in MS broth with shaking at 100 rpm at 28 °C for 24 h. Plate counts of each strain (in triplicates) were carried out to calculate the average number of colony forming units per milliliter (CFU/mL) of bacteria used to vaccinate or to challenge fish as reported under each experiment.

Mutant safety and stability. The *in vitro* stability of genomovar II mutant 17-23 had already been tested [14] but its *in vivo* stability was assayed by inoculating five channel catfish fingerlings (of approximately 15 cm in length) with ca. 10^5 CFU/mL of 17-23. Fish were inoculated by intraperitoneal injection and monitored for 2 days for signs of disease. After 2 days, fish were euthanized with 300 ppm of tricaine methanesulfonate, and 17-23 mutant was recovered onto MS supplemented with 300 µg/mL of rifampicin. The newly recovered isolate was used as inoculum for the second round of intraperitoneal injections. This iterative process was repeated six times. No mortalities or sings of columnaris disease were observed. In addition, and to confirm the safety of 17-23 by immersion, 30 channel catfish fingerlings (equally divided into 3 replicates) were immersed in ca. 10^8 CFU/mL of 17-23. This experiment was repeated twice. No mortalities or signs of columnaris disease were observed

Comparison between vaccine efficacies of genomovar II mutants – Experiment 1. Channel catfish fingerlings (n=540) were vaccinated with Genomovar II mutants (Table 4-1) by immersion following previously described protocols [16]. Briefly, fish (15 fish/tank; 4 replicates per treatment) were vaccinated in a 2 L bath at room temperature (25°C) containing the experimental vaccine (see Table 4-1 for each individual dose). Control treatment fish (15 fish/tanks; 4 replicates) were sham vaccinated using MS broth as inoculum in the vaccination

bath. After 30 min in the vaccination bath, fish were returned to their tanks and maintained under normal husbandry conditions. At 28 days post-vaccination, fish were challenged by immersion with genomovar II strain BGFS-27, a highly virulent strain of *F. columnare* as per Shoemaker et al. [17]. Fish were returned to their tanks and monitored twice a day for signs of disease. Moribund and dead fish were promptly removed from the tanks. *F. columnare* was isolated from the dead fish by plating on MS agar medium containing 1 µg/mL tobramycin [18]. Putative *F. columnare* colonies were presumptively identified based on their pigmentation and characteristic colony morphology on agar plates and selected colonies were confirmed by specific PCR [19].

Zebrafish vaccination – Experiment 2. A total of 336 adult zebrafish were vaccinated by immersion in a 2 L bath for 30 min with the following treatments: 17-23, 16-534 and FCRR at concentrations of 7.8×10^6, 1.5×10^6 and 9.1×10^6 CFU/mL. The genomovar II mutant 16-534 was included in this study because preliminary data (not shown) on *F. columnare* virulence in zebrafish concluded that the parental strain of this mutant was the most virulent strain for this species. Each treatment consisted of 3 replicates (tanks) with 28 zebrafish per tank. A control treatment (3 tanks) was sham vaccinated by immersion in sterile MS broth. Post-vaccination and challenge protocols followed those described in Experiment 1.

Channel catfish fry vaccination – Experiment 3. A total of 1,500 channel catfish fry were distributed in three treatment groups: 17-23, FCRR and control. Each treatment group consisted of 10 replicates (tanks) with 50 fish per tank. Fish were vaccinated as described above with 17-23 and FCRR at concentrations of 6.5×10^6 and 6.3×10^6 CFU/mL, respectively. Fish in the control treatment were sham vaccinated as described above. Following vaccination, fry in each treatment group were placed back into their holding tanks and maintained under normal husbandry conditions. At 28 days post-vaccination, the 10 replicates per treatment were

randomly divided into two groups: A and B, with 5 replicates each. The fish in group A were challenged with *F. columnare* strain BGFS-27 (genomovar II) while fish in group B were challenged with strain ARS-1 a genomovar I strain of lower virulence potential [20] at concentrations of 7.6×10^6 and 9.8×10^6 CFU/mL, respectively. Post-challenge protocols followed those described in Experiment 1.

Nile tilapia vaccination and challenge – Experiment 4. A total of 510 Nile tilapia juveniles were distributed into three treatment groups, 170 fish were used in each group (17 fish per tank, ten replicates). Tilapia were vaccinated as described above using the experimental vaccine 17-23 and FCRR at concentrations of 2.2×10^6 and 7.3×10^6 CFU/mL, respectively for 30 min. A control group (10 replicates) was sham vaccinated as described previously. After vaccination, the fish in each treatment group were removed from the vaccine bath, replaced back into their original holding aquaria and maintained under normal husbandry conditions. At 28 days post-vaccination, the 10 replicates per treatment were randomly divided into two groups: A and B, with 5 replicates each. The fish in group A were challenged with *F. columnare* strain BGFS-27 (genomovar II) while fish in group B were challenged with strain ARS-1 as in Experiment 3. Challenge doses were 2.7×10^6 and 5.5×10^6 CFU/mL for BGFS-27 and ARS-1, respectively. Post-challenge protocols followed those described in Experiment 1.

Statistical analysis. Results of challenges were presented as cumulative percent mortality and RPS [21]. Cumulative percent mortality was calculated by dividing the number of dead fish per time period by the total number of fish per treatment and multiplying by 100. RPS was calculated according to the following formula: RPS = [1 − (vaccinated mortality/control mortality)] ×100. Mortality data were analyzed by one-way analysis of variance (ANOVA) using general linear model (PROC GLM) followed by Duncan's multiple range test (SAS Institute,

Cary, NC.) to determine significant differences ($p< 0.05$) between the mean mortality of treatment groups and replicates (tanks) in trials 1, and 2. For trials 3 and 4, Tukey's Studentized Range (HSD) test for all-pairwise comparisons was used to determine significant ($p< 0.05$) difference between vaccinated (either with the genomovar II experimental vaccines or FCRR) and non-vaccinated fish challenged with either genomovar I or II.

Results

The stability and safety of the rifampicin-resistant genomovar II mutants were confirmed *in vivo*. No mortalities due to 17-23 occurred after immersion or intraperitoneal challenges. Fish vaccinated with 17-23 did not displayed signs of columnaris disease prior to challenge with virulent strains in any of the experiments carried out to date.

Comparison of genomovar II mutants – Experiment 1. Table 4-1 shows the results obtained when channel catfish fingerlings were vaccinated with 8 genomovar II mutants. All mutants conferred some level of protection against *F. columnare* but the degree of protection varied based on the individual mutant. Cumulative percent mortality ranged from 53% in the control treatment to 24.0% when fish were vaccinated with mutant 17-23. Four mutants provided significantly higher ($p<0.05$) cumulative percent survival than the control treatment: 11-131, 15-133, 16-534, and 17-23. Following challenge, all the dead fish were confirmed positive for *F. columnare* by culturing. Based on these results, mutant 17-23 was selected for further experiments in channel catfish and tilapia.

3.1. Zebrafish vaccination and challenge – Experiment 2. The cumulative percent mortality observed in fish vaccinated with (17-23) was 63% and significantly lower than the cumulative percent mortality of the control treatment (88%) (Table 4-2). The cumulative percent mortality

(70%) observed if fish vaccinated with 16-534 mutant was not statistically significant than that observed in the control treatment (88%). Genomovar I mutant, FCRR, showed less protection (cumulative mortality of 81%) than genomovar II mutant 17-23 and it was not significant different from the control treatment. Although all vaccinated fish exhibited a lower cumulative mortality than the sham vaccinated fish, only fish vaccinated with 17-23 showed a significant lower cumulative mortality than the control group. RPS was 28.4, 20.3 and 8.1% in the fish vaccinated with 17-23, 16-534 and FCRR, respectively (Table 4-2). Following challenge, all the dead fish were confirmed positive for *F. columnare*.

Channel catfish fry vaccination and challenge – Experiment 3. Table 4-3 shows the results of vaccination in channel catfish fry. No significant differences were found between control treatment and fish vaccinated with 17-23 and FCRR when genomovar I ARS-1 strain was used for challenge. The low mortality induced by this strain in channel catfish fry (only 16.5% cumulative mortality in the control group) likely masked any potential benefit of the vaccines. Conversely, when genomovar II BGFS-27 strain was used for challenge, significant differences ($p< 0.05$) in survival were found between fish vaccinate with 17-23 and FCRR. Fish vaccinated with 17-23 had a 37.0% RPS in contrast with only 4.4% RPS in fish vaccinated with FCRR. Cumulative percent mortality in control fish challenged with BGFS-27 was 65.9% (significantly higher than in control fish challenged with ARS-1).

Nile tilapia vaccination and challenge – Experiment 4. Nile tilapia were equally susceptible to columnaris disease caused by genomovar I (ARS-1) and II (BGFS-27) strains with a cumulative percent mortality of 62.4 and 65.9%, respectively (Table 4-4). However, significant differences in vaccine efficacy were observed in Nile tilapia. When Nile tilapia were vaccinated with 17-23 and challenged with BGFS-27 and ARS-1, both groups showed a cumulative percent

mortality significantly lower than that in the control group. Conversely, when Nile tilapia were vaccinated with FCRR and challenged with BGFS-27 and ARS-1, the cumulative percent mortalities between both groups were significantly different. However, the survival of fish vaccinated with FCRR and challenged with BGFS-27 was not statistically different from control fish. Figure 4-1 shows the cumulative mortality in all treatments over time. RPS reflected similar results to cumulative mortality. High RPS (>80%) were obtained when Nile tilapia were vaccinate with 17-23, regardless of which strain was used for challenge.

In summary, 17-23 conferred significant protection against columnaris disease caused by genomovar II in all species tested. Vaccine efficacy of 17-23 was higher than that of genomovar I mutant FCRR in channel catfish fry and Nile tilapia exposed to genomovar II but was not significantly different in zebrafish. In both channel catfish fry and Nile tilapia a certain degree of cross-protection was observed when fish were vaccinated with 17-23 and challenged with ARS-1 although it was only significant in Nile tilapia due to the low mortality observed in channel catfish fry challenged with ARS-1. The opposite scenario, fish vaccinated with genomovar I and challenged with genomovar II, did not result in significant protection.

Discussion

Infectious diseases are one of the main factors limiting aquaculture productivity [22]. Continuous efforts have been devoted over the last two decades to develop effective means of vaccination against bacterial infections including columnaris disease. At present, a modified live vaccine, AQUAVAC-COL™, is commercially available to protect US farm raised catfish against columnaris disease. However, the efficacy of AQUAVAC-COL™ has been questioned in some forums. To date, only one study has attempted to characterize the efficacy of

AQUAVAC-COL™ under field conditions [23]. According to Kirkland (2010) no significant differences were found in survival rates or production traits of pond-raised hybrid catfish (female channel catfish X male blue catfish, *Ictalurus furcatus*) between vaccinated and unvaccinated fish.

Strain selection is a critical step in vaccine development. AQUAVAC-COL™ contains an avirulent rifampicin-resistant mutant of *F. columnare* genomovar I, the less virulent group for catfishes [8, 13]. In addition, epidemiological data on columnaris disease showed that genomovar II is more broadly distributed among catfishes than genomovar I [24]. These facts lead me to hypothesize that a rifampicin-resistant mutant derived from highly virulent genomovar II will confer better protection against columnaris disease than the current commercial vaccine. Previous efforts have failed to obtain rifampicin-resistant mutants from genomovoar II strains [8]. Our group recently succeeded in developing new rifampin-resistant mutants from genomovar II strains [25] that were tested as vaccines in the present study. Not all the genomovar II mutants tested in this study behaved equally further enhancing the notion that rifampicin-induced mutations are unique events in *F. columnare* as previously reported by whole-genome fingerprinting of these mutants [14].

Overall, the results showed that genomovar II mutant 17-23 confers better protection against columnaris disease than FCRR in channel catfish and Nile tilapia. Percent mortalities in sham vaccinated fish varied depending on the experiment but ranged from 53% (channel catfish fingerlings) to 88% (zebrafish) when genomovar II was use for challenge. Differences in genetic stocks, physiological state and rearing conditions can clearly influence fish mortality but the mortality values I obtained were in range with those from previous studies on catfishes [16, 20]. The RPS observed when fish were vaccinated with 17-23 and challenged with genomovar II

vastly differed depending on fish species and ranged from 37% (catfish fry) to 82% (Nile tilapia). Catfish fry showed the lowest RPS with both tested vaccines but 17-23 coffered significantly greater protection than FCRR. Although fish present a non-adaptive immune system prior to hatching, adaptive immunity requires around 28 days to develop (depending on fish species) [17]. It is plausible that fry at 21 days post hatch lacked a fully developed immune system. However, vaccination at this age is the standard practice for the catfish industry.

Recently, Shoemaker et al. [17] showed that AQUAVAC-COL™ conferred protection against challenge with genomovar I and II strains. However, the RPS in fish challenged with genomovar II was 57% *versus* an RPS of 87-96% in fish challenged with genomovar I. The authors suggested that AQUAVAC-COL™ presents core antigens found in both genomovar and thus elicits a protective response against either type of columnaris disease. My data shows that indeed common antigens are likely shared among genomovars as our 17-23 mutant confers equal protection against columnaris induced by genomovar I and II in Nile tilapia. In channel catfish I observed the same trend: fish vaccinated with genomovar II were better protected against both genomovars than those vaccinated with genomovar I that was not protective against genomovar II. However, and due to the low mortality induced by genomovar I ARS-1 strain, vaccinated and unvaccinated fish had no significant differences in cumulative mortalities after challenge with ARS-1. Our group has previously used ARS-1 in experimental challenges with cumulative mortalities ranging from 4 to 32% [16]. Clearly, BGFS-27 provides a more robust challenge model to determine vaccine efficacy against columnaris disease.

Interestingly, 17-23 outperformed FCRR even when a genomovar I was used for challenge although this was only significant in Nile tilapia. This may be due to the fact that genomovar II strains are more chemotactic towards fish than genomovar I [26]. Furthermore,

genomovar II strains are known to persist longer in fish tissues than genomovar I [27]. Both characteristics, better chemotaxis and longer survival on the host, can act synergistically allowing genomovar II mutants to colonize the host in a more efficient manner. Since 17-23 mutant conferred higher protection in channel catfish, and Nile tilapia than FCRR, I speculate that the routes of entry, rates of adhesion and duration of attachment of this new mutant are similar in all fish species tested.

Conclusion

In summary, this study reports the efficacy of a new modified live genomovar II vaccine in channel catfish, zebrafish and Nile tilapia. This mutant, 17-23, conferred higher protection than that provided by the genomovar I mutant FCRR in channel catfish and Nile tilapia. The results suggest that genomovar II mutant 17-23 will generate a better commercial vaccine than FCRR, particularly when high virulent columnaris or co-infection with more than one genomovar of *F. columnare* occur in aquaculture settings.

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References

[1] Austin B., Austin D.A., Bacterial fish pathogens: disease of farmed and wild fish, Springer Verlag, 1999.

[2] Bernardet J.F., Bowman J.P., The genus *Flavobacterium*, in: Dworkin M., Falkow S., Rosemberg E., Schleifer K.-H., Stackerbrant E. (Eds.), The Prokaryotes, Springer, New York, 2006, pp. 481-531.

[3] Wagner B.A., Wise D.J., Khoo L.H., Terhune J.S., The epidemiology of bacterial diseases in food-size channel catfish, Journal of Aquatic Animal Health. (2002) 14:263-272.

[4] Plumb J.A., Health maintainence and principal microbial diseases of cultured fish, Iowa State University Press, Ames, IA, 1999.

[5] Gudding R., Lillehaug A., Evensen O., Recent developments in fish vaccinology, Veterinary Immunology and Immunopathology. (1999) 72:203-212.

[6] Shoemaker C.A., Klesius P.H., Evans J.J., Arias C.R., Use of modified live vacccine in aquaculture, Journal of the World Aquaculture Society. (2009) 40:573-585.

[7] Woodrow K.A., Bennett K.M., Lo D.D., Mucosal vaccine design and delivery, Annual Review of Biomedical Engineering. (2012) 14:17-46.

[8] Shoemaker C.A., Klesius P.H., Evans J., Modified live *Flavobacterium columnare* against columnaris disease in fish, US Patent Number 6881412B1. (2005).

[9] Wise D.j., Greenway T., Byars T., Oral vaccination of fish with live attenuated *Edwardsiella ictaluri* vaccines, US Patent Number 20130164331 A1. (2011).

[10] Klesius P.H., Shoemaker C.A., Development and use of modified live *Edwardsiella ictaluri* vaccine against enteric septicemia of catfish, Advanced Veterinay Medicine. (1999) 41:523-537.

[11] Schurig G.G., Roop R.M., Bagchi T., Boyle S., Buhrman D., Sriranganathan N., Biological properties of RB51; a stable rough strain of *Brucella abortus*, Veterinary Microbiology. (1991) 28:171-188.

[12] Shoemaker C.A., Klesius P.H., Evans J.J., Immunization of eyed channel catfish, *Ictalurus punctatus*, eggs with monovalent *Flavobacterium columnare* vaccine and bivalent *F. columnare* and *Edwardsiella ictaluri* vaccine, Vaccine. (2007) 25:1126-1131.

[13] Zhang Y., Arias C.R., Shoemaker C.A., Klesius P.H., Comparison of lipopolysaccharide and protein profiles between *Flavobacterium columnare* strains from different genomovars, Journal of Fish Diseases. (2006) 29:657-663.

[14] Olivares-Fuster O., Arias C.R., Development and characterization of rifampin-resistant mutants from high virulent strains of *Flavobacterium columnare*, Journal of Fish Diseases. (2011) 34:385-394.

[15] Thoesen J.C., Suggested procedures for the detection and identification of certain finfish and shellfish pathogens, American Fisheries Society-Fish Health Section, Bethesda, ML, 2004.

[16] Arias C.R., Cai W., Peatman E., Bullard S.A., Catfish hybrid *Ictalurus punctatus* x *I. furcatus* exhibit higher resistance to columnaris disease than the parental species, Diseases of Aquatic Organisms. (2012) 100:77-81.

[17] Shoemaker C.A., Klesius P.H., Drennan J.D., Evans J.J., Efficacy of a modified live *Flavobacterium columnare* vaccine in fish, Fish & Shellfish Immunology. (2011) 30:304-308.

[18] Decostere A., Haesebrouck F., Devriese L.A., Shieh medium supplemented with tobramycin for selective isolation of *Flavobacterium columnare* (*Flexibacter columnaris*) from diseased fish, Journal of Clinical Microbiology. (1997) 35:322-324.

[19] Welker T.L., Shoemaker C.A., Arias C.R., Klesius P.H., Transmission and detection of *Flavobacterium columnare* in channel catfish *Ictalurus punctatus*, Diseases of Aquatic Organisms. (2005) 63:129-138.

[20] Shoemaker C.A., Olivares-Fuster O., Arias C.R., Klesius P.H., *Flavobacterium columnare* genomovar influences mortality in channel catfish (*Ictalurus punctatus*), Veterinary Microbiology. (2008) 127 353-359.

[21] Amend D.F., Potency testing of fish vaccines, Karger, Basel, 1981.

[22] Hanson T.R., The economic implications of feeding fingerlings during outbreaks of Enteric Septicemia in Catfish, Animal Health Aquaculture, 2006.

[23] Kirkland S., Evaluation of the Live-Attenuated Vaccine AquaVac-COL® on Hybrid Channel x Blue Catfish Fingerlings in Earthen Ponds, 2010.

[24] Olivares-Fuster O., Baker J.L., Terhune J.S., Shoemaker C.A., Klesius P.H., Arias C.R., Host-specific association between *Flavobacterium columnare* genomovars and fish species, Systematic and Applied Microbiology. (2007) 30:624-633.

[25] Olivares-Fuster O., Arias C.R., Development and characterization of rifampicin-resistant mutants from high virulent strains of *Flavobacterium columnare*, Journal of Fish Diseases. (2011) 34:385-394.

[26] Klesius P.H., Shoemaker C.A., Evans J.J., *Flavobacterium columnare* chemotaxis to channel catfish mucus, Fems Microbiology Letters. (2008) 288:216-220.

[27] Olivares-Fuster O., Bullard S.A., McElwain A., Llosa M.J., Arias C.R., Adhesion dynamics of *Flavobacterium columnare* to channel catfish *Ictalurus punctatus* and zebrafish *Danio rerio* after immersion challenge, Diseases of Aquatic Organisms. (2011) 96:221-227.

Table 4-1. Comparison of the vaccine efficacy of rifampicin-resistant mutants obtained from different genomovar II parent strains. Vaccinated fish were challenged with virulent genomovar II strain BGFS-27 (challenge dose of 1.6×10^{7} CFU/mL). Results from channel catfish vaccination (Experiment 1) are shown as cumulative percent mortality and RPS. Within a column, different superscript letters means significant difference ($p < 0.05$).

Parent Strain[a]	**Mutant Strain**	**Vaccination Dose (CFU/mL)**	**Cumulative Percent Mortality (mean ±SE)**	**RPS**
AL-CC-11	11-131	7.0×10^{6}	27.0 ± 6.8[a]	49.1
	11-132	9.1×10^{6}	39.0 ±5.0[b,c]	26.4
AL-CC-15	15-132	2.0×10^{7}	34.0 ±13.3[b,c]	27.3
	15-133	2.5×10^{7}	28.0 ±8.6[a]	49.1
AL-CC-16	16-532	1.7×10^{7}	34.0 ±13.3[b,c]	32.0
	16-534	2.1×10^{7}	30.0 ±7.7[a]	40.0
AL-CC-17	17-13	2.1×10^{7}	36.0 ±14.2[b,c]	32.1
	17-23	1.7×10^{7}	24.0 ±5.7[a]	54.7
	Control	-	53.0 ±8.6[c]	-

a, see Olivares-Fuster and Arias (2011) reference for complete details on the parent strains

Table 4-2. Results from Experiment 2 shown as cumulative percent mortality and RPS of vaccinated zebrafish after immersion challenge with virulent genomovar II strain BGFS-27 (challenge dose of 6.4 x10^6 CFU/mL). Within a column, different superscript letters means significant difference ($p < 0.05$).

Mutant Strain	**Vaccination Dose (CFU/mL)**	**Cumulative Percent Mortality (mean ±SE)**	**RPS**
17-23	7.8×10^6	63.1 ± 0.38^a	49.1
16-534	1.5×10^6	$70.2 \pm 0.87^{a,b}$	27.3
FCRR	9.1×10^6	$81.0 \pm 0.32^{a,b}$	32.0
Control	-	88.1 ± 1.86^b	-

Table 4-3. Cumulative percent mortality and RPS of vaccinated channel catfish fry after immersion challenge with virulent *F. columnare* strains either genomovar I (ARS-1, challenge dose of 9.8×10^6 CFU/mL) or genomovar II (BGFS-27, challenge dose of 7.6×10^6 CFU/mL) at 28 dpv (Experiment 3). Within a column, different superscript letters means significant difference ($p < 0.05$).

Mutant Strain	Vaccination Dose (CFU/mL)	Challenge Strain	Cumulative Percent Mortality (mean ±SE)	RPS
17-23	6.5×10^6	ARS-1	1.5 ± 0.01[a]	90.9
		BGFS-27	41.5 ± 0.08[b]	37.0
FCRR	6.3×10^6	ARS-1	4.5 ± 0.01[a]	72.7
		BGFS-27	63.0 ± 0.05[c]	4.4
Control	-	ARS-1	16.5 ± 0.03[a]	-
		BGFS-27	65.9 ± 0.03[c]	-

Table 4-4. Experiment 4 results. Cumulative percent mortality and RPS of immersion vaccinated Nile tilapia fingerlings after immersion challenge with genomovar I (ARS-1, challenge dose of 5.5×10^6 CFU/mL) or genomovar II (BGFS-27, challenge dose of 2.7×10^6 CFU/mL). Within a column, different superscript letters means significant difference ($p < 0.05$).

Mutant Strain	**Vaccination Dose (CFU/mL)**	**Challenge Strain**	**Cumulative Percent Mortality (mean ±SE)**	**RPS**
17-23	2.2×10^6	ARS-1	8.2 ± 0.63^a	86.9
		BGFS-27	11.8 ± 1.58^a	82.1
FCRR	7.3×10^6	ARS-1	15.3 ± 1.20^a	75.5
		BGFS-27	55.3 ± 0.36^b	16.1
Control	-	ARS-1	62.4 ± 0.85^b	-
		BGFS-27	65.9 ± 0.47^b	-

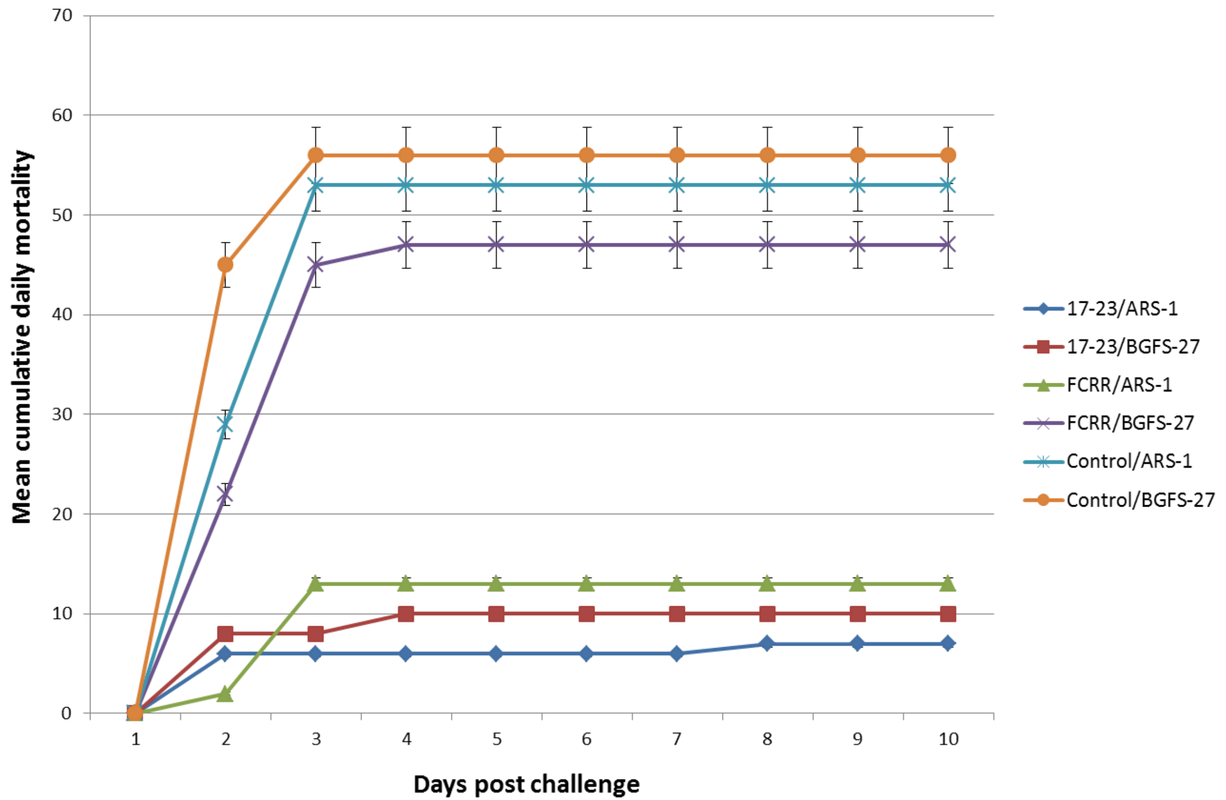

Figure 4-1. Mean cumulative daily mortality of Nile tilapia immersion vaccinated with 17-23, FCRR or sham vaccinated and challenged by immersion with either BGFS-27 (genomovar II) or ARS-1 (genomovar I). Note: numbers represent cumulative mean mortality of an average of 5 tanks, error bars shows standard deviation from the mean.

CHAPTER 5. POTASSIUM PERMANGANATE ELICITS A SHIFT OF THE EXTERNAL FISH MICROBIOME AND INCREASES HOST SUSCEPTIBILITY TO COLUMNARIS DISEASE

Abstract

The external microbiome of fish is thought to benefit the host by hindering the invasion of opportunistic pathogens and/or stimulating the immune system. Disruption of those microbial communities could increase susceptibility to diseases. Traditional aquaculture practices include the use of potent surface-acting disinfectants such as potassium permanganate (PP, $KMnO_4$) to treat external infections. This study evaluated the effect of PP on the external microbiome of channel catfish and investigated if dysbiosis leads to an increase in disease susceptibility. Columnaris disease, caused by *Flavobacterium columnare,* was used as disease model. Four treatments were compared in the study: (I) negative control (not treated with PP nor challenged with *F. columnare*), (II) treated but not challenged, (III) not treated but challenged, and (IV) treated and challenged. Ribosomal intergenic spacer analysis (RISA) and pyrosequencing were used to analyze changes in the external microbiome during the experiment. Exposure to PP significantly disturbed the external microbiomes and increased catfish mortality following the experimental challenge. Analysis of similarities of RISA profiles showed statistically significant changes in the skin and gill microbiomes based on treatment and sampling time. Characterization of the microbiomes using 16S rRNA gene pyrosequencing confirmed the disruption of the skin

microbiome by PP at different phylogenetic levels. Loss of diversity occurred during the study,even in the control group, but was more noticeable in fish subjected to PP than in those challenged with *F. columnare*. Fish treated with PP and challenged with the pathogen exhibited the least diverse microbiome at the end of the study.

Introduction

Fish are in intimate contact with the aquatic environment which harbors pathogenic and opportunistic organisms [1]. As a result, cutaneous diseases are more common in fish than in terrestrial vertebrates [2] and the external epithelial surfaces are often the major route of entry for infectious agents in aquatic animals [3]. Skin and gills of fish are extremely important as the first line of defense against invasion by opportunistic pathogens and subsequent infections that may result in disease. In addition to being mechanical barriers, skin and gills represent a biologically active environment [4, 5] that is colonized by a diverse, complex and dynamic microbial communities that constitutes the fish external microbiome [6-10]. A healthy microbiome exerts antagonistic effects against pathogens by competitive exclusion for nutrient and/or synthesis of antimicrobial compounds and promotes host homeostasis [11, 12]. Suppression of pathogenic organisms by the resident microbiota has been reported in birds, fish, crustaceans, and other aquatic organisms [10, 13, 14]. Thus, preserving the integrity of the normal protective microbiome is key for excluding potential invaders and maintaining health [15].

Intensive production practices used in fish farms can result in environmental stressors such as low dissolved oxygen or high organic loads that favor opportunistic pathogens and are stressful to fish [16]. Moreover, the use of chemical treatments to control or prevent specific pathogens can alter the normal healthy fish microbiome making the fish more vulnerable to

infections [17]. The effect of these intensive culture practices on the fish external microbiome is for the most part unknown. I hypothesized that the use of harsh chemicals as treatment against external bacterial, parasitic and fungal infections disrupts the skin and gill microbiome and increases susceptibility to opportunistic bacterial pathogens. To test this hypothesis, I chose to use PP ($KMnO_4$), a potent oxidizing agent commonly used in aquaculture to treat external infections, and *Flavobacterium columnare* as the causative agent of columnaris disease, a very common bacterial infection in freshwater aquaculture farms.

Columnaris disease courses primarily as an external infection and the bacteria frequently attack the fins, skin, and gills of fish causing frayed fins, depigmented or ulcerated skin and necrotic gills [18, 19]. Skin and gills are believed to be the point of entry and the primary site of infection for *F. columnare* [3, 20] and bacterial competition is considered one of the factors determining the degree of the infection [21]. Previous studies have shown that survival and infectivity of *F. columnare* decline in presence of competitive bacteria species such as *Aeromonas hydrophila* (an opportunistic fish pathogen) and *Citrobacter freundii* (nonpathogenic to fish) [22] or when the density of *F. columnare* was too low relative to total bacterial counts [23]. Thus, it has been suggested that when *F. columnare* is present in low numbers, it may not be able to compete with other naturally occurring bacteria on the fish skin and gills [24].

To prove if PP altered the composition of the fish external microbiome and, subsequently, increased susceptibility to columnaris disease I applied culture-independent methods to characterize and compare the channel catfish (*Ictalurus puntactus*) external microbiome before and after exposure to PP and challenge with *F. columnare.* Our model has direct implications for commercial aquaculture as channel catfish is the main aquaculture species

in the U.S. and is highly susceptible to columnaris disease. In addition, PP is routinely used in freshwater fish farms around the world to control external infections.

Materials and Methods

Fish husbandry. Channel catfish fingerlings (n = 199, average weight ± SD was 15 ± 1.7 g and average length ± SD was 14.3 ± 0.7 cm) were purchased from Osage Catfisheries Inc. (Osage Beach, MO, USA) (the fish were inspected by University of Arkansas at Pine Bluff Fish Diseases Laboratory and found to be free of pathogens, Case ID#:PB11-233) and express shipped to the E. W. Shell Fisheries Center (EWSFC) at North Auburn Fisheries Experiment Station, Auburn, AL, USA. Fish were kept in a 250 gallons plastic tank supplied with dechlorinated city water for 4 weeks prior to the experiment. Fish were then transferred in aerated containers to the Aquatic Microbiology Laboratory (AML) located on main campus at Auburn University. Upon arrival to AML and prior to stocking in the glass aquaria/tanks, mucus, skin and gill samples of ten randomly caught fingerlings were sampled, examined following standard procedures [25] and proved culture negative for *F. columnare*. Before fish were transferred to the glass aquaria, DNA was extracted from the skin and gills of 9 randomly caught fingerlings from the stock tank (t_0). Fish were then stocked into 12 tanks, 37 L each at a stocking rate of 15 fish/tank and maintained as previously described [26]. Water quality was monitored daily and parameters were maintained at 80 ppm alkalinity, 40 ppm hardness, 0.1 ppt salinity, 26 ± 1°C, pH 7.7 ± 0.2 [mean ± SD], ammonia and nitrites were kept at non-detectable levels and a dark and light period of 12:12 h was maintained throughout the experiment. Fish were acclimated for 7 days before treatment with PP. Fish were fed daily to apparent satiation with commercial pellets, AQUAMAX Grower 400 (Purina Mills, Inc., St. Louis, MO, USA). All

animal protocols were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC number 2012-2141).

Experimental design. The study design is shown in Figure 5-1. Four treatments with 3 replicates each (replicate=tank) were set up as follows: (I) Non-treated non-challenged fish acted as controls (not exposed to PP and not challenged with *F. columnare*), (II) treated with PP and not challenged with *F. columnare*, (III) not treated with PP and challenged with *F. columnare*, and (IV) treated with PP and challenged with *F. columnare*. Tanks were randomized and assigned blindly to each treatment. For PP treatment, a dose of 5 mg/L above 15-minute PP demand (PPD) of the tank water was applied [27, 28]. PPD is a measure of the amount of PP required to react with organic matter in a 15-min time frame [29]. PPD was determined [29] prior to the treatment and the average was 0.4 mg/L. The final PP dose was calculated as the PPD (0.4 mg/L) + 5 mg/L. Two of the treatment groups (II and IV) were treated with PP for 30 min in buckets containing 5 L aerated water by adding 27 milliliters of the stock solution to each bucket (A stock PP solution was prepared by dissolving 1 g of PP in 1 L of water). Fish in treatments I and III were similarly handled but were not exposed to PP (received a sham treatment). At the conclusion of the 30 min treatment, fish were removed from the buckets and returned to their respective tanks. Fish were not fed during PP exposure, but were offered food afterwards. Fish were allowed 3 days of recovery time after exposure to PP and before challenge with *F. columnare*. Challenge with *F. columnare* was carried out as previously described [26]. Briefly, fish were exposed for 30 min to pathogenic strain ALG-00-530 (genomovar II) at a concentration in the challenge bath of 3.2×10^6 CFU/ml. Fish in treatments I and II were similarly handled but sham challenged using sterile modified Shieh (MS) broth as inoculum in the challenge suspension. After the challenge, fish were removed from the challenge buckets,

returned to their respective tanks and maintained under normal husbandry conditions. Fish were not fed on the challenge day, but were offered food on the next day after challenge and throughout the rest of the study. Fish were observed for clinical signs of columnaris disease and mortality was recorded twice daily. Columnaris infection was confirmed in moribund and dead fish by isolation of *F. columnare* as previously described [30].

Sampling. Skin and gills were sampled for DNA extraction at time 0 (t_0 = fish from stock tank), at time 10 days (t_{10} = three days after treatment with PP and immediately before the challenge) and at time 25 days (t_{25} = from the survivors at the end of the experiment). Three fish were sampled at each time point per tank except from the stock tank at t_0 (9 fish were sampled) and from treatment IV (at the end of the experiment t_{25}, all the fish died in a tank and in another tank, only 2 catfish survived). To analyze the data, I further subdivided the samples from the four treatments into seven groups based on designated time points (Figure 5-1). Group 1 (G1), samples from the stock tank at t_0; Group 2 (G2), samples from treatments I&III (non-treated with PP) at t_{10}; Group 3 (G3), samples from treatments II&IV (treated with PP) at t_{10}; Group 4 (G4), samples from treatment I (system control) at t_{25}; Group 5 (G5), samples from treatment II (treated with PP) at t_{25}; Group 6 (G6), samples from treatment III (challenged with *F. columnare*) at t_{25}; Group 7 (G7), samples from treatment IV (treated with PP and challenged with *F. columnare*) at t_{25}.

DNA extraction. All skin (n=77) and gill (n=77) samples for DNA extraction (~30 mg from each tissue) were taken from the tip of the lower lobe of the caudal fin and from the second right gill arch, respectively. To account for variability associated with DNA extraction and downstream nucleic acid analysis, three fish were sampled per tank at each sampling time. All samples were immediately subjected to DNA extraction using the DNeasy Blood & Tissue kit

(Qiagen, Valencia, CA) following manufacturer's instructions (Total DNA from Animal Tissues, Spin-Column Protocol), including pretreatment with lysozyme for lysis of Gram positive bacteria. DNA was eluted with 100 µL elution buffer and was quantified using a NanoDrop *ND-1000* spectrophotometer (Thermo Scientific, Nanodrop Technologies, Wilmington, DE, USA).

Ribosomal intergenic spacer analysis (RISA). Extracted DNA was used as a template for RISA which was performed as previously described by Arias et al. (2006) with some modifications. The primer sequences ITS-FEub (5'-GTCGTAACAAGGTAGCCGTA-3') and ITS-REub (5'-GCCAAGGCATCCACC-3') were used for PCR amplification of the internal transcribed spacer region [31]. The PCR master mix contained 1x Taq buffer, 0.4 mM dNTPs (Promega, Madison, WI), 2 mM $MgCl_2$, 0.4 µM ITS-FEub primer, 0.2 µM ITS-REub primer, 2 µM ITS-REub labeled primer, 1 U of Taq polymerase (5 PRIME, Inc., Gaithersburg, MD), and 10 ng of template DNA in a final volume of 50 µL. The samples were amplified in a PTC-200 DNA-Engine thermocycler (PTC-200, MJ Research, Watertown, MA, USA) and the PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, 55 °C for 1 min, and 68 °C for 2 min, with a final extension step at 68 °C for 7 min. To prepare samples for gel loading, 10 µL of each PCR product were diluted with 10 µL AFLP® Blue Stop Solution (LI-COR). Diluted samples were denatured at 95 °C for 5 min followed by quick cooling (to prevent reannealing) prior to gel loading (0.6 µL of sample was loaded into each well). PCR products were electrophoresed on a LI-COR 4300 DNA Analyzer (LI-COR Biosciences, Lincoln, NE) following manufacturer's instructions. RISA gel images in TIFF format were exported to Bionumerics v. 7 (Applied Maths, Austin, TX) and were analyzed as previously described [32].

Pyrosequencing. To identify the predominant bacterial species on catfish skin, DNA of 21 skin samples (3 samples per group) were randomly selected for sequencing. The variable V1-V3 region of the 16S rRNA gene was amplified by PCR using the universal Eubacterial primer set 27F (5'-AGRGTTTGATCMTGGCTCAG-3') and 519R (5'-GWATTACCGCGGCKGCTG-3') as described before [33]. Amplicons were then subjected to Roche 454 FLX titanium sequencing following manufacturer's guidelines. The resulting sequences were processed using a proprietary analysis pipeline (MR DNA, Shallowater, TX). Barcodes and primers were removed from the sequences, followed by removal of short sequences <200 base pairs in length, ambiguous base calls, and homopolymer runs longer than 6 base pairs. Afterwards, sequences were denoised and chimeras and singleton sequences were removed. Operational taxonomic units (OTUs) were defined at a cutoff value of 3% divergence (97% similarity) in agreement with the current accepted prokaryote species concept [34-39]. Final OTUs were taxonomically assigned using BLASTn against the Greengenes database [40]. Since species richness and evenness can be compared only between samples with equal sample sizes [41], I randomly normalized the sequences so as to standardize to the samples with the least number of sequences obtained (N = 1,813) (the number of reads for each sample was normalized by randomly subsampling from the larger sample to the number of reads of the smallest one). Rarefaction curves, Good´s coverage, abundance-based coverage estimation (ACE), Chao1, Shannon evenness, and shared OTUs based on defined OTUs were generated using Mothur v.1.33.3 package [42]. Sample-by-OTU abundance data matrices from mothur were subsequently transposed and multivariate analysis was performed with the PRIMER 6 (Plymouth Routines In Multivariate Ecological Research) software package.

Data analyses. Bionumerics v. 7 (Applied Maths, Austin, TX) was used to process RISA images. Following normalization and background subtraction with mathematical algorithms, similarity levels between fingerprints were calculated by Pearson product-moment correlation coefficient. Cluster analysis was performed according to Arias et al. (2006) using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Multidimensional scaling (MDS) was performed using optimized positions to visualize the similarities or dissimilarities of the samples. Analysis of similarities (ANOSIM) was run on the similarity matrix generated from Bionumerics using PRIMER v6 (Primer-E Ltd, Plymouth, UK). Mortality data was analyzed using analysis of variance (ANOVA) with general linear model (PROC GLM) followed by Tukey's Studentized Range (HSD) test for all-pairwise comparisons to determine significant ($p < 0.05$) differences between the mean mortality of the different treatments (SAS Institute, Cary, NC.). A one-way ANOVA was performed on all diversity indexes, followed by a Tukey's post hoc test where significance ($P < 0.05$). A genera abundance table was loaded into PRIMER v6 [43] and similarity percentages (SIMPER) analysis was performed to determine the genera responsible for differences between groups. Cut-off for low contributions was set at the default 90%.

Results

Mortality. The mean cumulative percent mortality of the four treatments is shown in (Figure 5-2). Control (treatment I) and PP treated but non-challenged fish (treatment II) did not show any mortality throughout the experiment. Fish non-treated with PP and challenged with *F. columnare* (treatment III) had a mean percent mortality of 61.1 ±1.5 (SD), which was significantly different ($P<0.05$) from the mortality observed in fish treated with PP and

challenged with *F. columnare* (treatment IV) that was 86.1± 1.5 (SD). Mortalities of both challenged treatments significantly differed from that of the non-challenged treatments (0%). Channel catfish fingerlings in challenged tanks (treatments III and IV) exhibited clinical signs typical of columnaris disease. *F. columnare* was isolated from skin lesions, gills and kidneys of dead or moribund fish. Anecdotal observations at day 1 post-challenge, suggested that fish treated with PP and challenged with *F. columnare* (treatment IV) were more lethargic with rapid opercular movement than those challenged but not PP treated (treatment III). Mortality persisted for 8 d with the majority of fish deaths occurring on days 2 and 3 post-challenge. The study was concluded on day 15 after 7 consecutive days without mortalities.

RISA. A total of 154 (77 skin & 77 gill) samples were analyzed by RISA representing all seven groups (see Figure 5-3). RISA profiles averaged 25 bands that ranged in size between 50 to 700 bp. Similarities between microbial community profiles ranged from a maximum of 99% to a minimum of 17.5% based on Pearson correlation coefficient analysis followed by UPGMA clustering. For better visualization of the clusters observed by RISA, MDS was used to display skin and gills microbiome profiles using the variables treatment, time, tissue and group. Figure 5-3 shows the MDS plot of skin and gill samples based on group ascription. ANOSIM directly compared the clusters based on the following variables: treatment (I through IV), time (t_0, t_{10}, t_{25}), tissue (skill and gill) and group (G1 through G7). Samples clustered significantly ($p = 0.001$) by all factors considered, although there was some overlap among them (Table 5-1). The least significant factor for the cluster separation was tissue (skin or gill) with an R value of 0.093. Separation was most significant when samples were assigned to clusters based on group with an R value of 0.387 and 14 out of 21 pairwise comparisons were significant while only 7 were not significant. The R values for treatment and time were 0.214 and 0.304, respectively. Seven out of

10 pair and 10 out of 10 pairwise comparisons were significantly different by treatment and by time, respectively. These global R values indicate that RISA-based clusters are significantly correlated with all the factors although group (group = treatment + time combined) was the most significant variable and played the main role determining the change in composition of the skin and gill microbiome.

Pyrosequencing. Twenty one skin samples, 3 replicates per group, were subjected to 16S rRNA gene pyrosequencing. No gill samples were sequenced as diversity on fish gills was previously found to be lower than that on fish skin [44-46] and the skin and gill RISA results in my study were in agreement. Pyrosequencing yielded a total of 236,697 bacterial sequences and 483 OTUs. After sample normalization, 38,073 sequences and 454 OTUs were included in the analysis. Sequence coverage was ≥98% in all sequenced samples (Good's coverage, Table 5-2). Rarefaction curves (Figure 5-4) confirmed that G3 (3 days post-treatment with PP) was the group with the least diverse bacterial population. G1 (fish prior tank stocking) displayed the most diverse microbiome. Total expected richness as calculated by ACE and Chao1 was significantly different between groups and the Shannon evenness index was significantly different as well (Table 5-2).

When sequences were ascribed at the phylum level, each group returned a unique bacterial composition. Eight bacterial phyla (Actinobacteria, Planctomycetes, Firmicutes, Thermi, Verrucomicrobia, Acidobacteria, Proteobacteria and Bacteroidetes) were identified from the skin samples of all groups (Figure 5-5). Proteobacteria accounted for 73.1% of all sequences obtained, whereas, Firmicutes represented 17.1% of the total sequences. Other less common phyla like Bacteroidetes, Verrucomicrobia and Actinobacteria formed 6.9%, 2.6% and 0.2%, respectively. The phylum Proteobacteria was the most predominant phylum in six groups and

comprised the majority of all sequences (49.8% in G1, 80.5% in G2, 93.9% in G4, 88.8% in G5, 98.4% in G6 and 99.9% in G7) while in G3, the phylum Firmicutes was the most abundant phylum forming 99.5% of all sequences. Bacteroidetes was identified in varying levels in five groups (29.6% in G1 and 18.7% in G2, 0.05% in G3, 0.003% in G6 and 0.002% in G7). The less common phyla varied in abundances between groups. Planctomycetes, Thermi and Verrucomicrobia were unique to G1 (0.3%, 0.1% and 18.4%, respectively). Acidobacteria was identified merely in G2 (0.2%). Sequences for Actinobacteria were detected only in G1 and G2 representing 0.7% and 0.5%, respectively.

The skin microbiome of all groups was composed of a total of 105 genera; only genera accounting for more than 5% of all identified sequences in at least one group are presented in Table 5-3. Proteobacteria was represented by many genera but the most common were *Aeromonas, Vogesella, Stenotrophomonas, Klebsiella, Trabulsiella*, *Citrobacter*, *Enterobacter*, *Rheinheimera, Pseudomonas, Acinetobacter* and *Herbaspirillum.* The majority of all Firmicutes sequences correspond to members of the genus *Bacillus*. Bacteroidetes was represented by the genera *Chryseobacterium* and *Runella.* The genus *Flavobacterium* accounted for only 0.4% of all genera identified in all groups. Verrucomicrobia and Actinobacteria were mostly represented by *Puniceicoccaceae* and *Propionibacterium*, respectively. Out of the 105 genera identified, only 3 genera (*Enterobacter, Raoultella and Citrobacter*) were shared between the microbiome of the seven groups, suggesting significant dissimilarity in the bacterial composition of the skin between groups at the genus level. Predominant genera varied between groups with *Bacillus* being the most abundant genus in G3, *Aeromonas* in G4 and G6, *Vogesella* in G5, *Stenotrophomonas* in G2 and *Klebsiella* in G7. Other relatively abundant genera included *Trabulsiella*, *Citrobacter* and *Enterobacter* in G7, *Chryseobacterium* in G1 and G2,

Rheinheimera in G2, and *Puniceicoccaceae*, *Pseudomonas*, *Acinetobacter*, *Runella* and *Herbaspirillum* in G1.

Similarity Percentage (SIMPER) analysis by bacterial genera between replicates (within each group) showed high similarities within group. Figure 5-6 summarizes the clustering analysis of all 21 skin samples analyzed. Conversely, SIMPER analysis showed high pairwise dissimilarities between groups (Table 5-4). The majority of the differences between groups were due to different relative abundances of the genera *Stenotrophomonas*, *Chryseobacterium*, *Puniceicoccaceae*, *Bacillus*, *Aeromonas*, *Klebsiella*, *Trabulsiella* and *Vogesella* (Table 5-4). Based on genus composition, SIMPER analysis indicated that G3 and G7 were the most dissimilar (99.94%), followed by G2 and G4 (99.86%), while G6 and G4 were the least dissimilar (26.62%).

Discussion

In the aquatic environment, both saprophytic and pathogenic organisms can infect fish when the conditions suit favorable for their multiplication [47]. However, under normal conditions, fish use a repertoire of innate and specific defense mechanisms to maintain healthy status and defend themselves against potential invaders [48]. The microbiome is now considered an essential extra organ of the host, and recent studies using gnotobiotic animals have shown the profound impact of bacteria on the anatomical, physiological and immunological development of the host [49, 50]. Therefore, colonization of the fish surface by a healthy microbiome results in a protective barrier that enhances host fitness [15, 51-54]. The microbiome can protect the host by outcompeting pathogens for living space, adhesion sites, energy and essential nutrients, or by producing inhibitory compounds and enhancing the immune response [55, 56]. Disturbance of

these functions by dysbiosis (an imbalanced or disrupted microbiome) may contribute to development of diseases. Stressful settings such as those occurring under intensive aquaculture production induce dysbiosis to the healthy fish microbiome, thus allowing pathogens to establish infections [17].

My results show that PP treatment dramatically altered the community composition of the catfish external microbiome, as G3 (3 days post-exposure to PP) had the least diverse microbiome in terms of species richness. Furthermore, the phylum Proteobacteria was the predominant phylum on the skin microbiome of all groups except G3, which was dominated by the phylum Firmicutes (99.5% of all OTUs). This disruption in microbiome structure was correlated with a significant increase in mortality of fish treated with PP (86.1%) compared to those with intact external microbiome (61.1%) after pathogen exposure. Hence, dysbiosis of the external microbiome significantly increased catfish susceptibility to columnaris disease. This increase in susceptibility could be attributed to chemical injuries induced by exposure to PP; however, fish were allowed to recover from PP exposure for 3 days prior challenge. Previous studies reported that exposure to PP at therapeutic dose (as the one used in this study) can cause mild hypertrophy and spongiosis in gills but channel catfish recovered within 48-h post-treatment [57]. Similarly, when channel catfish were granted 3 days between physical injury and *F. columnare* exposure, regardless of the method of injury, no mortality was reported [58]. In my study, I could not separate the negative effect of PP on the external tissues from its effect on the external microbiome. However, based on previous studies [57], the integrity of the external tissues was restored soon after PP treatment while, based on my results, the microbiome was not. Therefore, the observed increase in susceptibility to bacterial infection is likely due to disruption of the normal beneficial microbiome caused by exposure to PP.

The phylum Proteobacteria dominated the skin microbiome of channel catfish, followed by the phylum Firmicutes, which was in agreement with previous studies on bacterial communities associated with fish skin in other species, regardless of the method used for identification [5, 9, 10, 17, 59]. After PP treatment, the external microbiome dramatically changed and all Proteobacteria were eliminated and substituted by Firmicutes. It was expected that Proteobacteria and other Gram-negative bacteria were less resistant to the action of PP than Gram-positive bacteria. A previous study showed that up to 32 mg/L PP is needed to reduce *Bacillus* sp. viable cells by 99% [60], a dose much higher than the one used in this study. Interestingly, members of the phylum Proteobacteria (*Aeromonas*, *Citrobacter*, *Pseudomonas* and *Luteimonas*) that were removed by PP treatment and replaced by Firmicutes, have shown antagonism to *F. columnare* in earlier studies [22, 24, 61, 62]. On the other hand, although most probiotics proposed as biological control agents in aquaculture belong to the phylum Firmicutes, (*Bacillus*, *Lactobacillus*, etc.) [11], a thorough literature review revealed no antagonism between any Firmicutes (mainly *Bacillus*) and *F. columnare*. Our findings suggest that the observed shift from a 'Proteobacteria dominated' to a 'Firmicutes dominated' external microbiome results in the loss of key antagonistic species against *F. columnare*.

The variable 'group' (Group = treatment + time combined) was the most influential factor affecting the skin microbiome composition. Each group presented a significantly distinct microbiome with a fairly low sample-to-sample variability within each group. At the phylum level, G1 displayed the most diverse microbiome with 7 out of 8 phyla found in the study present in this group. Interestingly, the microbiome composition differed significantly over the time during the study period even in the control treatment. Groups G2 and G4 significantly differed from G1 and from each other even though no treatment was applied to those fish except for

handling. The phylum Verrucomicrobia was present in G1 but was not detected in G2. While the numbers of Bacteriodetes were significantly reduced from G1 to G2, the numbers of Proteobacteria increased. This trend continued over time and at day 25, control group G4 was overwhelmingly dominated by Proteobacteria (93.9%).

It is well known that moving fish is a source of stress and disease outbreaks are not uncommon after fish had been handled [63-65]. However, this is the first report in where significant changes in the external microbiomes of fish that were transferred between apparently similar environments have been documented. Our group has previously shown that skin microbiome is species-specific [9] but environmental factors and resident bacteria within an ecological niche can alter the bacterial communities associated with skin and mucus [17, 44, 66].

Differences in external microbiomes based on time were more apparently between G3 and G5 where the only difference between groups was sampling time after treatment with PP. For G3 at t_{10}, Firmicutes represented 99.5% of the bacterial phyla percentages while Proteobacteria were 0.4%. At t_{25}, G5 was dominated by Proteobacteria (88.2%) and the percentage of Firmicutes decreased drastically to 11.2%. Normally, the skin microbiome is dynamic and its composition fluctuates/shifts (community adaptation) over time and in response to changes in the environmental conditions [17, 59, 67-70]. Groups subjected to only one treatment (G5=PP and G6=pathogen) seemed to recover and shared a similar microbiome to that found in control group G4. Conversely, after two treatments (PP and pathogen) group G7 external microbiome was entirely reduced to Proteobacteria.

Overtime Proteobacteria became the predominant phylum regardless of the composition at earlier time points. However, not all microbiomes dominated by Proteobacteria were comprised of the same genera. At the genus level, only 3 genera were present in all the groups

out of 105 total genera identified and genera abundance within Proteobacteria differed dramatically between groups (Table 5-3). The microbiome of fish in treatment IV (PP treated) was dominated by the genera *Bacillus* before challenge at t_{10} (G3) and by *Klebsiella*, *Trabulsiella*, *Citrobacter* and *Enterobacter* at t_{25} (G7). The microbiome of fish in treatment III (*F. columnare* challenged) was dominated by the genera *Stenotrophomonas* before challenge at t_{10} (G2) and by *Aeromonas* at t_{25} (G6). This substantial difference in genera abundance between PP-treated fish compared to the untreated fish microbiome may have determined the increased susceptibility to *F. columnare* infection. However, further studies under field conditions are needed to fully understand the resilience of the fish microbiome to PP treatments in aquaculture ponds. Future studies should explore if manipulation of the fish microbiome by using pre- or probiotics will lead to a more natural and sustainable approach to prevent columnaris disease in aquaculture farms .

In conclusion, the data proved that harsh chemical treatments commonly used in fish farms induce dysbiosis to the fish's healthy microbiome, reducing the numbers of beneficial bacteria and potentially increase susceptibility to pathogens. My study emphasizes the fundamental importance of maintaining the integrity of the external microbiome as front-line defender against opportunistic pathogens like *F. columnare*. In the context of mutualism, fish in aquaculture could benefit from manipulating the composition of their external microbiome in order to decrease the incidence of columnaris disease. To the best of our knowledge, this is the first study to identify the skin microbiome composition of channel catfish. Further research would be necessary to select potential probiotic candidates from the fish external microbiome that can be used efficiently as biocontrol agents in a durable prophylactic management regime against columnaris disease.

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References

[1] Magnadottir B., Immunological control of fish diseases, Marine biotechnology. (2010) 12:361-379.

[2] Groff J., Cutaneous biology and diseases of fish, The veterinary clinics of North America. (2001) 4:321-411.

[3] Esteban M.Á., An overview of the immunological defenses in fish skin, ISRN Immunology. (2012) 2012.

[4] Bullock A.M., Roberts R.J., The dermatology of marine teleost fish. I. The normal integument, Oceanogr. Mar. Biol. Annu. Rev. (1974) 13:383-411.

[5] Horsley R.W., A review of the bacterial flora of teleosts and elasmobranchs, including methods for its analysis, Journal of fish biology. (1977) 10:529-553.

[6] Hawkes J.W., The structure of fish skin, Cell and Tissue Research. (1974) 149:159-172.

[7] Austin B., The bacterial microflora of fish, revised, The Scientific World Journal. (2006) 6:931-945.

[8] Xu Z., Parra D., Gómez D., Salinas I., Zhang Y.-A., von Gersdorff Jørgensen L., Heinecke R.D., Buchmann K., LaPatra S., Sunyer J.O., Teleost skin, an ancient mucosal surface that elicits gut-like immune responses, Proceedings of the National Academy of Sciences. (2013) 110:13097-13102.

[9] Larsen A., Tao Z., Bullard S.A., Arias C.R., Diversity of the skin microbiota of fishes: evidence for host species specificity, FEMS microbiology ecology. (2013) 85:483-494.

[10] Boutin S., Sauvage C., Bernatchez L., Audet C., Derome N., Inter individual variations of the fish skin microbiota: host genetics basis of mutualism?, PloS one. (2014) 9:e102649.

[11] Verschuere L., Rombaut G., Sorgeloos P., Verstraete W., Probiotic bacteria as biological control agents in aquaculture, Microbiology and Molecular Biology Reviews. (2000) 64:655-671.

[12] Dillon R., Charnley K., Mutualism between the desert locust *Schistocerca gregaria* and its gut microbiota, Research in Microbiology. (2002) 153:503-509.

[13] Nurmi E., Rantala M., New aspects of *Salmonella* infection in broiler production, Nature. (1973) 241:210-211.

[14] Balcázar J., Vendrell D., Blas I.d., Ruiz-Zarzuela I., Muzquiz J., Probiotics: a tool for the future of fish and shellfish health management, Journal of Aquaculture in the Tropics. (2004) 2004.

[15] Balcázar J.L., Decamp O., Vendrell D., De Blas I., Ruiz-Zarzuela I., Health and nutritional properties of probiotics in fish and shellfish, Microbial ecology in health and disease. (2006) 18:65-70.

[16] Wakabayashi H., Effect of environmental conditions on the infectivity of *Flexibacter columnaris* to fish, Journal of Fish Diseases. (1991) 14:279-290.

[17] Boutin S., Bernatchez L., Audet C., Derôme N., Network Analysis Highlights Complex Interactions between Pathogen, Host and Commensal Microbiota, PloS one. (2013) 8:e84772.

[18] Bernardet J.F., Immunization with bacterial antigens: *Flavobacterium* and *Flexibacter* infections, Dev Biol Stand. (1997) 90:179-188.

[19] Plumb J.A., Health maintenance and principal microbial diseases of cultured fishes, Iowa State University Press, Ames, IA, 1999.

[20] Tripathi N.K., Latimer K.S., Gregory C.R., Ritchie B.W., Wooley R.E., Walker R.L., Development and evaluation of an experimental model of cutaneous columnaris disease in koi *Cyprinus carpio*, Journal of veterinary diagnostic investigation. (2005) 17:45-54.

[21] Wakabayashi H., Egusa S., Preliminary experiments on environmental factors influencing the prevalence of columnaris disease, Fish Pathol. (1972) 7:58-63.

[22] Chowdhury M.B., Wakabayashi H., A study on the mechanism of the bacterial competitive effects on *Flexibacter columnaris* infection: Effects of the time-lag between the exposures of fish to *F. columnaris* and its competitor, Fish Pathology. (1989) 24:105-110.

[23] Chowdhury M.B., Wakabayashi H., Effects of competitive bacteria on the survival and infectivity of *Flexibacter columnaris*, Fish Pathology. (1989) 24:9-15.

[24] Suomalainen L.R., Tiirola M.A., Valtonen E.T., Effect of Pseudomonas sp MT5 baths on Flavobacterium columnare infection of rainbow trout and on microbial diversity on fish skin and gills, Diseases of Aquatic Organisms. (2005) 63:61-68.

[25] Thoesen J.C., Suggested procedures for the detection and identification of certain finfish and shellfish pathogens, Vol. American Fisheries Society-Fish Health Section, Bethesda, ML, American Fisheries Society, 2004.

[26] Mohammed H., Olivares-Fuster O., LaFrentz S., Arias C.R., New attenuated vaccine against columnaris disease in fish: Choosing the right parental strain is critical for vaccine efficacy, Vaccine. (2013) 31:5276-5280.

[27] Darwish A.M., Mitchell A.J., Hobbs M.S., In vitro and in vivo evaluation of potassium permanganate treatment efficacy for the control of acute experimental infection by *Flavobacterium columnare* in channel catfish, North American Journal of Aquaculture. (2008) 70:314-322.

[28] Martins M.L., Xu D.H., Shoemaker C.A., Klesius P.H., Temperature effects on immune response and hematological parameters of channel catfish *Ictalurus punctatus* vaccinated with live theronts of *Ichthyophthirius multifiliis*, Fish & Shellfish Immunology. (2011) 31:774-780.

[29] Boyd C.E., Water quality in warmwater fish ponds, Auburn University, Agricultural Experiment Station Auburn, Alabama, 1979.

[30] Mohammed H., Arias C., Epidemiology of columnaris disease affecting fishes within the same watershed, Diseases of Aquatic Organisms. (2014) 109:201-211.

[31] Cardinale M., Brusetti L., Quatrini P., Borin S., Puglia A.M., Rizzi A., Zanardini E., Sorlini C., Corselli C., Daffonchio D., Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities, Applied and Environmental Microbiology. (2004) 70:6147-6156.

[32] Arias C.R., Abernathy J.W., Liu Z., Combined use of 16S ribosomal DNA and automated ribosomal intergenic spacer analysis to study the bacterial community in catfish ponds, Letters in Applied Microbiology. (2006) 43:287-292.

[33] Larsen A.M., Mohammed H.H., Arias C.R., Characterization of the gut microbiota of three commercially valuable warmwater fish species, Journal of Applied Microbiology. (2014) 116:1396-1404.

[34] Rosselló-Mora R., Amann R., The species concept for prokaryotes, FEMS Microbiology Reviews. (2001) 25:39-67.

[35] Dowd S.E., Callaway T.R., Wolcott R.D., Sun Y., McKeehan T., Hagevoort R.G., Edrington T.S., Evaluation of the bacterial diversity in the feces of cattle using 16S

rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP), BMC microbiology. (2008) 8:125.

[36] Edgar R.C., Search and clustering orders of magnitude faster than BLAST, Bioinformatics. (2010) 26:2460-2461.

[37] Capone K.A., Dowd S.E., Stamatas G.N., Nikolovski J., Diversity of the human skin microbiome early in life, Journal of Investigative Dermatology. (2011) 131:2026-2032.

[38] Eren A.M., Zozaya M., Taylor C.M., Dowd S.E., Martin D.H., Ferris M.J., Exploring the diversity of *Gardnerella vaginalis* in the genitourinary tract microbiota of monogamous couples through subtle nucleotide variation, PloS one. (2011) 6:e26732.

[39] Swanson K.S., Dowd S.E., Suchodolski J.S., Middelbos I.S., Vester B.M., Barry K.A., Nelson K.E., Torralba M., Henrissat B., Coutinho P.M., Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals similarities with humans and mice, The ISME journal. (2010) 5:639-649.

[40] DeSantis T.Z., Hugenholtz P., Larsen N., Rojas M., Brodie E.L., Keller K., Huber T., Dalevi D., Hu P., Andersen G.L., Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB, Applied and Environmental Microbiology. (2006) 72:5069-5072.

[41] Hurlbert S.H., The nonconcept of species diversity: a critique and alternative parameters, Ecology. (1971) 52:577-586.

[42] Schloss P.D., Westcott S.L., Ryabin T., Hall J.R., Hartmann M., Hollister E.B., Lesniewski R.A., Oakley B.B., Parks D.H., Robinson C.J., Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities, Applied and Environmental Microbiology. (2009) 75:7537-7541.

[43] Clark K., Gorley R., PRIMER v6: user manual, Plymouth Marine Laboratory, Plymouth, United Kingdom. (2006).

[44] Horsley R., The bacterial flora of the Atlantic salmon (*Salmo salar* L.) in relation to its environment, Journal of Applied Bacteriology. (1973) 36:377-386.

[45] Mudarris M., Austin B., Quantitative and qualitative studies of the bacterial microflora of turbot, *Scophthalmus maximus* L., gills, Journal of fish biology. (1988) 32:223-229.

[46] Wang W., Zhou Z., He S., Liu Y., Cao Y., Shi P., Yao B., Ringø E., Identification of the adherent microbiota on the gills and skin of poly-cultured gibel carp (*Carassius auratus gibelio*) and bluntnose black bream (*Megalobrama amblycephala* Yih), Aquaculture Research. (2010) 41:e72-e83.

[47] Gómez G.D., Balcázar J.L., A review on the interactions between gut microbiota and innate immunity of fish, FEMS Immunology & Medical Microbiology. (2008) 52:145-154.

[48] Ellis A.E., Innate host defense mechanisms of fish against viruses and bacteria, Developmental & Comparative Immunology. (2001) 25:827-839.

[49] Rawls J.F., Samuel B.S., Gordon J.I., Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota, Proceedings of the National Academy of Sciences of the United States of America. (2004) 101:4596-4601.

[50] O'Hara A.M., Shanahan F., The gut flora as a forgotten organ, EMBO reports. (2006) 7:688-693.

[51] Nikoskelainen S., Ouwehand A.C., Bylund G., Salminen S., Lilius E.-M., Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*), Fish & Shellfish Immunology. (2003) 15:443-452.

[52] Kim D.-H., Austin B., Innate immune responses in rainbow trout (*Oncorhynchus mykiss*, Walbaum) induced by probiotics, Fish & Shellfish Immunology. (2006) 21:513-524.

[53] Balcázar J.L., De Blas I., Ruiz-Zarzuela I., Vendrell D., Gironés O., Muzquiz J.L., Enhancement of the immune response and protection induced by probiotic lactic acid bacteria against furunculosis in rainbow trout (*Oncorhynchus mykiss*), FEMS Immunology & Medical Microbiology. (2007) 51:185-193.

[54] Salminen S.J., Gueimonde M., Isolauri E., Probiotics that modify disease risk, The Journal of nutrition. (2005) 135:1294-1298.

[55] Gatesoupe F.J., The use of probiotics in aquaculture, Aquaculture. (1999) 180:147-165.

[56] Irianto A., Austin B., Probiotics in aquaculture, Journal of Fish Diseases. (2002) 25:633-642.

[57] Darwish A.M., Griffin B.R., Straus D.L., Mitchell A.J., Histological and hematological evaluation of potassium permanganate exposure in channel catfish, Journal of Aquatic Animal Health. (2002) 14:134-144.

[58] Bader J.A., Moore S.A., Nusbaum K.E., The effect of cutaneous injury on a reproducible immersion challenge model for *Flavobacterium columnare* infection in channel catfish (*Ictalurus punctatus*), Aquaculture. (2006) 253:1-9.

[59] Wilson B., Danilowicz B.S., Meijer W.G., The diversity of bacterial communities associated with Atlantic cod *Gadus morhua*, Microbial ecology. (2008) 55:425-434.

[60] Tucker C.S., Boyd C.E., Relationships between potassium permanganate treatment and water quality, Transactions of the American Fisheries Society. (1977) 106:481-488.

[61] Tiirola M., Valtonen E.T., Rintamäki-Kinnunen P., Kulomaa M.S., Diagnosis of flavobacteriosis by direct amplification of rRNA genes, Diseases of Aquatic Organisms. (2002) 51:93-100.

[62] Boutin S., Bernatchez L., Audet C., Derôme N., Antagonistic effect of indigenous skin bacteria of brook charr (*Salvelinus fontinalis*) against *Flavobacterium columnare* and *F. psychrophilum*, Veterinary Microbiology. (2012) 155:355-361.

[63] Plumb J.A., Hanson L.A., Health maintenance and principal microbial diseases of cultured fishes, John Wiley & Sons, 2011.

[64] Schreck C.B., Solazzi M.F., Johnson S.L., Nickelson T.E., Transportation stress affects performance of coho salmon, *Oncorhynchus kisutch*, Aquaculture. (1989) 82:15-20.

[65] Urbinati E.C., de Abreu J.S., da Silva Camargo A.C., Landinez Parra M.A., Loading and transport stress of juvenile matrinxã (*Brycon cephalus*, Characidae) at various densities, Aquaculture. (2004) 229:389-400.

[66] Cahill M.M., Bacterial flora of fishes: a review, Microbial ecology. (1990) 19:21-41.

[67] Larsen A.M., Arias C.R., More Than Mucus: The Hidden world of the Fish Microbiota, Fisheries. (2014) 39:154-154.

[68] Tomic-Canic M., Perez-Perez G.I., Blumenberg M., Cutaneous microbiome studies in the times of affordable sequencing, Journal of Dermatological Science. (2014) 75:82-87.

[69] de Vos W., Engstrand L., Drago L., Reid G., Schauber J., Hay R., Mendling W., Schaller M., Spiller R., Gahan C., Human Microbiota in Health and Disease, SelfCare Journal. (2012) 3:1-68.

[70] Verner-Jeffreys D.W., Shields R.J., Bricknell I.R., Birkbeck T.H., Changes in the gut-associated microflora during the development of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae in three British hatcheries, Aquaculture. (2003) 219:21-42.

Table 5-1. Analysis of similarities (ANOSIM) values obtained when RISA profiles were ascribed to the variables tested in the study.

Variable	Global R	P value	# Significant pairwise comparisons
Tissue	0.093	0.001	-
Treatment	0.214	0.001	7 out of 10
Time	0.304	0.001	3 out of 3
Group	0.387	0.001	14 out of 21

Table 5-2. Diversity indices as calculated by MOTHUR software (ver. 1.33.3). Operational taxonomic units (OTUs) were defined at 97% sequence similarity. Significance among total values for each fish species was determined by a one-way ANOVA followed by Tukey's post hoc test. Within a column, different superscript letters means significant difference (ANOVA: $p < 0.01$).

Group	Sobs[a]	Good's coverage	ACE[b]	Chao1	Shannon evenness
G1	96 ^{A}	0.988	110 ^{AB}	113 ^{AB}	0.731 ^{AB}
G2	38 ^{C}	0.996	44 ^{C}	44 ^{C}	0.659 ^{BC}
G3	23 ^{C}	0.996	29 ^{C}	27 ^{C}	0.191 ^{D}
G4	93 ^{AB}	0.986	123 ^{A}	125 ^{A}	0.760 ^{A}
G5	56 ^{BC}	0.991	71 ^{BC}	68 ^{BC}	0.636 ^{C}
G6	112 ^{A}	0.985	132 ^{A}	131 ^{A}	0.768 ^{A}
G7	42 ^{C}	0.994	54 ^{C}	52 ^{C}	0.584 ^{C}

[a], Sobs, the total number of species observed in the community
[b], ACE, abundance-based coverage estimation

Table 5-3. Genus identity of sequences represented by percentage from the total sequences. Only genera accounting for more than 5% of sequences in at least one group are displayed

Genus	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**
Bacillus	0	0	99.469	6.104	11.236	1.613	0
Aeromonas	0.593	0.004	0.008	91.264	0	80.043	0
Vogesella	0.477	0.132	0	0	76.524	1.935	0.004
Stenotrophomonas	0.542	65.116	0.320	0	0.024	0	0.104
Klebsiella	0	0.189	0.004	0.036	5.041	5.177	41.45
Trabulsiella	0	0.057	0	0	3.258	2.599	26.952
Chryseobacterium	13.163	18.145	0.0328	0	0	0	0.002
Puniceicoccaceae	17.936	0	0	0	0	0	0
Citrobacter	0.051	0.552	0.007	0.260	1.724	2.685	15.273
Enterobacter	0.324	0.060	0.002	0.032	1.679	1.909	12.293
Rheinheimera	0.358	8.904	0	0	0	0	0
Pseudomonas	8.555	1.984	0.066	0	0.002	0	0
Acinetobacter	8.546	0.399	0.004	0	0	0	0
Runella	8.534	0	0	0	0	0	0
Herbaspirillum	5.191	0.208	0	0	0	0	0

Table 5-4. 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
G1 & G2 = 90.98	*Stenotrophomonas*	15	2132	11.63
	Chryseobacterium	1147	1519	10.27
	Puniceicoccaceae	1684	0	9.25
G1 & G3 = 99.75	*Bacillus*	0	9072	45.47
	Puniceicoccaceae	1684	0	8.44
G2 & G3 = 99.61	*Bacillus*	0	90.72	45.54
	Stenotrophomonas	2132	10	10.66
G1 & G6 = 99.18	*Puniceicoccaceae*	1684	0	8.49
	Chryseobacterium	1147	0	5.78
	Aeromonas	0	971	4.89
G2 & G6 = 99.35	*Stenotrophomonas*	2132	0	10.73
	Chryseobacterium	1519	0	7.64
G3 & G6 = 98.39	*Bacillus*	9072	142	45.38
	Aeromonas	0	971	4.93
G1 & G7 = 99.81	*Klebsiella*	0	3811	19.09
	Puniceicoccaceae	1684	0	8.44
	Trabulsiella	0	1387	6.95
G2 & G7 = 99.12	*Klebsiella*	13	3811	19.16
	Stenotrophomonas	2132	4	10.74
G3 & G7 = 99.94	*Bacillus*	9072	0	45.39
	Klebsiella	0	3811	19.06
G6 & G7 = 87.65	*Klebsiella*	474	3811	19.04
	Trabulsiella	144	1387	7.09
G1 & G5 = 99.44	*Vogesella*	10	2071	10.36
	Puniceicoccaceae	1684	0	8.47
G2 & G5 = 99.38	*Stenotrophomonas*	2132	0	10.73
	Vogesella	2	2071	10.41
G3 & G5 = 88.74	*Bacillus*	9072	1024	45.34
	Vogesella	0	2071	11.67
G6 & G5 = 90.21	*Vogesella*	50	2071	11.20
	Aeromonas	971	0	5.38
G7 & G5 = 87.97	*Klebsiella*	3811	453	19.09
	Vogesella	0	2071	11.77
G1 & G4 = 99.40	*Puniceicoccaceae*	1684	0	8.47
	Chryseobacterium	1147	0	5.77
G2 & G4 = 99.86	*Stenotrophomonas*	2132	0	10.68
	Chryseobacterium	1519	0	7.61
G3 & G4 = 93.90	*Bacillus*	9072	559	45.33
	Aeromonas	0	1079	5.75

G6 & G4 = 26.62	*Klebsiella*	474	2	8.86
	Bacillus	142	559	8.57
G7 & G4 = 99.68	*Klebsiella*	3811	2	19.11
	Trabulsiella	1387	0	6.95
G5 & G4 = 95.29	*Vogesella*	2071	0	10.87
	Aeromonas	0	1079	5.66

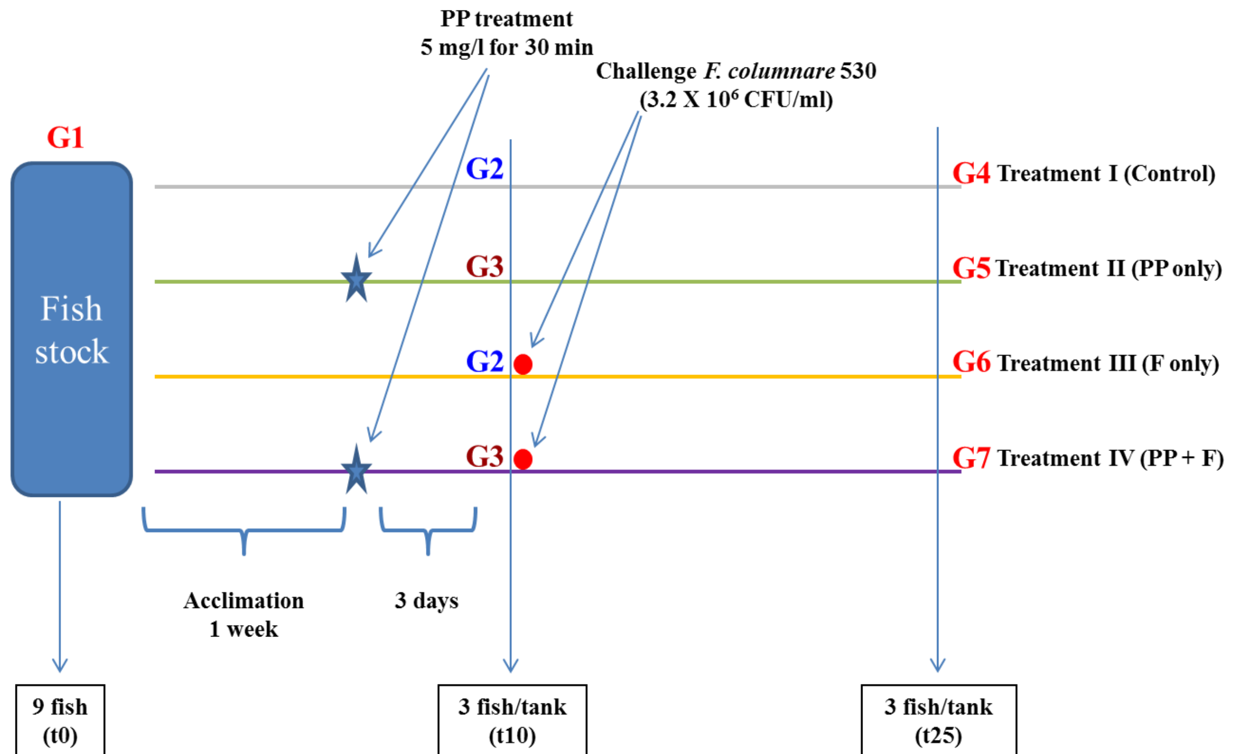

Figure 5-1. Experimental design showing the different treatments, time points and groups used in the study. (I) control = not treated nor challenged, (II) PP = treated with PP and not challenged, (III) F = not treated with PP and challenged with *F. columnare*, and (IV) PP+F = treated with PP and challenged with *F. columnare*. Treatments and DNA collection points (t_0, t_{10}, and t_{25}) are indicated on the timeline. Groups (G1 to G7) are indicated on each treatment.

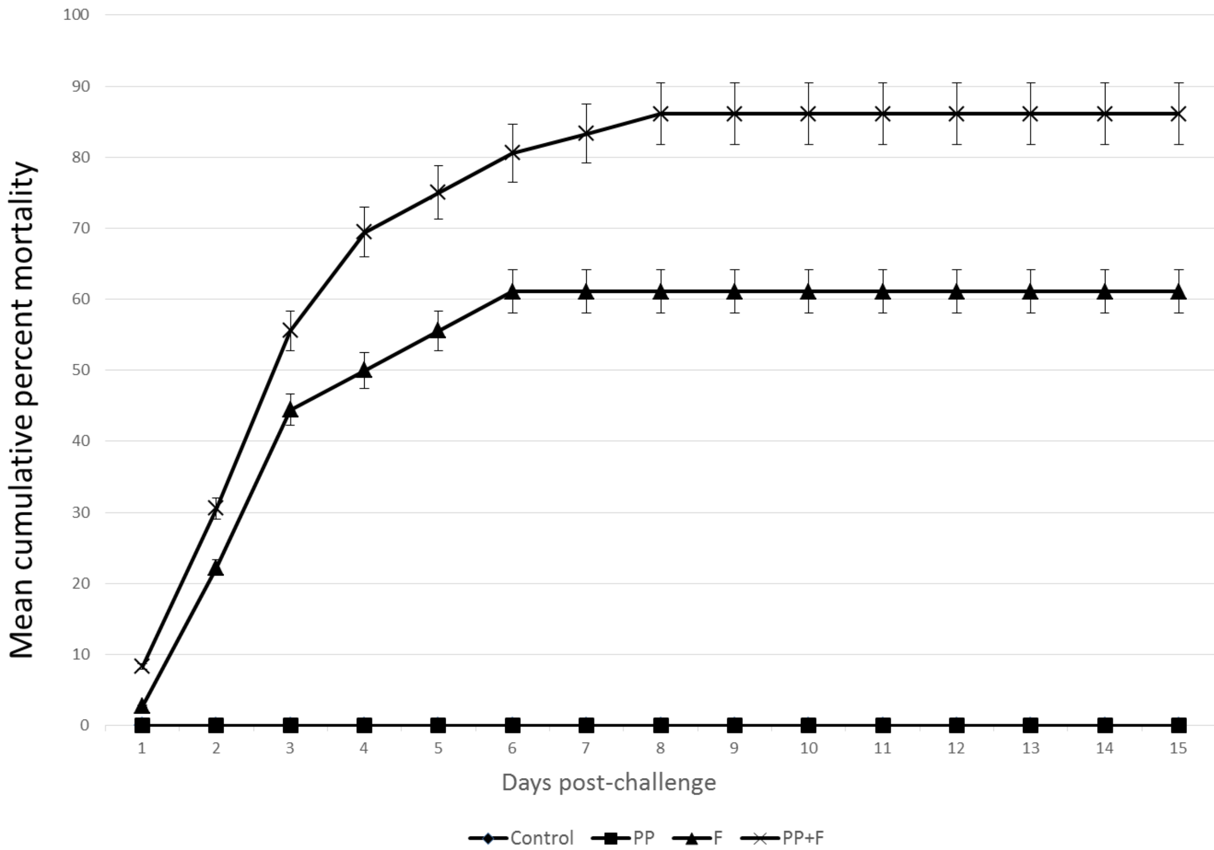

Figure 5-2. Mean cumulative percent mortality of channel catfish challenged with *Flavobacterium columnare*. (I) control = not treated nor challenged, (II) PP = treated with PP and not challenged, (III) F = not treated with PP and challenged with *F. columnare*, and (IV) PP+F = treated with PP and challenged with *F. columnare*. (Note: treatments I and II had 0% mortality, so the mortality curves are superimposed).

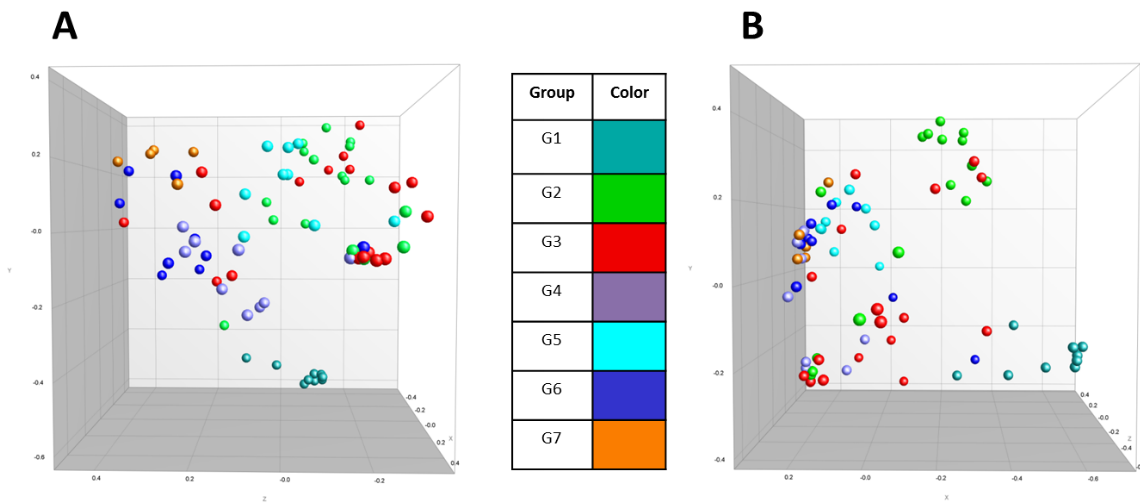

Figure 5-3. Multidimensional scaling (MDS) plot of skin (panel A) and gill (panel B) samples. The similarity matrix obtained was used to compare RISA fingerprints based on groups. Distance between entries represents graphical dissimilarities obtained from the similarity matrix.

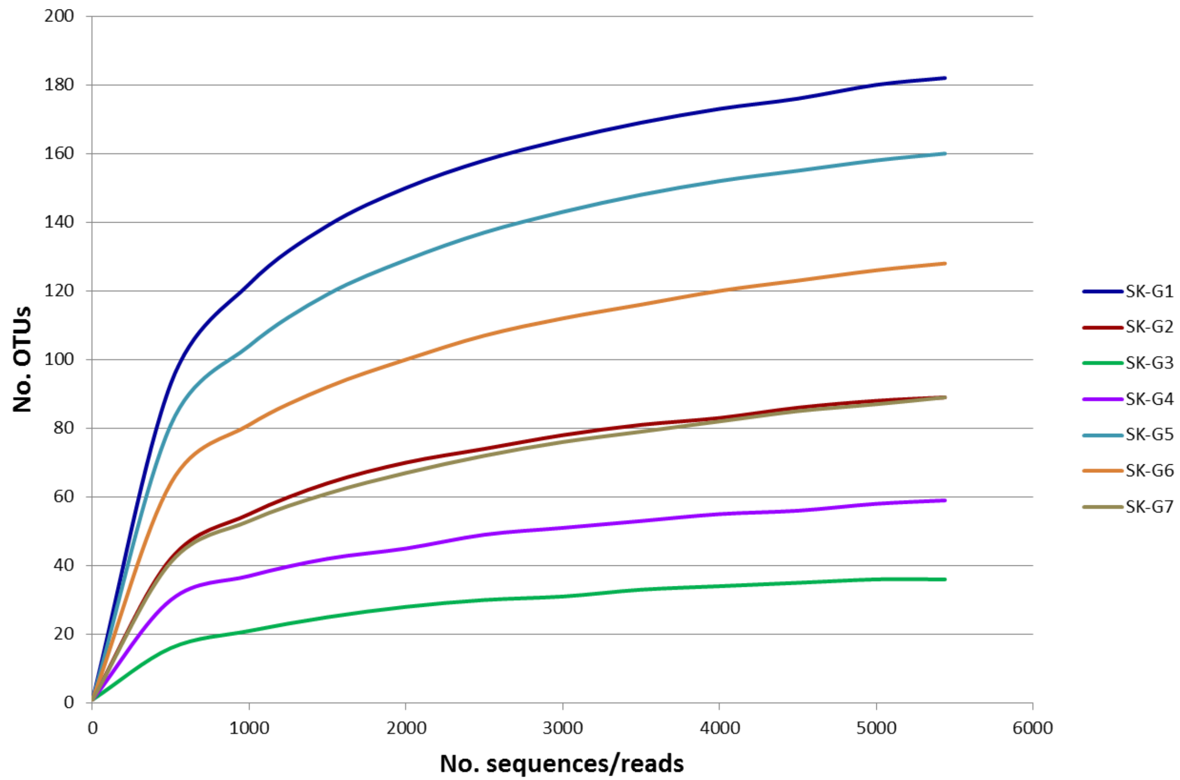

Figure 5-4. Rarefaction curves of skin samples when OTUs where defined at 97% sequence similarity. Samples were standardized to the least number of sequences obtained.

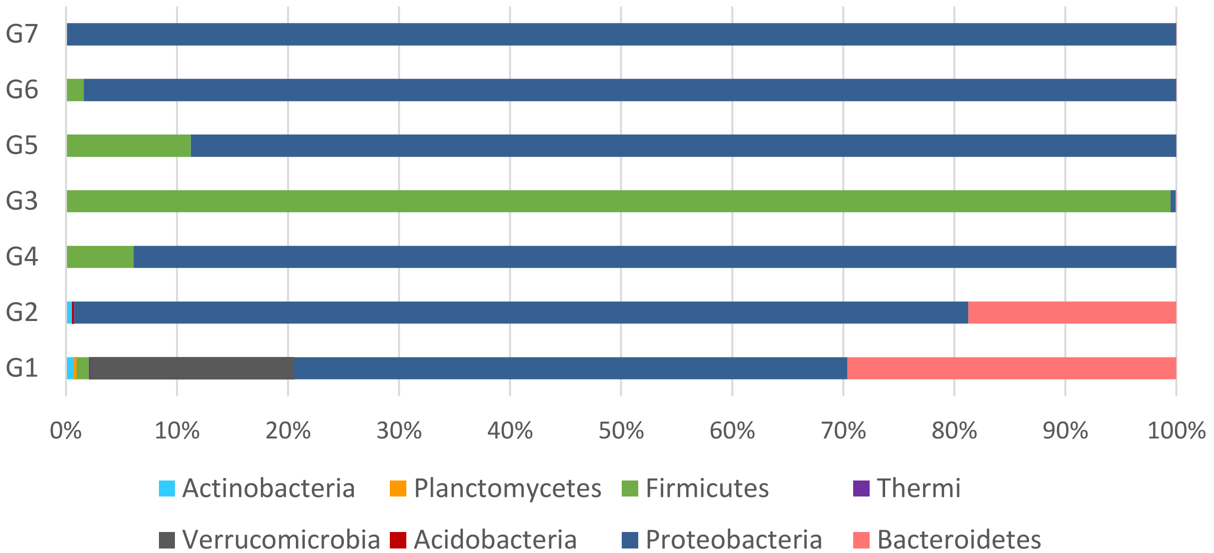

Figure 5-5. Bacteria phyla composition for each group, representing average of all replicates, obtained by pyrosequencing. Bacterial diversity at the phylum level based on pyrosequencing of 16S rRNA gene showing the differences in the skin microbiome structure between groups and the percent of detected sequences belonging to the different bacterial phyla in each group.

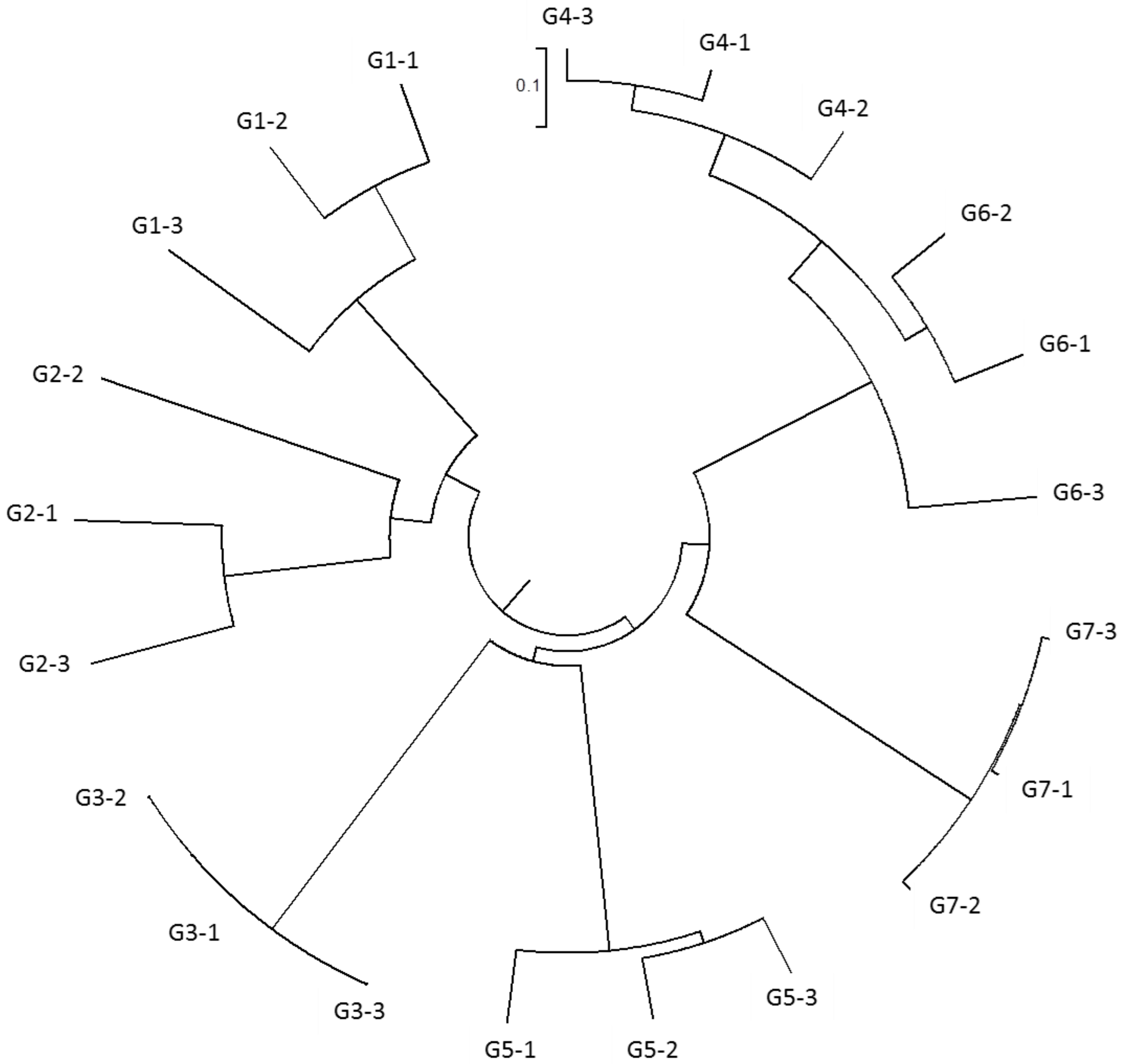

Figure 5-6. A dendrogram illustrating the hierarchical arrangement of the sequenced samples showing all replicates per group. The scale bar on the dendrogram represents the percentage of dissimilarity between two samples.

CHAPTER 6. PROTECTIVE EFFICACY OF *NIGELLA SATIVA* SEEDS AND OIL AGAINST COLUMNARIS DISEASE IN FISHES

Abstract

Columnaris disease, caused by the bacterium *Flavobacterium columnare*, is currently the most frequently reported bacterial disease affecting farm-raised channel catfish in the USA. Common treatments against the disease include the use of medicated feed that has led to emergent antibiotic resistant strains of *F. columnare*. *Nigella sativa* (Black cumin) is a medicinal herb commonly used by many cultures as a natural remedy for numerous disorders. Recently, I have discovered the antibacterial activity of *N. sativa* and its oil extract against *F. columnare*. In this study, I showed *N. sativa* oil (NSO) strongly inhibited the growth of all of the strains of *F. columnare* tested and yielded significantly larger zones of inhibition than those produced by oxytetracyclin. I tested the protective effect against columnaris disease *in vivo* by incorporating NSO (5%) or *N. sativa* seeds (NSS) (5%) into fish feeds. Fishes (*Ictalurus punctatus* and *Danio rerio*) fed amended diets displayed significantly lower mortality than those fed control diets. Percent mortalities in control groups ranged from 77% to 44% and from 70% to 18% in zebrafish and channel catfish, respectively. A dose study using different NSS concentrations showed that 5% NSS offered the most protection against columnaris disease in channel catfish.

Introduction

Over the last few decades, global aquaculture has been the world's fastest growing animal food-producing sector and the main source of the increase in fish supply [1, 2]. Unfortunately, the development of super-intensive culture systems required to maximize production are concomitant with fish stress and a subsequent higher incidence of infectious diseases [3]. Disease outbreaks, such as those of columnaris, are considered a major limiting factor to aquaculture production and account for a significant source of economic losses [4-6]. Columnaris disease, caused by the Gram-negative bacterium *Flavobacterium columnare*, continues to be a serious threat to freshwater finfish species resulting in extensive mortalities worldwide [7, 8]. This ubiquitous, opportunistic fish pathogen can be extremely virulent to ictalurids such as the commercially valuable food fish, channel catfish (*Ictalurus punctatus*, Rafinesque). Currently, columnaris disease costs the US catfish industry millions of dollars annually [9-13]. The disease also assails many ornamental fishes, including zebrafish, *Danio rerio*, [11, 14-16]. Despite the worldwide distribution and the significant economic losses of columnaris disease, safe and effective remedies are not yet available.

Due to the growing concern over the presence of antibiotic residues in foods and the emergence of drug resistant microbes, modern aquaculture industry desperately demands environmentally friendly alternative practices for sustainable disease management [17, 18]. The use of natural plant products as an alternative to chemical or antibiotic treatments for disease control in aquaculture has attracted great attention in recent years. *Nigella sativa* (also known as black cumin, black seeds or black caraway), belongings to family *Ranunculacea,* is an important medicinal herb growing in many Mediterranean, African, and Asian countries and has been proven to effectively treat a wide range of diseases. *N. sativa* oil (NSO) has been used all over

the world for thousands of years as a spice, condiment, carminative, food additive, and food preservative as well as a natural remedy for many disorders in traditional folk medicine [19, 20]. *N. sativa* seeds (NSS) contain 36-38% fixed oil, with proteins, alkaloids, saponins, and 0.4-2.5% essential oils making up the rest of the composition, and the main pharmacologically active substances include thymoquinone, thymohydroquinone, dithymoquinone, thymol and carvacol [21, 22]. Although NSO has been described to possess antimicrobial activity [23], antioxidant activity [21], anticancer activity [24], and immunostimulatory effect [25], a thorough literature search did not reveal any published report on the antibacterial properties of NSO towards *F. columnare*.

Recently, I have discovered the antibacterial activity of NSO against *F. columnare*. Therefore, the objectives of this study were to (1) fully evaluate the antibacterial effect of NSO against 25 strains of *F. columnare* representing three genomovars (I, II and III) by disc diffusion assay, (2) determine the protective efficacy of dietary supplementation with NSS or NSO against experimental columnaris disease in zebrafish and channel catfish for potential application as an antimicrobial feed additive, and (3) optimize the therapeutic dose in fish feed. I chose channel catfish and zebrafish to test these plant supplements based on their economic importance in the US [26, 27] and the fact that, in recent years, zebrafish has become a favorite and popular model species for studying vertebrate diseases and drug discovery [28-30].

Material and Methods

Bacterial cultures and inoculum preparation. Twenty five strains of *F. columnare* (Table 6-1) previously characterized by our group [13, 31, 32] were chosen for this study based on their

genetic diversity. The type strain of *F. columnare* (ATCC 23463) was used as a reference. All bacterial strains were stored as glycerol stocks at -80 °C and grown in modified Shieh (MS) [33] broth with continuous shaking at 100 rpm at 28 °C for 24 h. Bacterial inocula were prepared following a previously described protocol [34]. Briefly, the cultures were centrifuged in a refrigerated centrifuge at 2,900 rpm for 10 min at 10 °C to harvest the bacterial cells. Bacterial pellets were then re-suspended in 5 ml sterile saline solution (0.9% NaCl). To prevent excessive clumping, centrifugation and resuspension of the pellets in sterile saline were repeated to achieve a density of McFarland #2 standard. The optical density (OD) of the adjusted suspensions was measured at 600 nm using an Eppendorf Biophotometer (UV spectrophotometer, Eppendorf, Hamburg, Germany). The average OD_{600} of the adjusted inocula at McFarland #2 standard was 0.3. Immediately after preparation of bacterial saline suspensions, plate counts (in triplicates) of 5 suspensions/strains were carried out on MS agar plates to calculate the average number of colony forming units per milliliter (CFU/mL) of bacteria resulting in an average count of 2.3×10^7 CFU/ml.

Susceptibility testing. Antimicrobial susceptibility testing by disc diffusion was used to evaluate the susceptibility of all strains towards pure (solvent free, alcohol free) cold-pressed NSO (SaaQin, Inc., Hicksville, NY) on both improved diluted Mueller Hinton Agar (IDMHA) and on MS agar. *Escherichia coli* ATCC 25922 strain was used as a quality control organism. Dilute Mueller Hinton agar (DMHA) containing 4 grams of Mueller Hinton broth and 17 grams of agar per liter (pH 7.2), and improved by equine serum was found to support maximum *F. columnare* growth and was therefore recommended for susceptibility testing of *F. columnare* [34]. MS has been previously used by our group to determine antibiotic susceptibility in *F. columnare* [32]. IDMHA and MS agar (4 mm depth) plates were allowed to warm up to room

temperature to eliminate any excess moisture in the medium. Then, a fresh, sterile cotton-tipped swab was dipped into the standardized suspension for each strain, and the excess liquid was removed from the swab by pressing against the side of the tube. The surface of the test media (3 replicates (plates) per strain per medium) was inoculated by streaking with the saturated cotton swab to completely cover the surface of the plate with bacteria and to ensure the inoculum was evenly distributed following the manual on antimicrobial susceptibility testing procedures [35]. Plates were allowed to dry at room temperature for 15 min prior to the addition of discs. Oxytetracyclin (T30 µg, as positive control) and blank commercial Sensi-Disc cartridges were obtained from (Becton Dickinson (BD), Franklin Lakes, NJ, USA). Blank discs were impregnated with either 10 µl of NSO, 10 µl of vegetable oil (VO, oil control), or with 10 µl of sterile, distilled water (B, blank/negative control) [19] and were allowed to dry for 15 min before use. Four discs (T30, NSO, VO, and B) were distributed equidistantly on each plate. Discs were pressed gently against the agar surface to ensure contact and subsequent diffusion of the active principle. The plates were incubated at 28 °C for 48 h before the diameters of the inhibition zones around the paper discs were accurately measured in millimeters (mm) to the nearest millimeter using a metric ruler and recorded. The mean diameter of inhibition zones for each strain was calculated for both oxytetracycline and NSO on both test media and statistically compared.

Fish husbandry. Six month old zebrafish (n = 385, mean weight ± SD = 0.2 ± 0.01 g) were purchased from Aquatica Tropicals (Plant City, FL, USA), and channel catfish fingerlings (n = 310, mean weight ± SD = 2.2 ± 0.3 g) were purchased from Osage Catfisheries Inc. (Osage Beach, MO, USA). Purchased fishes were express shipped to the Aquatic Microbiology Laboratory (AML) located on main campus at Auburn University, Auburn, AL, USA. For the

dose determination study, a second batch of channel catfish fingerlings (n = 330, mean weight ± SD = 7.0 ± 0.5 g) was supplied by the E. W. Shell Fisheries Center (EWSFC) at North Auburn Fisheries Experiment Station, Auburn, AL, USA. Upon arrival to AML and prior to stocking into the glass tanks, mucus, skin, and gill samples of ten randomly caught fish from the initial pool of each batch were sampled, examined following standard procedures [36], and determined culture negative for *F. columnare*. Fish were then stocked into 37 L tanks (stocking rate is reported under each experiment), acclimatized to experimental tank conditions for 2 weeks before commencement of the experiment, and maintained as previously described [37]. Water quality parameters were monitored daily and were kept at 80 ppm alkalinity, 40 ppm hardness, 0.1 ppt salinity, 26 ± 1°C, pH 7.7 ± 0.2 [mean ± SD]. Ammonia and nitrites were kept at non-detectable levels. A dark-light photoperiod of 12:12 h was maintained throughout the experiment. Fish were fed daily to apparent satiation with commercial pellets, AQUAMAX Grower 100 for zebrafish and 400 for catfish (Purina Mills, Inc., St. Louis, MO, USA).

Zebrafish challenge. A total of 375 zebrafish were divided into 3 treatments and fed: (I) control diet, (II) diet containing 5% NSS, and (III) diet containing 5% NSO. Each treatment group consisted of 5 replicates (tanks) with 25 fish per tank. Tanks were assigned blindly to each treatment group. The control diet was prepared by mixing the food pellets with 3% menhaden fish oil (MFO) (Omega Protein, Houston, TX, USA). The experimental diets were formulated by mixing the food pellets with 5% ground NSS (Indus Organics, Inc., San Ramon, CA) or 5% NSO. MFO was added (3%) to the experimental diets to coat the ground seeds and to make the diets palatable to the fish. Dietary ingredients were thoroughly mixed together until a homogenous mixture was achieved. Diets were allowed to dry and then were stored frozen at −20 °C until use. Fish were fed daily on the experimental feeds at 2% of their body weight for a

period of 21 days prior to bacterial challenge. Then, fish were challenged by immersion for 30 min with a virulent strain of *F. columnare* (ALG-00-530) at a concentration of 9.6×10^6 CFU/mL following previously described procedures [37]. After challenge, fish were removed from the challenge suspensions, returned to their respective tanks, and maintained under normal husbandry conditions. Fish were not fed on the challenge day but were offered the experimental diets on day one after the challenge and throughout the remainder of the study. Fish were monitored for signs of columnaris disease, and mortality was recorded twice daily. Isolation of *F. columnare* from moribund and dead fish to confirm columnaris as cause of death was conducted as previously described [32]. The study was terminated 21days post-challenge after 10 consecutive days with no mortalities. In total, the experiment lasted for a total of 42 days, from the first day of feeding the experimental diets until the conclusion of the experiment.

Channel catfish challenge. A total of 300 channel catfish fingerlings were randomly distributed into three treatment groups (20 fish/tank, 5 replicates per treatment) following the same experimental design described in the zebrafish challenge experiment: (I) fed control diet, (II) fed diet containing 5% NSS, and (III) fed diet containing 5% NSO. Fish were fed 2% of their body weight on the experimental diets (prepared as described earlier) for 21 days before being challenged by immersion in a 2 L bath at room temperature (25°C) containing a virulent *F. columnare* strain (BGFS-27) at a concentration of 9.7×10^5 CFU/mL for 30 min following standard protocols. The strains of *F.* columnare used for challenge (BGFS-27 and ALG-00-530) were selected based on their virulence properties towards channel catfish and zebrafish, respectively [10, 11, 37]. At the conclusion of the 30 min challenge, fish from each treatment group were placed back into their holding tanks and maintained under normal husbandry conditions. Post-challenge protocols followed those described above in the zebrafish experiment.

Dose determination study. To determine the optimum therapeutic dietary concentration of NSS in channel catfish, a total of 320 fingerlings were blindly divided into 4 treatments (16 fish/tank, 5 replicates per treatment): (I) fed control diet, (II) fed diet containing 1% NSS, (III) fed diet containing 5% NSS, and (IV) fed diet containing 10% NSS. The control diet for treatment (I) was prepared as mentioned earlier. The experimental diets were prepared by mixing the food pellets with either 1%, 5%, or 10% ground NSS for treatments (II), (III), and (IV), respectively. MFO was added (3%) to all diets. Similar to the previous experiments, fish were fed the treatment diets for 21 days before the fish were exposed to *F. columnare* BGFS-27 for 30 min at a concentration of 8.5×10^5 CFU/ml in the challenge bath. The challenge was carried out following the standard protocols previously described [37]. After 30 min in the challenge suspension, fish were removed, returned to their respective tanks, and maintained under normal husbandry conditions. Fish were not fed on the day of the challenge, but feeding of the experimental diets was restored on the next day and continued for the remainder of the experiment. Fish were observed for clinical signs of columnaris disease twice daily, and mortalities were recorded for three weeks. *F. columnare* was isolated from the dead fish and confirmed as described above.

Statistical analysis. Statistical analysis was performed by the SAS software package (version 9.2, SAS Institute, Cary, NC, USA). Susceptibility and mortality data were analyzed by one-way analysis of variance (ANOVA) using the general linear model (PROC GLM) followed by Tukey's Studentized Range (HSD) test for all pairwise comparisons to determine significant differences ($p < 0.05$) between the means.

Results

Antibacterial susceptibility. Results of the antimicrobial susceptibility of 25 strains of *F. columnare* towards NSO are shown in Table 6-2. NSO displayed a strong antibacterial activity against all *F. columnare* strains used in the study. NSO yielded significantly larger zones of inhibition than those produced by oxytetracyclin. The average zones of inhibition (±SD) around NSO on IDMHA ranged from 33.7 ± 3.0 mm (BZ-1-02) to 53.7 ± 2.0 mm (ALM-05-114), while the average zones of inhibition around oxytetracyclin ranged from 9.0 ± 0.0 mm (BGFS-25) to 41.3 ± 1.7 mm (ATCC 23463). On MS agar, NSO resulted in average zones of inhibition ranging from 25.3 ± 1.0 mm (Grizzle) to 78.0 ± 1.0 mm (ATCC 23463), while oxytetracyclin resulted in average zones of inhibition ranging from 10.0 ± 0.0 mm (ALM-05-121) to 51.0 ± 1.0 mm (ATCC 23463). Although the 25 strains of *F. columnare* varied in their susceptibility to oxytetracyclin, none of the strains were resistant to NSO. In the case of oxytetracyclin resistant strains, the inhibitory effect of NSO was more than 4 times that of oxytetracyclin. No zones of inhibition were observed around the VO (vegetable oil control) or B (blank) discs indicating they did not possess any inhibitory effects toward *F. columnare*. Figure 6-1 shows the zones of inhibition for NSO, oxytetracyclin (T30), VO, and B discs on MS agar when ALG-00-530 (oxytetracyclin sensitive) and ALM-05-21 (oxytetracyclin resistant) strains were tested. Generally, zones of inhibition of NSO were much wider on MS agar medium than on IDMHA for the same strain. Moreover, zones of inhibition were better defined on MS agar than on IDMHA.

Challenge experiments. Table 6-3 shows the cumulative percent mortality values of all treatments when zebrafish and channel catfish were challenged with *F. columnare* after being fed the experimental diets. Supplemented diets were protective to zebrafish with cumulative percent

mortality values (±SD) of 76.8 ± 2.9, 52.8 ± 3.6, and 44 ± 4.4% for control (I), 5% NSS (II), and 5% NSO (III), respectively. The 44.0 ± 4.4 cumulative percent mortality of zebrafish fed 5% NSO (III) was significantly lower (*P<0.05*) than the 76.8 ± 2.9 cumulative percent mortality observed in control fed fish (I). The cumulative percent mortality of fish fed 5% NSS (II, 52.8 ± 3.6) was lower but not significantly different (*P<0.05*) from the cumulative percent mortality observed in control fed fish (I, 76.8± 2.9).

In channel catfish, amended feed yielded a higher survival than in zebrafish, with cumulative percent mortality values (±SD) of 70 ± 1.0, 18 ± 3.0, and 32 ± 2.5% for control (I), 5% NSS (II), and 5% NSO (III), respectively. The cumulative percent mortalities of both treatments (18 ± 3.0 in fish fed 5% NSS diet (II) and 32 ± 2.5 in fish fed 5% NSO diet (III)) were significantly (*P<0.05*) lower than the cumulative percent mortality of the control (70 ± 1.0) fed fish (I).

Following challenges, moribund zebrafish and channel catfish displayed clinical signs typical of columnaris disease, and all the dead fish were confirmed positive for *F. columnare* by culturing and PCR. Anecdotal observations at the end of the catfish challenge experiment (after 42 days of fish being fed the experimental diets) suggested that the average weight of the fish fed diets containing NSS and NSO was higher than the average weight of the control fed fish. The average weights ± SD per fish were 6.1 ± 0.4 g, 6.7 ± 0.5 g, and 6.9 ± 0.5 g for control (I), 5% NSS (II), and 5% NSO (III), respectively. Based on the channel catfish challenge results, supplementation with NSS was selected for further investigation in catfish to determine the optimum dietary concentration.

Dose study. The mean cumulative percent mortality of the four treatments is shown in Figure 6-2. When catfish were challenged with *F. columnare* BGFS-27 after being fed the experimental diets, the cumulative percent mortality values (±SD) were 73.75 ± 1.6, 43.75 ± 1.8, 33.75 ± 3.2, and 60 ± 2.9 for control diet (I), 1% NSS diet (II), 5% NSS diet (III), and 10% NSS diet (IV), respectively. Cumulative percent mortalities of both the 1% NSS diet (II, 43.75 ± 1.8) and the 5% NSS diet (III, 33.75 ± 3.2) were significantly ($P<0.05$) lower than that of the control diet (I, 73.75 ± 1.6). Although the cumulative percent mortality of fish fed 10% NSS diet (IV, 60 ± 2.9) was lower than that of the group fed the control diet (I, 73.75 ± 1.6), there was no significant difference ($P<0.05$) statistically. The average weight/fish (± SD) at the end of the experiment (after 42 days) was 14.2 ± 0.7, 15.8 ± 0.9, 16.1 ± 1.0, and 14.9 ± 0.8 g for control (I), 1% NSS (II), 5% NSS (III), and 10% NSS (IV), respectively.

Discussion

Disease management in fish farms is one of the most complex challenges to the development of productive and sustainable aquaculture. To date, fish farmers rely routinely on heavy use of chemotherapy and antimicrobial agents for treatment of disease outbreaks [17, 38]. However, using chemotherapeutants in aquaculture has multiple drawbacks including the development and spread of antibiotic resistance [39-41]. In the present work, I showed the potential of NSO and NSS as biologicals against columnaris disease.

NSO displayed a potent antimicrobial activity against all tested strains of *F. columnare* with mean inhibition zone diameters substantially larger than those of oxytetracyclin. I used oxytetracyclin as a positive control because it is the most commonly used antibiotic and one of

the three FDA approved drugs for use in US aquaculture [42]. Although the different strains of *F. columnare* used in this study varied in their susceptibility to oxytetracyclin (some strains were resistant as previously reported [32]), NSO was effective in inhibiting the growth of all tested strains regardless of their genetic differences, geographic origins, host species, and dates of isolation. Conversely, none of the 25 strains showed resistance to NSO. Research has shown that both the crude extract and the water extract of *N. sativa* have antimicrobial effects on multiple drug-resistant bacteria isolated from clinical cases of human patients [23, 43, 44]. In preliminary tests, I have confirmed that NSO effectively inhibited the growth of other members of the genus *Flavobacterium* including *F. psychrophilum*, *F. enshiense*, *F. macrobrachae*, and *F. soliperosum* (data not shown). However, I did not observe a similar suppression against other fish pathogens (*Aeromonas hydrophila*, *Edwardsiella ictaluri*, *Edwardsiella tarda*, and *Yersinia ruckeri*) (data not shown). These results suggest the inhibition mechanism induced by NSO is not universal but clearly has a deleterious effect against members of the genus *Flavobacterium*.

My results demonstrate that dietary supplementation with 5% NSS or NSO protects zebrafish and channel catfish against experimental infection with *F. columnare* in controlled laboratory challenges. For each species, the cumulative percent mortality of fish fed with experimental diets was lower than that of their respective control fed fish. A previous study in which rainbow trout were fed 1, 2.5, and 5% NSS showed immunostimulatory effects as increased serum protein and total immunoglobulin levels; however, no challenge experiments were carried out for verification in that study [45]. Similarly, diets with 1, 2 and 3% NSO fed for 14 days enhanced the immune response (lysozyme, antiprotease, total protein, myeloperoxidase, bactericidal activity, and IgM titers) of rainbow trout [46]. It has been reported that feeding Nile tilapia with 3% NSS for 30 successive days significantly increased white blood cell counts and

significantly decreased the mortality rate when fish were challenged with *A. hydrophila* compared to those that received the control diet [47]. However, my *in vitro* preliminary screenings did not detect an inhibitory effect for NSO against *A. hydrophila* isolates. This reported reduction in mortality after exposure to *A. hydrophila* could be due to the obvious immunostimulatory benefits of feeding *N. sativa*. Additionally, thymoquinone, a constituent of NSO, showed significant antimycotic activity against water molds, particularly *Saprolegnia* spp. pathogenic to fish [48]. In the case of columnaris, fish could benefit from both the direct antibacterial activity of *N. sativa* against *F. columnare* as well as its immune enhancement properties; however, no immunological parameters were measured in my study.

To determine if there was a dose-response relationship, I compared 3 different dietary concentrations of 1, 5, and 10% NSS. Unexpectedly, only the cumulative percent mortalities of fish fed 1% NSS diet (II, 43.75 ± 1.8) and 5% NSS diet (III, 33.75 ± 3.2) were significantly ($P<0.05$) lower than that of the control fed fish (I, 73.75 ± 1.6). Whereas, the cumulative percent mortality of fish fed 10% NSS diet (IV, 60 ± 2.9) was lower but not significantly different ($P<0.05$) from that of the group fed control diet (I, 73.75 ± 1.6). Some therapeutic agents may exert more antibacterial action at lower than at higher concentrations due to physicochemical properties (for example, solubility and diffusion) [22]. Previously, the effects of different dietary levels (0%, 1%, 2% and 3%) of NSS on performance, intestinal *E. coli* count, and morphology of jejunal epithelium in laying hens were investigated after a 10 week period. Although all supplementation levels decreased the *E. coli* numbers and improved eggshell quality, morphological and histological alterations in the small intestine were observed after dietary supplementation with at least 2% NSS. Moreover, the best feed conversion ratio (FCR) was obtained when diets were supplemented with 2% NSS [49]. Similarly, when the effects of 0.5%,

1%, 2% or 3% NSS were evaluated as a natural growth promoting substance to replace antibiotics in broiler diets, supplementation of 1% NSS was the best to improve body weight, FCR, and carcass yield after a growing period of 6 weeks, followed by the other concentrations and the control (the supplementation of NSS in excess of 1% did not have any additional benefits). Accordingly, supplementation of diets with 1% NSS was recommended to be used as a natural alternative growth promoter for poultry instead of antibiotics [50]. My results are in agreement with previously reported data regarding NSS dose administration.

The mortality I obtained by dietary 5% NSS (33.75 ± 3.2%) in the dose study was different from the observed mortality when the same concentration was used in the previous catfish experiment (18 ± 3.0%). These differences in mortality could be attributed to the fact that the catfish used for the dose study were neither from the same genetic stock nor the same age as the fish used for the previous catfish challenge experiment. Different lots of fish were previously shown to vary in susceptibility to *F. columnare* [51]. Moreover, differences in genetic stocks, physiological state, rearing conditions, and/or prior exposure can certainly result in variations in fish susceptibility as previously suggested [37, 51-53].

Although I did not carry out FCR experiments, I observed higher average body weight in catfish fed the experimental diets than that of the control fed catfish. Several studies have found that NSS enhances growth performance and immunity of fish [46, 54]. These results were also in accordance with earlier reports of significantly improved weight gain and FCR in broilers receiving diets supplemented with NSS due to the more efficient use of nutrients, improved intestinal health, and/or reduced intestinal infections [50, 55-57]. Interestingly, meat taste acceptability revealed no abnormal odor or flavor induced by inclusion of NSS in broiler diet (NSS have traditionally been used as condiment and flavoring agents in cookery) following

sensory evaluation of broiler's meat [58]. Thus, supplementing fish diets with NSS might not influence meat culinary properties although sensory evaluation of the meat will have to be conducted.

In summary, considering the rapid growth and importance of aquaculture industry worldwide and the widespread, intensive, and often unregulated use of chemotherapeutants, all modern approaches for prevention and treatment of aquaculture diseases are now shifting away from conventional therapies to much ecologically safer and more economical alternative management practices. Overall, I found NSO to be a potent antibacterial against *F. columnare*. The results also showed the protective potential of dietary supplementation with NSS or NSO against columnaris disease. Further research should be conducted to determine if amended feeds with NSS or NSO improve other culture traits such as FCR, resistance to other pathogens, lower stress response, etc. Although more work still needs to be carried out to define the pharmacokinetics, pharmacodynamics, and possible toxicity of the pharmacoactive ingredient(s) in targeted fish species, the use of NSS or NSO to prevent bacterial infections in aquaculture is a promising alternative to the use of antibiotics.

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References

[1] Olsen R.L., Hasan M.R., A limited supply of fishmeal: Impact on future increases in global aquaculture production, Trends in Food Science & Technology. (2012) 27:120-128.

[2] Mathiesen A., The State of the World Fisheries and Aquaculture 2012. (2012).

[3] Iwama G.K., Pickering A., Sumpter J., Schreck C., Fish stress and health in aquaculture, Cambridge University Press, 2011.

[4] Dorsey D., Robertson W., Recent Advances in Fish Diseases Treatment: Probiotics as Alternative Therapy to Antibiotics in Aquaculture, European Journal of Ocean and Marine. (2013) 11:20-27.

[5] Verschuere L., Rombaut G., Sorgeloos P., Verstraete W., Probiotic bacteria as biological control agents in aquaculture, Microbiology and Molecular Biology Reviews. (2000) 64:655-671.

[6] Balcázar J.L., Blas I.d., Ruiz-Zarzuela I., Cunningham D., Vendrell D., Múzquiz J.L., The role of probiotics in aquaculture, Veterinary Microbiology. (2006) 114:173-186.

[7] Beck B.H., Li C., Farmer B.D., Barnett L.M., Lange M.D., Peatman E., A comparison of high- and low-virulence *Flavobacterium columnare* strains reveals differences in iron acquisition components and responses to iron restriction, Journal of Fish Diseases. (2015):n/a-n/a.

[8] Pulkkinen K., Suomalainen L.R., Read A., Ebert D., Rintamäki P., Valtonen E., Intensive fish farming and the evolution of pathogen virulence: the case of columnaris disease in Finland, Proceedings of the Royal Society B: Biological Sciences. (2010) 277:593-600.

[9] Arias C.R., Welker T.L., Shoemaker C.A., Abernathy J.W., Klesius P.H., Genetic fingerprinting of *Flavobacterium columnare* isolates from cultured fish, Journal of Applied Microbiology. (2004) 97:421-428.

[10] Shoemaker C.A., Olivares-Fuster O., Arias C.R., Klesius P.H., *Flavobacterium columnare* genomovar influences mortality in channel catfish (*Ictalurus punctatus*), Veterinary Microbiology. (2008) 127:353-359.

[11] Olivares-Fuster O., Bullard S.A., McElwain A., Llosa M.J., Arias C.R., Adhesion dynamics of *Flavobacterium columnare* to channel catfish *Ictalurus punctatus* and zebrafish *Danio rerio* after immersion challenge, Diseases of Aquatic Organisms. (2011) 96:221-227.

[12] Wagner B.A., Wise D.J., Khoo L.H., Terhune J.S., The epidemiology of bacterial diseases in food-size channel catfish, Journal of Aquatic Animal Health. (2002) 14:263-272.

[13] Olivares-Fuster O., Baker J.L., Terhune J.S., Shoemaker C.A., Klesius P.H., Arias C.R., Host-specific association between *Flavobacterium columnare* genomovars and fish species, Systematic and Applied Microbiology. (2007) 30:624-633.

[14] Decostere A., Haesebrouck F., Devriese L.A., Characterization of four *Flavobacterium columnare* (*Flexibacter columnaris*) strains isolated from tropical fish, Veterinary Microbiology. (1998) 62:35-45.

[15] Roberts H.E., Palmeiro B., Weber Iii E.S., Bacterial and Parasitic Diseases of Pet Fish, Veterinary Clinics of North America: Exotic Animal Practice. (2009) 12:609-638.

[16] Bullard S.A., McElwain A., Arias C.R., Scanning Electron Microscopy of "Saddleback" Lesions Associated with Experimental Infections of *Flavobacterium columnare* in Channel Catfish, *Ictalurus punctatus* (Siluriformes: Ictaluridae), and Zebrafish, *Danio rerio* (Cypriniformes: Cyprinidae), Journal of the World Aquaculture Society. (2011) 42:906-913.

[17] Ángeles Esteban M., An overview of the immunological defenses in fish skin, International Scholarly Research Notices. (2012) 2012.

[18] Pandiyan P., Balaraman D., Thirunavukkarasu R., George E.G.J., Subaramaniyan K., Manikkam S., Sadayappan B., Probiotics in aquaculture, Drug Invention Today. (2013) 5:55-59.

[19] Nair M.K.M., Vasudevan P., Venkitanarayanan K., Antibacterial effect of black seed oil on *Listeria monocytogenes*, Food Control. (2005) 16:395-398.

[20] Merfort I., Wray V., Barakat H., Hussein S., Nawwar M., Willuhn G., Flavonol triglycosides from seeds of *Nigella sativa*, Phytochemistry. (1997) 46:359-363.

[21] Burits M., Bucar F., Antioxidant activity of *Nigella sativa* essential oil, Phytotherapy Research. (2000):323-328.

[22] Ali B.H., Blunden G., Pharmacological and toxicological properties of *Nigella sativa*, Phytotherapy Research. (2003) 17:299-305.

[23] Morsi N.M., Antimicrobial effect of crude extracts of *Nigella sativa* on multiple antibiotics-resistant bacteria, Acta Microbiologica Polonica. (1999) 49:63-74.

[24] Worthen D.R., Ghosheh O.A., Crooks P., The in vitro anti-tumor activity of some crude and purified components of blackseed, *Nigella sativa* L, Anticancer research. (1997) 18:1527-1532.

[25] Salem M.L., Hossain M.S., *In vivo* acute depletion of CD8+ T cells before murine cytomegalovirus infection upregulated innate antiviral activity of natural killer cells, International Journal of Immunopharmacology. (2000) 22:707-718.

[26] Crim M.J., Riley L.K., Viral Diseases in Zebrafish: What Is Known and Unknown, ILAR Journal. (2012) 53:135-143.

[27] USDA, United States Department of Agriculture Catfish 2010 Part 1: reference of catfish health and production practices in the United States, 2009, USDA-APHIS-VS, CEAH. (2010):580-1210.

[28] Zon L.I., Peterson R.T., In vivo drug discovery in the zebrafish, Nat Rev Drug Discov. (2005) 4:35-44.

[29] Rubinstein A.L., Zebrafish: from disease modeling to drug discovery, Current Opinion in Drug Discovery and Development. (2003) 6:218-223.

[30] Dahm R., Geisler R., Learning from small fry: the zebrafish as a genetic model organism for aquaculture fish species, Marine biotechnology. (2006) 8:329-345.

[31] Olivares-Fuster O., Shoemaker C.A., Klesius P.H., Arias C.R., Molecular typing of isolates of the fish pathogen, *Flavobacterium columnare*, by single-strand conformation polymorphism analysis, Fems Microbiology Letters. (2007) 269:63-69.

[32] Mohammed H., Arias C., Epidemiology of columnaris disease affecting fishes within the same watershed, Diseases of Aquatic Organisms. (2014) 109:201-211.

[33] Shoemaker C.A., Arias C.R., Klesius P.H., Welker T.L., Technique for identifying *Flavobacterium columnare* using whole-cell fatty acid profiles, Journal of Aquatic Animal Health. (2005) 17:267-274.

[34] Farmer B., Improved methods for the isolation and characterization of *Flavobacterium columnare*, 2004.

[35] Lalitha M., Manual on antimicrobial susceptibility testing, Performance standards for antimicrobial testing: Twelfth Informational Supplement. (2004) 56238:454-456.

[36] Thoesen J.C., Suggested procedures for the detection and identification of certain finfish and shellfish pathogens, Vol. American Fisheries Society-Fish Health Section, Bethesda, ML, American Fisheries Society, 2004.

[37] Mohammed H., Olivares-Fuster O., LaFrentz S., Arias C.R., New attenuated vaccine against columnaris disease in fish: Choosing the right parental strain is critical for vaccine efficacy, Vaccine. (2013) 31:5276-5280.

[38] Haya K., Burridge L., Davies I., Ervik A., A review and assessment of environmental risk of chemicals used for the treatment of sea lice infestations of cultured salmon, Environmental effects of marine finfish aquaculture, Springer, 2005, pp. 305-340.

[39] Boyd C.E., Massaut L., Risks associated with the use of chemicals in pond aquaculture, Aquacultural Engineering. (1999) 20:113-132.

[40] Sapkota A., Sapkota A.R., Kucharski M., Burke J., McKenzie S., Walker P., Lawrence R., Aquaculture practices and potential human health risks: Current knowledge and future priorities, Environment international. (2008) 34:1215-1226.

[41] Baquero F., Martínez J.-L., Cantón R., Antibiotics and antibiotic resistance in water environments, Current opinion in biotechnology. (2008) 19:260-265.

[42] Romero J., Feijoó C.G., Navarrete P., Antibiotics in aquaculture-use, abuse and alternatives, INTECH Open Access Publisher, 2012.

[43] Ferdous A., Islam S., Ahsan M., Hasan C., Ahmed Z., In vitro antibacterial activity of the volatile oil of *Nigella sativa* seeds against multiple drug resistant isolates of *Shigella* spp. and isolates of *Vibrio cholerae* and *Escherichia coli*, Phytotherapy Research. (1992) 6:137-140.

[44] Salman M.T., Khan R.A., Shukla I., Antimicrobial activity of *Nigella sativa* Linn. seed oil against multi-drug resistant bacteria from clinical isolates, Natural Product Radiance. (2008) 7:10-14.

[45] Dorucu M., Colak S.O., Ispir U., Altinterim B., Celayir Y., The effect of black cumin seeds, *Nigella sativa*, on the immune response of rainbow trout, *Oncorhynchus mykiss*, Med. Aquacult. J. (2009) 2:1-7.

[46] Awad E., Austin D., Lyndon A.R., Effect of black cumin seed oil (*Nigella sativa*) and nettle extract (Quercetin) on enhancement of immunity in rainbow trout, *Oncorhynchus mykiss* (Walbaum), Aquaculture. (2013) 388–391:193-197.

[47] Elkamel A.A., Mosaad G.M., Immunomodulation of Nile Tilapia, *Oreochromis niloticus*, by *Nigella sativa* and *Bacillus subtilis*, Journal of Aquaculture Research & Development. (2012) 3.

[48] Hussein M.M.A., El-Feki M.A., Hatai K., Yamamoto A., Inhibitory Effects of Thymoquinone from *Nigella sativa* on Pathogenic *Saprolegnia* in Fish, Biocontrol science. (2002) 7:31-35.

[49] Boka J., Mahdavi A.H., Samie A.H., Jahanian R., Effect of different levels of black cumin (*Nigella sativa* L.) on performance, intestinal *Escherichia coli* colonization and jejunal morphology in laying hens, Journal of Animal Physiology and Animal Nutrition. (2014) 98:373-383.

[50] Guler T., Dalkilic B., Ertas O., Ciftci M., The effect of dietary black cumin seeds (*Nigella sativa L.*) on the performance of broilers, ASIAN AUSTRALASIAN JOURNAL OF ANIMAL SCIENCES. (2006) 19:425.

[51] Shoemaker C.A., LaFrentz B.R., Lack of association between *Flavobacterium columnare* genomovar and virulence in hybrid tilapia *Oreochromis niloticus* (L.) × *Oreochromis aureus* (Steindachner), Journal of Fish Diseases. (2014):n/a-n/a.

[52] Beck B.H., Farmer B.D., Straus D.L., Li C., Peatman E., Putative roles for a rhamnose binding lectin in *Flavobacterium columnare* pathogenesis in channel catfish *Ictalurus punctatus*, Fish & Shellfish Immunology. (2012).

[53] LaFrentz B.R., Shoemaker C.A., Booth N.J., Peterson B.C., Ourth D.D., Spleen index and mannose-binding lectin levels in four channel catfish families exhibiting different susceptibilities to *Flavobacterium columnare* and *Edwardsiella ictaluri*, Journal of Aquatic Animal Health. (2012) 24:141-147.

[54] John G., Aly S., Rezk M., El Naggar G., Mohamed Fathi M., Effect of some immunostimulants as feed additives on the survival and growth performance of Nile Tilapia *Oreochromis niloticus*: and their response to artificial infection, Egypt Journal of Aquatic Biology & Fish. (2007) 11:1299-1308.

[55] Al-Beitawi N., El-Ghousein S., Effect of feeding different levels of *Nigella sativa* seeds (Black cumin) on performance, blood constituents and carcass characteristics of broiler chicks, International Journal of Poultry Science. (2008) 7:715-721.

[56] Al-Beitawi N.A., El-Ghousein S.S., Nofal A.H., Replacing bacitracin methylene disalicylate by crushed *Nigella sativa* seeds in broiler rations and its effects on growth, blood constituents and immunity, Livestock Science. (2009) 125:304-307.

[57] Erener G., Altop A., Ocak N., Aksoy H., Cankaya S., Ozturk E., Influence of Black Cumin Seed (*Nigella sativa* L.) and Seed Extract on Broilers Performance and Total Coliform Bacteria Count, Asian Journal of Animal & Veterinary Advances. (2010) 5:128–135.

[58] Toghyani M., Toghyani M., Gheisari A., Ghalamkari G., Mohammadrezaei M., Growth performance, serum biochemistry and blood hematology of broiler chicks fed different levels of black seed (*Nigella sativa*) and peppermint (*Mentha piperita*), Livestock Science. (2010) 129:173-178.

Table 6-1. List of *F. columnare* strains used in the study n= 25 strains

Strain Name	Genomovar	Species	Geographic origin	Year isolated
ALM-05-30	II	Channel catfish	Alabama	2005
ALM-05-35	II	Fresh water drum	Alabama	2005
ALM-05-58	II	Blue catfish	Alabama	2005
ALM-05-68	I	Threadfin shad	Alabama	2005
ALM-05-114	I	Threadfin shad	Alabama	2005
ALM-05-121	II	Channel catfish	Alabama	2005
ALM-05-122	I	Blue catfish	Alabama	2005
ALM-05-173	II	Blue catfish	Alabama	2005
ALM-05-202	II	Blue catfish	Alabama	2005
ALG-00-530	II	Channel catfish	Alabama	2000
ALG-00-527	II	Channel catfish	Alabama	2000
ATCC23463	I	Chinook salmon	Washington	1972
ARS-1	I	Channel catfish	Alabama	1996
AU-98-24	III	Channel catfish	Alabama	1998
BGFS-25	II	Channel catfish	Alabama	2005
BGFS-27	II	Channel catfish	Alabama	2005
BioMed	I	Channel catfish	Alabama	1996
BZ-1-02	II	Nile tilapia	Brazil	2002
Dickerson-I	III	Channel catfish	Unknown	1995
Grizzle	I	Channel catfish	Alabama	Unknown
GA-02-14	I	Rainbow trout	Georgia	2002
CL-gill	II	Nile tilapia	Alabama	2011
LT-ulcer	II	Nile tilapia	Alabama	2011
TL-S 2	II	Nile tilapia	Alabama	2010
TL-G	II	Nile tilapia	Alabama	2010

Table 6-2. Antimicrobial susceptibility of the *F. columnare* strains showing the mean diameter (in mm) of inhibition zones (±SD).

Strain name	IDMHA[A]				MSA[B]			
	NSO[C]	T30[D]	VO[E]	B[F]	NSO[C]	T30[D]	VO[E]	B[F]
ALM-05-30	47.0 ± 1.7	35.0 ± 1.0	0	0	64.3 ± 2.1	32.7 ± 1.2	0	0
ALM-05-35	39.7 ± 1.2	38.0 ± 1.0	0	0	45.0 ± 3.5	28.7 ± 0.6	0	0
ALM-05-58	46.7 ± 0.6	36.7 ± 0.6	0	0	64.7 ± 0.6	30.3 ± 1.2	0	0
ALM-05-68	49.7 ± 1.2	38.7 ± 1.2	0	0	69.0 ± 1.7	34.7 ± 1.5	0	0
ALM-05-114	53.7 ± 2.3	31.7 ± 1.2	0	0	60.7 ± 4.5	30.0 ± 0.0	0	0
ALM-05-121	53.7 ± 2.3	10.0 ± 0.0	0	0	61.0 ± 5.3	10.0 ± 0.0	0	0
ALM-05-122	49.3 ± 3.1	36.0 ± 0.0	0	0	56.0 ± 1.0	27.0 ± 0.0	0	0
ALM-05-173	50.0 ± 3.0	34.7 ± 1.5	0	0	71.0 ± 0.0	31.0 ± 0.0	0	0
ALM-05-202	46.3 ± 2.1	34.3 ± 0.6	0	0	48.3 ± 4.0	30.7 ± 1.5	0	0
ALG-00-530	47.0 ± 2.6	35.7 ± 1.5	0	0	71.7 ± 1.2	28.7 ± 0.6	0	0
ALG-00-527	50.0 ± 1.0	36.7 ± 1.5	0	0	54.3 ± 3.1	34.3 ± 1.2	0	0
ATCC23463	46.7 ± 5.0	41.3 ± 1.5	0	0	78.0 ± 1.0	51.0 ± 1.0	0	0
ARS-1	36.3 ± 1.2	33.0 ± 1.0	0	0	46.0 ± 2.6	25.3± 0.6	0	0
AU-98-24	43.7 ± 2.3	35.0 ± 0.0	0	0	63.7 ± 5.0	29.0 ± 0.0	0	0
BGFS-25	42.7 ± 7.1	9.0 ± 0.0	0	0	71.7 ± 2.3	13.7 ± 2.3	0	0
BGFS-27	43.3 ± 2.5	35.7 ± 2.3	0	0	73.0 ± 2.0	38.7 ± 0.6	0	0
BioMed	49.3 ± 2.1	38.3 ± 1.2	0	0	59.0 ± 7.2	31.3 ± 1.5	0	0
BZ-1-02	33.7 ± 5.5	30.0 ± 2.6	0	0	59.3 ± 1.2	31.3 ± 1.5	0	0
Dickerson I	53.3 ± 3.5	33.3 ± 1.5	0	0	77.0 ± 1.0	33.7 ± 0.6	0	0
Grizzle	40.0 ± 0.0	29.7 ± 0.6	0	0	25.3 ± 0.6	24.3 ± 1.2	0	0
GA-02-14	35.0 ± 4.9	32.0 ± 1.7	0	0	40.7 ± 2.3	25.3 ± 0.6	0	0
CL Gill	36.0 ± 2.0	36.3 ± 1.5	0	0	49.3 ± 2.1	26.7 ± 0.6	0	0
LT Ulcer	39.7 ± 0.6	36.3 ± 1.5	0	0	63.0 ± 10.5	27.7 ± 1.5	0	0
TL-S 2	41.7 ± 2.3	35.3 ± 1.2	0	0	41.7 ± 2.1	25.3 ± 1.2	0	0
TL-G	38 ± 2.0	36.3 ± 1.5	0	0	42.0 ± 2.6	25.3 ± 0.6	0	0

[A] IDMHA, improved diluted Muller Hinton Agar
[B] MS, Modified Shieh Agar
[C] NSO, *N. sativa* Oil
[D] T30, Oxytetracyclin discks 30µg
[E] VO, Vegetable Oil
[F] B, Blank

Table 6-3. Cumulative percent mortality (± SD) of zebrafish and channel catfish fed meal with control, NSS, or NSO and immersion challenged with *F. columnare* ALG-00-530 or BGFS-27, respectively. Means followed by the same superscript letter within a column are not significantly (*P<0.05*) different as determined by Tukey's Studentized Range (HSD) Test.

	Zebrafish		**Channel catfish**	
Treatment	% Mortality	% Survival	% Mortality	% Survival
Control	76.8 ± 2.9^{A}	23.2 ± 2.9^{A}	70 ± 1.0^{A}	30 ± 1.0^{A}
NSS (5%)	52.8 ± 3.6^{AB}	47.2 ± 3.6^{AB}	18 ± 3.0^{B}	82 ± 3.0^{B}
NSO (5%)	44.0 ± 4.4^{B}	56 ± 4.4^{B}	32 ± 2.5^{B}	68 ± 2.5^{B}

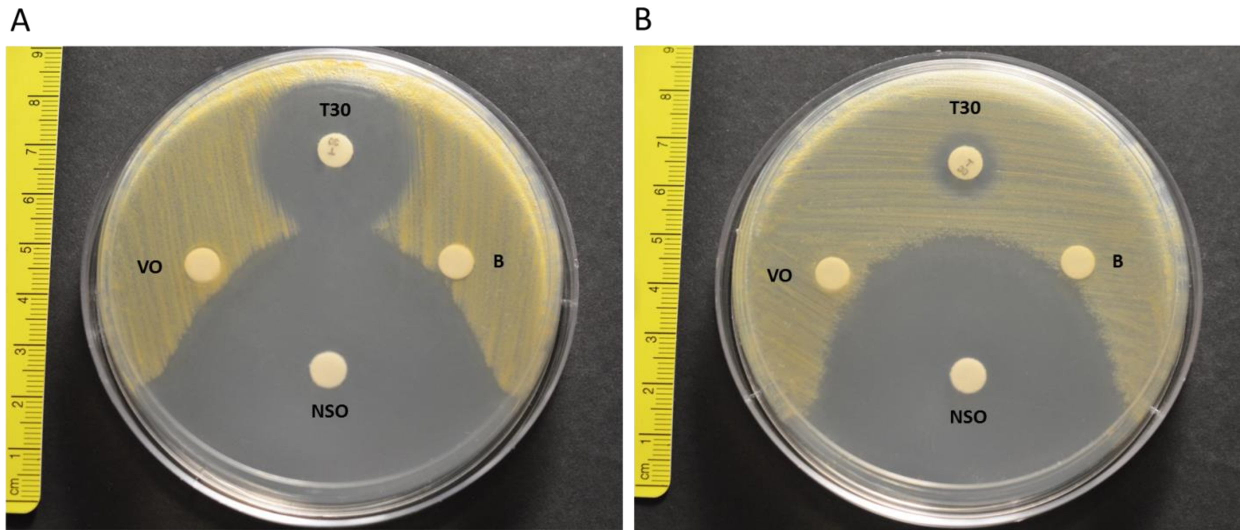

Figure 6-1. Zones of growth inhibition of oxytetracyclin sensitive (A, ALG-00-530) or resistant (B, ALM-05-21) *F. columnare* strains on MS agar medium around NSO, oxytetracyclin (T30), vegetable oil (VO), and blank (B).

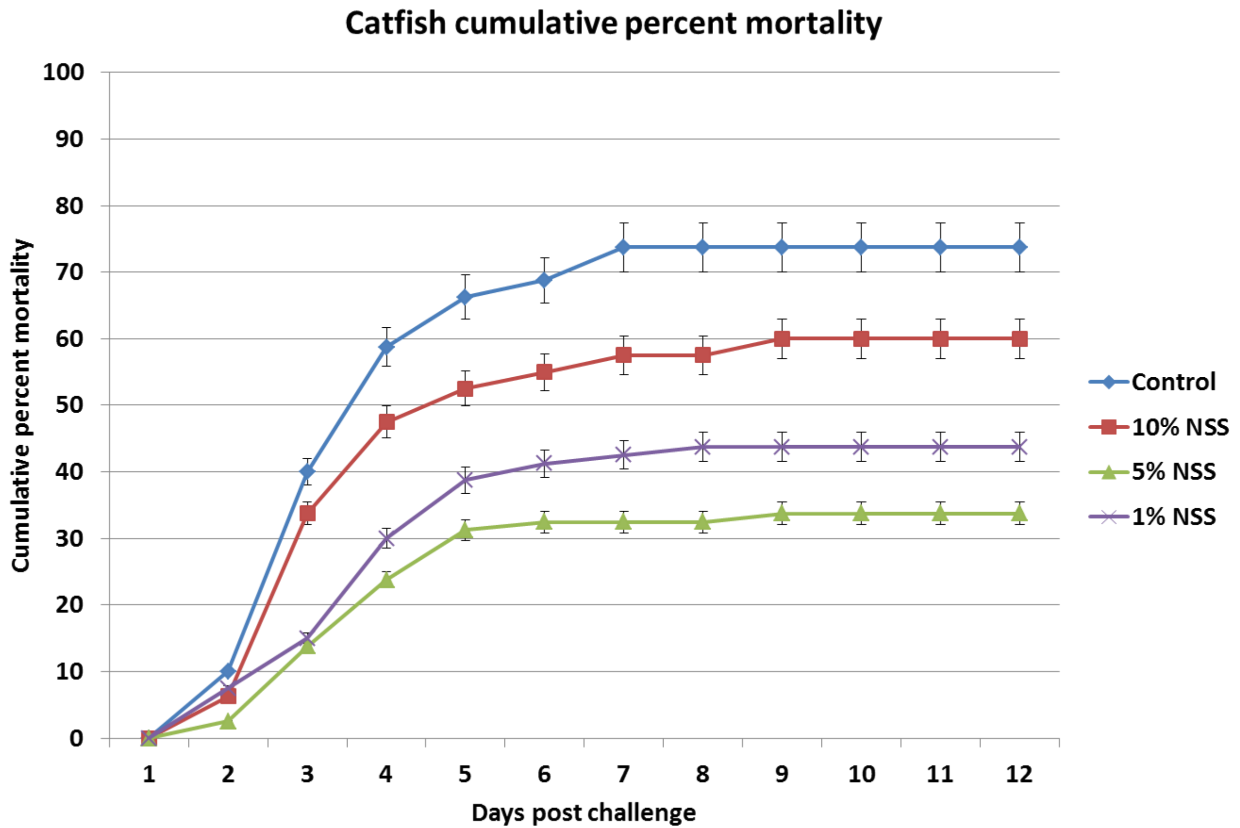

Figure 6-2. Mean cumulative percent mortality of channel catfish fed (I) control/basal diet, (II) 1% NSS diet, (III) 5% NSS diet, and (IV) 10% NSS diet then immersion challenged with genomovar II *F. columnare* BGFS-27.

CHAPTER 7. SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Based on the research performed in in Chapter 3 of this dissertation, genomovar II strains of *F. columnare* were responsible for the severe columnaris outbreaks affecting the E. W. Shell Fisheries Experiment Station from 2010 to 2012. Genomovar II was the only genotype in aquaculture and sport fishing ponds recovered during columnaris outbreaks in our experiment station (columnaris episodes occurred in 2013, 2014 and 2015 continued to be caused by genomovar II isoaltes; data not shown). I detected marked genetic diversity among the genomovar II strains and the strain relatedness was significantly influenced by collection site and date of isolation. Genomovar II strains were virulent to other aquaculture and sport fish species, not just catfishes. Moreover, I documented a co-infection by more than one strain of *F. columnare* in the same individual fish. According to my results in this study, florfenicol is the drug of choice and I recommend it for treatment of fish during columnaris outbreaks.

My vaccination studies in Chapter 4 showed the protective efficacy of the new genomovar II rifampicin-resistant mutants of *F. columnare* as vaccines in commercially important fishes. Genomovar II mutants resulted in higher relative percent survival values than those of the genomovar I mutant of the commercial vaccine in channel catfish, zebrafish and Nile tilapia. My results suggest that genomovar II mutants have the potential of formulating better commercial vaccine and that the administration of genomovar II mutants as modified-live vaccines is safe and elicits greater protection against columnaris disease than that provided by

FCRR, the genomovar I mutant in the currently licensed vaccine, particularly when highly virulent or co-infection by more than one genomovar of *F. columnare* is encountered in a columnaris outbreak.

The studies in Chapter 5 of this dissertation demonstrated the vital role played by the fish's external microbiome in reducing mortalities due to opportunistic pathogens like *F. columnare* and that the phylum Proteobacteria dominates the catfish skin microbiome. Exposure to chemical disinfectants commonly applied in fish farms like PP induced dysbiosis to the fish's healthy microbiome, altered the microbial community composition, reduced the numbers of beneficial bacteria and increased susceptibility to columnaris disease following experimental immersion challenge. Maintaining the integrity of the surface microbiome as a first line of defense against invading pathogens is crucial for combating infections. These results recommend paying careful attention in fish farms after application of PP or any other disinfectant to avoid the risk of subsequent columnaris infection and suggest manipulation of the fish's external microbiome composition as an ecological approach to decrease the incidence of columnaris disease. I hope that my results will stimulate further research to select potential probiotic candidates from the resident members of the catfish normal microbiome that can be used for biological control of columnaris disease in aquaculture.

Treatment of columnaris disease using chemotherapeutants and antimicrobial agents can be troublesome and has known different success rates. The results obtained in Chapter 6 of this dissertation revealed a potent antibacterial activity of *N. sativa* oil towards the three genomovars of *F. columnare* and a protective potential for dietary supplementation with *N. sativa* seeds or oil against columnaris disease in fishes. My data suggested that *N. sativa* could be an alternative curative and preventive measure against columnaris disease and encourage further investigations

to be carried out in order to isolate, purify and standardize the active antibacterial ingredients in *N. sativa* as the mechanism of action against *F. columnare* has not been exploited yet.