Biofilm formation by the fish pathogen *Flavobacterium columnare*:
a quantitative and qualitative study

by

Wenlong Cai

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Approved by

Covadonga R. Arias, Chair, Associate Professor of Fisheries
Leonardo De La Fuente, Assistant Professor of Plant Pathology
Stephen A. Bullard, Assistant Professor of Fisheries

Abstract

The objective of this study was to evaluate the effect that temperature, pH, salinity, hardness and carbohydrates have on biofilm formation by the fish pathogen *Flavobacterium columnare*. Nineteen *F. columnare* strains, including representatives from all genomovars, were compared in this study. Biofilm formation was quantified spectrophotometrically by allowing cells to attach and colonize polystyrene wells that were then stained with crystal violet. Although significant strain-to-strain variation was observed, differences in biofilm formation between genomovars were not significant. The two main factors influencing biofilm formation were salinity and hardness. Low salinity (5 ppt) and high hardness (360 ppm) favored biofilm proliferation. In addition, the carbohydrate mannose also promoted biofilm. On the contrary, warmer temperatures (30°C) inhibited biofilm formation. The ultrastructure of biofilm developed on glass slides was characterized by light, confocal laser and scanning electron microscopy. Surface colonization started within 6 h post-inoculation and microcolonies were observed within 24 h. Extracellular polysaccharide substances (EPS) and water channels were observed in mature biofilms (24-48 h). The virulence potential of biofilm was confirmed by cutaneous inoculation of channel catfish fingerlings with mature biofilm.

My results showed that *Flavobacterium columnare* can attach to inert surfaces and colonize them by producing biofilm. My data showed that some physicochemical parameters modulated biofilm formation by *F. columnare*. The values identified in this study could be used as reference values to prevent biofilm formation in aquaculture settings. I have experimentally

proved that it is possible to induce columnaris disease in channel catfish when compromised skin

is exposed to mature biofilm of *F. columnare.* This suggests that *F. columnare* could use biofilm

as reservoir in the natural environment but also in aquaculture facilities.

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List of Abbreviations

MS   Modified Shieh

FISH   Fluorescent *In Situ* Hybridization

SEM   Scanning Electron Microscopy

CLSM   Confocal Laser Scanning Microscopy

UV   Ultraviolet

BC   Bacterial Cell

SB   Surface Blebbing

EPS   Extracellar Polysaccharides

DIC   Differential Interference Contrast microscopy

LSD   Least Significant Difference

ANOVA   Analysis of Variance

CFU   Colony Forming Unit

ppt   parts per thousand

ppm   parts per million

USDA   United States Department of Agriculture

NSF   National Science Foundation

eDNA   extracellular DNA

NBF   neutral buffered formalin

I. LITERATURE REVIEW

1. *Flavobacterium columnare*

1.1. Phenotypic description

Flavobacterium columnare is a Gram-negative bacterium that belongs to Phylum
Bacteroidetes, Class Flavobacteria, Order Flavobacteriales and Family Flavobacteriaceae. This
species is widely distributed in fresh water environments and it is the causative agent of the
columnaris disease. *F. columnare* is one the oldest fish pathogens known as columnaris disease
was first described by Davis in 1922. The etiology of the disease refers to the column-like
aggregates that the bacteria form when infected gills are examined under the microscope (Davis
1922; Plumb 1999). Bacterial cells are long, thin bacilli of approximately 0.3 to 0.5 µm of width
and 3.0 to 10.0 µm in length (Buchanan and Bibbons 1974). *F. columnare* cells can be covered
with a capsule, which has been suggested as potential virulence factor (Decostere et al. 1999b).
Colonies of *F. columnare* are flat and rhizoid and have a characteristic yellow color due to the
pigment flexirubin (Bernardet and Bowman 2006). Typical diagnostic tests for this species
include the ability of *F. columnare* to absorb Congo red, production of catalase, and lack of acid
production from hydrolysis of carbohydrates (Buchanan and Bibbons 1974). This bacterium can
be selectively isolated from environmental samples by supplementing the culture medium with
neomycin and polymyxin B.

1.2. Taxonomy

Flavobacterium is the type genus of the Family Flavobacteriaceae, and has a wide
distribution in soil, aquatic environments and extreme polar habitats (Kirchman 2002). To date,
there are 114 species described within the genus *Flavobacterium* but new species are being
described every year (http://www.bacterio.cict.fr/f/flavobacterium.html). Three fish-pathogenic

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species: *F. psychrophilum, F. columnare* and *F. johnsoniae* have been extensively studied due to their economic significance worldwide (Plumb 1994; Austin and Austin 1999; Bernardet and Bowman 2006).

The taxonomy of *F. columnare* underwent a series of changes since its initial description. In 1922, David observed the bacteria on the external lesion of numerous fresh-water fishes in Mississippi River, and named it *Bacillus columnaris* (Davis 1922). Later, Ordal and Rucker (1944) isolated this organism from brown bullhead (*Ameiurus nebulosus*) and reclassified it as *Chondroccoccus columnaris* based on the wrong assumption that the cells were able to produce microcysts and fruiting bodies. In 1945, Garnjobst (1945) proved that the bacterium was indeed devoid of fruiting bodies and reclassified it as *Cytophaga columnaris*. However, further description of the genus *Flexibacter* and *Cytophaga* lead to another name change (*Flexibacter columnaris* (Buchanan and Bibbons 1974)). Finally, in 1996, Bernardet et al. (1996) restructured the Flavobacteriaceae family and reclassified the bacterium as *Flavobacterium columnare.*

1.3. Genetic diversity

F. columnare is a phenotypically homogeneous species but presents a wide intrinsic genetic diversity. In fact, Triyanto and Wakabayashi (1999) divided the species into three genomovars. The term "genomovar" was originally introduced by Ursing et al. (1995) to denote a genotypically distinct group of strains that lack the phenotypic properties required to erect new taxon. Triyanto and Wakabayashi (1999) ascribed *F. columnare* strains to the different genomovars based on 16S rDNA restriction fragment length polymorphism analysis (16S rDNA-RFLP), 16S rDNA sequencing, and DNA-DNA hybridization tests. The division of the species into three genomovars was further corroborated by using random amplified polymorphic DNA

(RAPD) (Thomas-Jinu and Goodwin 2004) and other various genotyping research (Wagner et al. 2002; Arias et al. 2004; Darwish and Ismaiel 2005; Suomalainen et al. 2005).

Since 2003 our group has characterized over 250 *F. columnare* from different origins using 16S-RFLP, 16S-single strand conformation polymorphism (SSCP) analysis, amplified fragment length polymorphism (AFLP) analysis, and multilocus sequence typing (MLST) that provided us with different levels of resolution for understanding the genetic diversity within the species (Arias et al. 2004; Olivares-Fuster et al. 2006; Olivares-Fuster et al. 2007; Zhang and Arias 2009). Regardless of the resolution level achieved by each method (e. g. AFLP provided us with strain-specific profiles), all our isolates can be clearly divided into two groups that correspond to genomovars I and II. Although genomovar III has been purposely reported in the USA (Darwish and Ismaiel 2005), we have never recovered this genomovar from clinical or environmental samples.

2. Columnaris disease

2.1. Epidemiology

Columnaris disease has been frequently reported from cultured, wild and ornamental fish of fresh water species worldwide (Plumb 1999; Wagner et al. 2002; Pulkkinen et al. 2010; Wang et al. 2010). It is considered the second most important bacterial pathogen (~ $30 million/year on economic losses) for the US channel catfish aquaculture, after enteric septicemia of channel catfish (Wagner et al. 2002). Mortality can reach 50-60% in food-size channel catfish but can be as high as 90% in catfish fingerlings (USDA 2003). Columnaris disease is also a serious threat for salmonid production with nearly 100% mortality in farmed salmon, if left untreated (Plumb 1994). Suomalainen (2005) investigated the influence of the rearing conditions of rainbow trout

(*Oncorhynchus mykiss*) and their susceptibility to columnaris disease. They found that mortality was associated with high rearing densities at high temperature (>23°C).

In general, fish are considered the main reservoir for *F. columnare* although limited data supports this statement (Plumb 1999). However, *F. columnare* can be isolated from water and biofilm in environments in where fish farming does not occur (Kunttu et al. 2012). Recently, our group (Arias et al. 2012b) has shown that *F. columnare* can survive in water for extended periods, a finding that has been confirmed by other authors (Kunttu et al. 2012). Moreover, columnaris disease can be water-borne and fish to fish contact is not required (Welker et al. 2005).These recent studies questioned the original paradigm by which the main reservoir for *F. columnare* was fish.

2.2. Host susceptibility

F. columnare can infect and colonize many freshwater fish species although some appear to be more susceptible to columnaris disease than others. The following species have been reported as susceptible to *F. columnare*: channel catfish *(Ictalurus punctatus)*, blue catfish (*I. furcatus*), common carp *(Cyprinus carpio)*, tilapia (*Oreochromis spp.*), rainbow trout (*Oncorhynchus mykiss*), black bass (*Micropterus salmoides*), brook trout (*Salvelinus fontinalis*), sunfish (*Lepomis incisor*), bluegill (*Lepomis macrochirus*), and eels (*Anguilla rostrata, A. japonica, A. anguilla*) (Plumb 1999). It is speculated that under favorable conditions nearly all fresh water fishes are susceptible to columnaris disease (Wakabayashi 1993). There are several factors that favor the onset and spread of the disease and those include physical and chemical stressors. For example, injured or mechanical abraded fish are more vulnerable to infection by *F. columnare* than intact fish (Bader et al. 2003). Similarly, stress induced by starvation significantly increases susceptibility to columnaris disease in experimental fish (Shoemaker et al.

2003). Other factors that favor columnaris disease include high organic loads (Chowdhury and Wakabayashi 1988), crowded conditions (Suomalainen et al. 2005), excessive handling (Wakabayashi 2006), low dissolved oxygen, and high ammonia and nitrite (Farmer et al. 2011).

2.3. Clinical signs

Columnaris disease is typically an external infection in where skin and gills are colonized. Specific lesions tend to vary with fish species and with the severity of the infection. Columnaris disease commonly causes extensive necrosis of the gills, which starts at the margins of the filaments and progresses toward the arches. On the body, small lesions start as areas of pale discoloration. These areas increase in size and may become as large as 3-4 cm in diameter, covering as much as 20-25% of total surface area of fish (Austin and Austin 1999). Skin, gills and fins are the most favored sites for bacteria attachment and colonization, however, on acute infections, *F. columnare* becomes systemic without obvious pathological signs on external organs (Plumb 1994). In catfish fingerlings, this disease is often associated with the presence of a so-called "saddleback lesion", a symmetrical area of body discoloration flanking the dorsal fin (Cone et al. 1980; Wakabayashi 1980). Morrison et al. (2006) described the morphology of the saddleback and its histopathological effect on the infected tissues in underyearling Atlantic salmon (*Salmo salar*). They found that *F. columnare* cells were mainly restricted to the dermis and underlying muscle of the saddle area. Bullard et al. (2011) investigated the ultrastructure of "saddleback" lesions of diseased channel catfish (*Ictalurus punctatus*) with scanning election microscopy, and described the saddleback area as characterized by epidermal sloughing intermingled with rod-shaped bacteria and cellular debris on strongly ridged, folded dermal connective tissue.

2.4. Early stages of colonization to gill tissue

In order for a pathogen to cause disease, it must gain access to the host, adhere to host tissues, resist or evade host defenses, and damage the host tissue. The attachment between pathogen and host takes place by means of surface projections (which are called adhesins or ligands on the pathogen), and complementary surface receptors on the host cells. Whereas the majority of ligands on bacteria thus far studied are glycoproteins or lipoproteins, the receptors are typically polysaccharides such as mannose residues (Ofek et al. 2003). Ligands on different strains of the same species of pathogen can vary and are frequently associated with microbial surface structures such as pili (Horvat 2008).

The mucosal surface of the host provides an interface between the body and the external environment. One of the main functions of the mucosal epithelium is to act as natural barrier to pathogenic microorganisms that can thrive in the nutrient-rich environment provided by the host. One of strategies that pathogenic bacteria used to cause disease is the colonization of epithelia. Skin epithelium is susceptible to colonization by pathogens and often serves as target through the pathogen life cycle (Brumell and Finlay 2000).

There are several different physiological strategies that a host can employ to prevent bacterial colonization to mucosal surfaces. These include entrapment in a thick blanket of mucus or clearance by peristalsis or cilia movement in the gill tissue (Brogden et al. 2000). Also, it is suggested that as water flows across the mucosal epithelium the bacteria are flushed away thus preventing attachment. However, the basic molecular mechanisms used by *F. columnare* to attach to the host are still unknown. Studies focused on attachment to host tissues have shown that *F. columnare* have a positive chemotactic response towards channel catfish mucus (Klesius et al. 2008) and that both gill and skin tissues can be rapidly colonized after exposure to the pathogen (Olivares-Fuster et al. 2011). However, significant differences of chemotaxis and

adhesion properties have been found between genomovar I, the least virulent type towards channel catfish, and genomovar II strains (Klesius et al. 2008; Olivares-Fuster et al. 2011).

Decostere reported that physicochemical factors (e.g. pH, salinity, hardness, etc.) played a significant role in attachment of *F. columnare* to gill tissues (Decostere 2002). These environmental factors may help the bacteria to attach to the gill epithelium of the host and help them to withstand the cleaning mechanisms of the body surface (Good et al. 2008; Kunttu et al. 2009a). Verma et al. (2011) studied the *in vivo* survival of *F. columnare* in Indian catfish (*Clarias batrachus*) and hypothesized that a pH of 7.0-7.5 was highly suitable for bacterial proliferation because none of the bacteria were isolated and recovered from the challenged fish at pH 8.0 or higher.

The ability to attach to host tissues has been evaluated in different fish species and correlated, to some degree, with virulence. Decostere et al. (1999a) compared the adhesion properties of strains of *F. columnare* to gill tissues of black mollies (*Poecilia sphenops*) and found a positive correlation between the number of cells that attached to gill and the virulence of the strain. Our group has studied the adhesion dynamics of *F. columnare* to different fish tissues using representative strains of genomovars I and II (Olivares-Fuster et al. 2011). Results from our study showed that both genomovars adhered to host tissues at high levels during the first 30 min post-challenge but genomovar I cells were eliminated at a faster rate than genomovar II cells. Clearly, not attachment but that ability to persist on the host during the first 24 h post-exposure is critical in displacing the host-pathogen equilibrium towards disease development (Olivares-Fuster et al. 2011).

The nature of the molecules that *F. columnare* uses for attachment is still unknown but proposed targets include components of the capsule, fimbriae and other appendages of the outer

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membrane (Ofek and Doyle 1994). A few studies have suggested carbohydrates as the main target for *F. columnare* adhesins. Decostere et al. (1999b) showed that adhesion to gill was significantly reduced when sodium metaperiodate or different sugars were added to the challenge bath. Because the ability of the bacteria to attach to gill tissue was reduced only when sodium metaperiodate was used but not proteases, the authors concluded that the major component of the receptor on fish tissue should be of carbohydrate nature (Decostere et al. 1999b). Recently, it has been proposed that a rhamnose-binding lectin (RBL) plays a direct role in columnaris pathogenesis. RBL are dramatically upregulated in gills of the fish infected with *F. columnare* and are constitutively upregulated in columnaris-sensitive fish families. Moreover, addition of RBL ligands such as L-rhamnose and D-galactose improved resistance against columnaris disease in channel catfish, which further suggested the bacterial ligands are sugars (Beck et al. 2012; Sun et al. 2012).

2.5. Diagnosis and prevention strategies

The presence of yellow lesions and brownish discoloration on the body surface or the presences of "hay stacks" of yellow bacteria colonization in a wet mount are typical signs of columnaris disease. The pathogen can be recovered by scraping away the epidermis from a small area on one side of the body and streaking the sample onto adequate culture media such as Shieh agar (Decostere et al. 1997), Hsu-Shotts agar (Bullock et al. 1986), or Cytophaga agar (Anacker and Ordal 1959). *F. columnare* colonies are yellow and rhizoid and the cells are long rods ranging from 6-15 µm usually. The presumptive isolates can be confirmed by molecular methods such as specific polymerase chain reaction (PCR) or by using classical bacteriology techniques (Thoesen 1994; Bader and Shotts Jr 1998). Griffin proposed a five-step method to distinguish *F. columnare* from other yellow pigment producing bacteria: 1) ability to grow in the presence of

antibiotics of neomycin sulfate and polymyxin B; 2) colonies rhizoid and yellow pigmented; 3) production of gelatin degrading enzymes; 4) colonies absorbing Congo red; 5) production of an enzyme that degrades chondroitin sulfate A (Griffin 1992).

Recently, new molecular diagnostic techniques have been applied to streamline the identification and/or detection of *F. columnare*, including fatty acid methyl ester analysis (FAME) (Shoemaker et al. 2005), whole-cell lipopolysaccharide and proteins profile test (Zhang et al. 2006), real-time PCR methods (Panangala et al. 2007), and loop-mediated isothermal amplification method (LAMP) (Yeh et al. 2006).

Due to the ubiquitous presence of the bacterium in aquaculture systems, eradication of the disease is unlikely to occur. Primary control and treatment strategies for columnaris disease have included oral antibiotic therapy and bathing in surface-active substances such as salt and formalin (Thomas-Jinu and Goodwin 2004). Immersion of the infected fish in salt bath of 3 ppt is an effect treatment for columnaris disease (Farmer 2004) as well as addition of the surface-acting disinfectants such as potassium permanganate (2 mg/L), hydrogen peroxide (75 ml/L), and copper sulfate (1 mg/L) (Wakabayashi 2006). There are also antibiotics in the form of medicated feeds such as Florfenicol (10 mg/Kg of body weight), Terramycin (80 mg/kg of body weight) and Romet (50 mg/kg of body weight) that can be used to treat outbreak of the disease (Hawke and Thune 1992; Gaunt et al. 2010). However, antibiotic therapy is not a sustainable option due to high application costs of medicated feed and the presence of *F. columnare* antibiotic-resistant strains, while bath treatments are not practical for pond-level implementation. A commercially vaccine is available to prevent columnaris (AQUAVAC-COL, Merck) but its efficacy has been questioned by producers and its use is somewhat limited. New strategies against columnaris need to be developed in order to control the incidence of the disease in catfish aquaculture. Oliver-

Fuster developed and characterized a rifampicin-resistant mutant from a high virulent genomovar II strains in an effort to improve the current commercial live vaccine which is originally obtained from Genomovar I strains (Olivares-Fuster and Arias 2011). Research on alternative methods like biological control with probiotics using *Bacillus* spp. have also been proposed and are under further evaluation (Ran et al. 2012).

3. Biofilm

Biofilm is generally defined as a community of microbes attached to a surface and embedded in an organic matrix. The first scientist to examine biofilm was Antonie van Leeuwenhoek who in 1676 realized that the dental plaque was made out of 'animalcules'. However, it was not until the early 1940s that microbiologist started to comprehend biofilm formation. It was ZoBell, a marine microbiologist, the first scientist to 'grow' biofilms by introducing glass slides into the ocean and allowing the bacteria to colonize the surfaces (ZoBell 1943). Since the 1990s, the study of biofilms has become one of the most important fields in microbiology (e.g., the NSF founded the Center for Biofilm Engineering at Montana State University in 1990). Biofilms are fascinating environments in where bacteria grow and interact with each other using patterns that differ from those employed by planktonic cells. From an evolutionary point of view, living in a biofilm offers many advantages to the cells including protection against desiccation, oxygen radicals, ultraviolet radiation, predators and harmful chemicals such as antibiotics (Flemming and Wingender 2010). Biofilms can have beneficial but also detrimental effects to many aspects of human life including food production, and biomedical and biotechnological processes (Simões et al. 2010).

3.1. Biofilm matrix

In most biofilms, the matrix can account for over 90% of the dried mass. It forms the scaffold for the three-dimensional architecture of biofilm and functions as the cohesive element for the biofilm components (Flemming and Wingender 2010). The matrix is mainly composed of extracellular polysaccharides, proteins, DNA and lipids. In some biofilm structures, there are channels within the matrix where water and nutrients can circulate and be distributed to all the layers (de Beer et al. 1994). Biofilms of well-studied organisms such as *Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus* usually present exopolysaccharides (EPS) as the main component of the matrix (Branda et al. 2005; Clark et al. 2007). However, other components such as proteins, DNA, lipids and outer membrane structures (i.e. pili, fimbriae) have also been found within the matrix of other bacteria (Sutherland 2001).

Polysaccharides, most of which are linear or branched long molecules, constitute a major fraction of the matrix. Those polysaccharides can be homopolysaccharides such as sucrose-derived glucans or heteropolysacchatides such as β-1, 6-linked N-acetylglucosamine (Branda et al. 2005). Extracellular proteins are also common in the matrix, which include enzymes and structural proteins. Enzymes have a very important role as they can degrade complex biopolymers (e.g. cellulose or chitin) generating smaller molecules that bacteria can then internalize. Typical enzymes reported from biofilm include chitinase, glucosidase, esterase, etc… DNA found in biofilms was initially regarded as residual materials from lysed cells but, it is now recognized as an integral part of the matrix since the addition of DNase during biofilm induction inhibited the formation of biofilm (Watanabe et al. 1998). Harmsen and colleagues further studied the role of extracellular DNA (eDNA) during biofilm formation by *Listeria monocytogenes* and found that the eDNA is an essential component during initial attachment of the cells and the early stages of biofilm formation (Harmsen et al. 2010). Finally, lipids are also

present in the matrix in where they provide the hydrophobicity required for some of the cell-inert surface interactions.

3.2. Enhanced resistance to antimicrobial agents

Bacteria within a biofilm have enhanced resistance to antimicrobial agents. There are generally three proposed mechanisms for this phenomena. One hypothesis is that biofilm can prevent transmission of antibiotics to the deeper layers of the biofilm. Polymeric substances such as those present in the matrix are known to lower the diffusion rate of antibiotics in solution. The second proposed mechanism postulates that the cells living in a biofilm could experience a situation of nutrient limitation, thus transitioning into a starved or slow-growing state. These slow-growing cells could possibly survive the presence of bacteriostatic antibiotics that halt growth but do not kill the bacteria. The third proposed mechanism hypothesizes that cells living in a biofilm present different gene expression profiles compared to those found in planktonic cells. This biofilm-specific gene expression pattern will confer a specific resistant phenotype to the cells embedded in the biofilm (Costerton et al. 1999).

3.3. Quorum sensing

Quorum sensing is the regulatory system for controlling gene expression in response to bacterial population (De Kievit and Iglewski 2000). This complex mechanism involves the production of chemical stimulus to determine the population density of a bacterial community. This special phenomena was discovered and described more than 30 years ago in species of the genus *Vibrio* (Miller and Bassler 2001). Cell to cell communication between bacteria is generally carried out by chemical signals that can diffuse away from one cell and be received by another. This chemical 'language' facilitates self-organization and regulation of microbial cells and allows bacteria to monitor and respond to their own population densities. High cell densities

usually result in high concentrations of signal, which in turns changes the expression of certain genes with the corresponding physiological changes in neighboring cells. The physiological activities stimulated by quorum sensing include symbiosis, virulence, competence, conjunction, antibiotic production, motility, and biofilm formation (Miller and Bassler 2001).

Common molecules employed in quorum sensing include oligopeptides and N-acylhomoserine lactones (AHL) (Simões et al. 2010). The vast majority of Gram-negative (e.g. *Pseudomonas aeruginosa, Erwinia carotovora* and *Brucella melitensis*) quorum-sensing systems utilize AHLs as signaling molecules (Jones et al. 1993; McClean et al. 1997; De Kievit and Iglewski 2000; Taminiau et al. 2002). In some cases, AHLs with differences in the structure of their N-acyl side chains are used by some Gram-negative bacteria such as the fish pathogen *Aeromonas hydrophila* (Swift et al. 1997). AHLs were first identified in marine bacteria, where they found it played a central role in the regulation of bioluminescence.

3.4. Biofilm formation

There are 5 general steps in biofilm formation: attachment, formation of microcolonies, production of extracellular matrix, maturation, and dispersion. Biofilm development starts when the bacteria of planktonic lifestyle attach to biotic or abiotic surfaces. This step is thought to be regulated by the nutritional status of the environment. The first interactions between the cells and the surface are maintained by weak bonds and can be reversed. However, after a few seconds, those links become permanent and the attachment phase is completed. In many species, the cell morphology changes from planktonic to sessile forms typically due to the loss of flagella. After this initial phase, the cells start to multiply and develop microcolonies. During this phase, attractant molecules can be release to recruit more planktonic cells. Once enough cells are attached to the substratum, the production of EPS starts and the biofilm acquires its three

dimensional structure. Microcolonies transform into larger aggregates to form mature biofilms. The cycle is completed when planktonic cells (or daughter cells) are shed from the biofilm into the aquatic environment (Costerton et al. 1999; An and Friedman 2000; Branda et al. 2005).

4. Biofilm & Aquaculture

Microbial activities have been found predominately at interfaces in natural environments (Costerton and Colwell 1979), as well as being closely related to human life, from both beneficial and troublesome sides. On one hand, biofilm is essential to develop good biofilters in waste-water treatment processes or in aquaculture facilities (Characklis et al. 1982). On the other hand, in many manufacturing processes, biofilms increase friction, decrease heat transfer rates and reduce flows, which translates in higher energy costs and mechanical failures such as clogged pipes (Characklis et al. 1982). The most common method for controlling biofilm development in pipes is periodic chlorination, which is added to the water directly and kills the microorganisms and/or helps to hydrolyze the extracellular polymers (Characklis et al. 1982).

In shellfish and algae farms, biofouling species tend to block nets and cages and ultimately outcompete the farmed species for space and food (Braithwaite and McEvoy 2004). Bacterial biofilms start the colonization process by creating microenvironments that more favorable for biofouling species. Also in the marine environment, biofilms could reduce the hydrodynamic efficiency of ships and propellers, lead to pipeline blockage and sensor malfunction, and increase the weight of appliances deployed in seawater (Qian et al. 2007). In addition, biofilms (usually algae biofilm) are also responsible for detriment of a variety of submerged structures including pipes, buoys and platforms. Algae biofilms can increase hydrodynamic drag and accelerate the corrosion of metals (Callow and Edyvean 1990), as well as impart undesirable tastes and odors to seafood products (Hutson et al. 1987).

Conversely, there are many good applications of biofilm in aquaculture settings. Thompson et al. found that biofilm is important for maintaining good water quality and nourishment in intensive shrimp cultures (Thompson et al. 2002). In environmental friendly zero-discharge marine aquaculture system, water is purified and recycled via microbial biofilters. This biofilters are usually dominated by bacteria of ammonia-oxidizing and nitrite-oxidizing communities, which keep the concentration of toxic organics like ammonia and nitrite within acceptable levels (Foesel et al. 2007). Boley (2000) studied the practice of using biodegradable polymers as biofilm carriers in the denitrification of recirculated aquaculture systems. He found that nitrate concentration was significantly lower in aquaria containing biofilm than in the untreated reference system. Additionally, biofilms helped maintain a stable pH without negatively affecting the fish. It has also been reported that biofilm could enhance the settlement of invertebrate larvae in aquatic environment. Biofilm could be used to reduce the levels of ammonium and phosphate (33% less phosphate) in water of rearing systems while serving as an important complementary food source for shrimp since primary producers like diatoms and cyanobacteria are common in these biofilms (Qian et al. 2007).

Nevertheless, numerous studies have shown that biofilm can be a reservoir for potentially pathogenic bacteria (King et al. 2004; Bourne et al. 2006; Wietz et al. 2009). As mentioned previously, biofilms can be difficult to eliminate even when antibiotics or chemicals are used in high doses (Karunasagar et al. 1994; Costerton et al. 1999). The role that biofilm plays as reservoirs of bacterial fish pathogens has not been explored in detail but it certainly deserves to be studied.

II. Objectives

Preliminary data obtained by our group showed that *F. columnare* can survive in water for extended periods of time suggesting that the aquatic environment could constitute the primary reservoir for this pathogen. As biofilms play key roles in the life cycle of many aquatic bacteria, I speculated that *F. columnare* could form biofilm and persists in it. To further clarify the life cycle of this bacterium, my overarching goal was to characterize biofilm formation by *F. columnare*. I hypothesized that genomovar I, and II will exhibit different adhesion properties based on their distinct genetic properties. The specific objectives for the present study were:

1. Qualitative characterization of biofilm formation by *F. columnare*.

    1.1. Induction of biofilm on glass slides: microcosm design

    1.2. Induction of biofilm under flow conditions

    1.3. Characterization of *F. columnare* biofilm using microscopy

2. Quantitative analysis of variables affecting biofilm proliferation.

    2.1. Attachment to plastic as proxy for quantification of biofilm

    2.2. Physical variable: temperature

    2.3. Chemical variables: pH, hardness and salinity

    2.4. Biological variables: sugars

Chapter 1. Biofilm formation by *Flavobacterium columnare* in artificial microcosms: an ultrastructure study

1.1. Introduction

Flavobacterium columnare is the causative agent of columnare disease that affects many freshwater fish species worldwide. The disease is particularly relevant for catfish, tilapia, and trout aquaculture where it causes major economic losses every year (Amin et al. 1988; Groff and Lapatra 2000; Wagner et al. 2002; Figueiredo et al. 2005; Suomalainen et al. 2005). Despite its great economic impact, the ecological niche of *F. columnare* has not been clearly identified although most studies point to fish as primary reservoirs for the disease (Wakabayashi 1993; Austin and Austin 1999). However, fish to fish contact is not always required for disease transmission (Welker et al. 2005) and the long-term survival of *F. columnare* in lake water suggests that the aquatic environment can be the main reservoir for this pathogen (Kunttu et al. 2009b). Previous data obtained by our group indicated that *F. columnare* can survive in water without nutrients for extended periods although cells underwent drastic morphological changes that result in loss of fitness overtime (Arias et al. 2012b). Therefore, it is likely that this pathogen utilizes other niches in the aquatic environment besides the water column.

Biofilm is referred to as a community of microbes embedded in an organic matrix and attached to a physical surface. Adopting such special life style provides the bacteria with benefits such as protection from desiccation, enhanced antibiotic resistance, concentration of nutrients and protection against predators (Branda et al. 2005). Many aquatic bacteria are capable of colonizing surfaces and forming biofilm that can act as reservoirs for those bacterial populations (King et al. 2004; Branda et al. 2005; Bourne et al. 2006; Wietz et al. 2009). However, very limited information (Staroscik and Nelson 2008) is available regarding the ability of *F.*

columnare to colonize surfaces and form biofilm. Aquaculture settings are particularly prone to the development of biofilms and biofouling thus, from an applied point of view, it is important to know whether *F. columnare* can form biofilm. The objectives of this study were: 1) to determine if *F. columnare* can induce biofilm on inert surfaces and, if so, 2) to investigate the ultrastructure of that biofilm.

1.2. Materials and Methods

1.2.1. *Flavobacterium columnare* **strain and maintenance**. Strain ALM-00-530, originally isolated from a diseased channel catfish in Alabama, was used in this study. This is a genomovar II strain that can cause high mortality (>70%) in channel catfish fingerlings under experimental conditions (Shoemaker et al. 2008). Cells were maintained as glycerol stocks in Modified Shieh (MS) broth (Table 1) supplemented with 20% glycerol at -80°C until used. After thawing, bacteria were cultured for 48 h at 28 °C in MS broth with gentle shaking, and maintained routinely by passing cultures into new MS broth every 24 h. Cultures were checked for purity on MS agar plates. The number of cells in the initial inoculum was determined by direct plate count on MS agar.

1.2.2. Microcosm model. To induce biofilm formation, I prepared a microcosm consisting of a glass beaker filled with MS broth and covered with permeable Parafilm[TM]. As physical substrate for the biofilm, I used glass slides that were suspended into the MS broth by paper binders that were attached to a glass rod on top of the beaker (Figure 1). All components, except for the Parafilm[TM], were sterilized by autoclaving prior assembly under aseptic conditions. Each microcosm contained 200 ml of MS broth and was inoculated with 200 µl of an 11 h-old ALM-00-530 culture to an approximately final concentration of 10^5 cells/ml. The

microcosm was kept at 28°C, to allow for bacterial attachment and biofilm formation. Slides were taken out at different time intervals for microscopic observation.

1.2.3. Microfluic chamber test. The bacterial attachment to plastic surfaces under fluid conditions were evaluated by using a microfluidic chamber as previously described (De La Fuente et al. 2007). Briefly, to fabricate the microfluidic chamber, photolithography was used to etch a pattern of two parallel microchannels on a silicon wafer for casting with polydimethylsiloxane (PDMS). The chamber is composed of a molded PDMS body, which is sandwiched by a cover glass and a supporting glass microscope slide. The resulting microchannels are 80 µm tall, 50 µm deep and 3.7 cm long. The microchannels include two inlets, for the entry of media and bacteria, and one outlet to allow the media to circulate out (Figure 2). The medium was injected by an automated syringe pump and the speed was set at 0.25 µl/min.

1.2.4. Light Microscope. Cell attachment and bacterial colonization on glass slides were examined using a light microscope (Leica Microsystems, USA) at 400 X magnification and with different microscopy techniques, including phase contrast and differential interference contrast (DIC).

In addition to visualize some of the characteristics of the induced biofilm, cells were treated with different chemicals including calcofluor white (10 mg/ml; sigma, CA), Congo red (0.1 % w/v; sigma, CA), and Dextran-Rhodamine (sigma, CA). Images were captured with a (charge-coupled device) CCD camera and processed with Zeiss Smart software (Version 3.0; Zeiss, Germany).

To observe bacterial attachment in the microfluidic chambers, the slides were mounted onto a Nikon Eclipse Ti inverted microscope (Nikon, Melville, NY) and observed at 40 X with

phase-contrast optics. Time-lapse microscopy images were acquired every 30 seconds with a

Nikon DS-Q1 digital camera (Nikon, Melville, NY) controlled by NIS-Elements software

(Nikon, Melville, NY).

1.2.5. Scanning Electron Microscopy (SEM). Samples were prepared for SEM

according to one of the following methods. For method one, the colonized slides were carefully

rinsed in ddH$_2$O to eliminate planktonic cells, fixed in 10% neutral formalin solution for 10 min,

and dehydrated in increasing concentrations of ethanol (30%, 50%, 80% and 100% for 10 min

each). Samples were then placed into 1:1 ethanol-hexamethydisilazane (HMDS) solution for 10

min and transferred to 100% HMDS for an additional 20 min. Samples were air dried in the hood

for another 10 min before sputter coating with gold palladium alloy in an EMS 550X (Electron

Microscopy Science, Hatfield, PA). For the second method, the slides were rinsed as stated

above, and then fixed in 4% osmium tetroxide overnight under a chemical hood. Slides were air

dried for 1 h and sputter coated for SEM observation using a Zeiss EVO 50 (Zeiss, Germany).

Planktonic cells were used as control for morphological observations. Briefly, 5 µl of a

24 h-old culture was inoculated in 5 ml of MS broth, immediately fixed in 2.5% glutaraldehyde

(vol/vol) and filtered through an Isopore^{TM} membrane (Millipore, USA). Filters were then

dehydrated in serial (50%, 70%, 90%, and 100%) ethanol and subject to critical point drying

before SEM microscopy as before.

1.2.6. Confocal Laser Scanning Microscopy (CLSM). Biofilm samples were prepared

as stated above and rinsed with distilled water three times. Twenty µl of mixture of propidium

iodide, SYTO 9 (Liver/Dead kit, Invitrogen, CA) and calcofluor white (10 mg/ml; sigma, CA)

was dropped on top of the biofilm directly and incubated in dark for 15 min before microscopy

observation. Slides were rinsed with distilled water again and observed with a Nikon Eclipse

confocal laser scanning inverted microscope (Nikon, Melville, NY), using 60X oil immersion objectives, with excitation wavelengths of 528 nm, 590 nm and 370 nm respectively. The three-dimensional architectures of biofilm were assessed by z-stack images with 0.9 µm increments. Images were acquired with CoolSNAP HQ2 camera (photometrics, Tucson, AZ) and analyzed with NIS-Elements AR software (Version 3.0; Nikon, Melville, NY).

1.2.7. Virulence study. To determine if *F. columnare* cells retained virulence when embedded in biofilm, I conducted a small virulence study using channel catfish fingerlings (for fish husbandry see (Arias et al. 2012a)). Briefly, 20 fish were stocked in a 37-L tank for each treatment. To recreate the conditions in which channel catfish fingerlings may encounter *F. columnare* in the form of biofilm, I eroded the skin of the fish (Bader et al. 2003) using a scalpel (this intended to mimic the cuts that fish suffer while rubbing themselves against the tank walls). Glass slides containing mature biofilms of strain BGFS-27 (about 72 h) was gently rubbed against the side of the fish for a few seconds. Glass slides in pure MS broth were served as negative control. Fish were monitored for signs of disease twice a day for 7 days.

1.3. Results

1.3.1. Cell attachment and biofilm formation: light microscopy. Observations under light microscopy showed cells attached to glass slides as early as 6 h post inoculation. Microcolonies started to appear at 12-24 h post-inoculation, and after 24 h the edges of the microcolonies merged into each other and the slides soon were completely covered with cells. Cell proliferation continued at 48 h post-inoculation and individual cells could not be longer visualized. At this time there was an observed changed in the refraction of the preparations, probably due to the production of EPS by the cells (Figure 3).

1.3.2. Components and structure of biofilm. Samples stained with calcofluor white showed the presence of polysaccharides within the biofilm while Congo red staining had a negative result (Figure 4). Biofilm was not of uniform in thickness and appeared as a heterogeneous matrix on the supporting slides. The surface of the biofilm appeared wrinkled with "mountain-and-valley" structures as revealed by phase contrast and fluorescent microscopy. The combined image using Dextran-Rhodamine revealed the presence of deep valleys in the biofilm that resembled water channels (Figure 5).

1.3.3. Biofilm ultrastructure under SEM microscope. SEM provided a higher resolution than light microscopy and was used to study the ultrastructure of biofilms. Figure 6 shows cells attached to the glass surface. Interestingly, cells were longer than those observed in planktonic forms, with most of the individual rods being 20 µm long although they ranged in size from 5 µm to more than 50 µm (Figure 6A). As microcolonies developed (Figure 6B) cells formed complex structural frameworks (Figure 6C). Blebbing-like vesicles were observed in some cells (Figure 6D) as well as on the control planktonic cells. Cells in biofilm produced EPS that were clearly visible by SEM (Figure 7). It is noteworthy that different fixations methods influenced the observed structure of the EPS. The formalin and HMDS fixation method seemed to eliminate more EPS and extracellular residues, and offered a better view of the individual cells (i.e. membrane blebbing, capsule) (Figure 7A). However, samples fixed with osmium tetroxide maintained a large portion of the extracellular matrix and seemed to present a less disturbed biofilm structure with cells completely embedded in the organic secretions (Figure 7B).

1.3.4. Biofilm ultrastructure using confocal microscopy. Confocal microscopy was used to observe two- and three- dimensional structures of biofilms. The use of the Live/Dead kit allowed to visualize cells that were active from those that had their cell membranes

compromised. In general, live cells tend to remain in the center of the microcolonies while "dead" cells (or cells with compromised membranes) appeared on the periphery of the cluster, and were enwrapped in EPS (Figure 8). An examination of the three dimensional image using the tilted view showed that the dead cells occupied the bottom layer of the biofilm (in contact with the glass) while the cells on the top of the microcolonies appeared to be metabolically active. Few or no dead cells were observed on the top layers of the clusters (Figure 9, panels A-C).

1.3.5. Bacterial attachment in microfluidic chamber. The microfluidic adhesion assay indicated of *F. columnare* can attach to organic polymer (polydimethylsiloxane) against effluent resistance. Cells tend to aggregate to each other and attach to the sidewalls. However, the attached cells flushed away when we increased the flow of medium to a speed of 1.0 µl/min, indicating a relative weak adhesion force between the cells and substrate. Not all the cells in the microchamber exhibit the same affinity for the microchannel. Most of the cells were flushed away, while just a small portion were left and attached firmly to the wall of the microchannel. The aggregation of biofilm seems tighter and tenser (Figure 10) under fluid water, presenting a different outlook than that formed on glass slides with no fluid motion. In a few instances, the cells glided for a short distance on the substratum (in the direction of flow) before mixing with other fixed cells or abruptly detaching from the surface.

1.3. 6. Biofilm virulence test. Channel catfish fingerlings challenged with strain BGFS-27 biofilm exhibited columnaris signs in less than 12 h post-challenge and reached nearly 100% mortality within 48 h post-challenge. *F. columnare* was recovered from all dead fish. Conversely, fish of the control tanks remained healthy and did not show any disease sign. These results showed that *F. columnare* in biofilm phase maintain the strong pathogenesis ability to infect the channel catfish under our experimental conditions.

1.4. Discussion

Attachment of bacteria to a surface is determined by the physical and chemical properties of the environment as well as by the nature of the bacterial ligands. Olivares-Fuster et al. (2011) showed that this pathogen can adhere and colonize gill tissue of channel catfish rapidly but no information was available regarding colonization of *F. columnare* to inert surfaces. In this study, I first investigated the timeline required for *F. columnare* cells to attach to glass surface. It took between 4 to 6 h to observe the first cells adhered to the glass slide. This contrasts with the less than 30 min time required for the cells to attach to live hosts (Olivares-Fuster et al. 2011). This time difference between *in vivo* and *in vitro* adhesion dynamics could be due to the positive chemotaxis that *F. columnare* displays towards host tissues (Klesius et al. 2008). It is also plausible that the cells need more time to recognize and adapt to the artificial surface and that they needed to induce the expression of different cell membrane receptors required to facilitate adhesion.

The importance of elucidating the dimensions and spatial arrangement of biofilms, including distribution and composition of matrix components, is considered essential in understanding the function of biofilms (Evans 2000). A broad range of microscopy techniques, including light microscope (differential interference contrast-, phase contrast- , epifluorescent- and confocal laser scanning laser microscopy), electron microscopy (transmission-, scanning-, and environmental scanning microscopy) have been applied to investigate biofilm structures in various stages of its development, with a different degree of sensitivity and level of resolution (Beech et al. 2000). Light microscope allows the observation of biofilm in its intact and original appearance and minimizes the artifacts produced by sample preparation (Lawrence et al. 1991; Keevil and Walker 1992; Walker and Keevil 1994). In my experiments, biofilm was observed

using a light microscope equipped with phase contrast and DIC. Phase contrast microscope allows biological samples to be observed in their unstained state by taking advantage of difference in refractive index between materials (Evans 2000). This technique is suitable for transparent, non light-absorbing, biological specimens. By employing a polarizing filter, DIC microscopy have the advantage to highlight edges of specimen structural details, provide high-resolution optical sections of thick specimen and avoid phase halos that typically appeared in phase-contrast image. Keevil al. reported that DIC could provide a 3-dimentional effect which allow the visualization of the bacterial glycocalyx over conventional phase contrast (Keevil and Walker 1992). For this study, phase contrast was suitable for visualizing the early colonization stages in where only a few cells were attached to the glass. However, later stages (particularly those in where EPS was already present) were better visualized with DIC. This technique has the additional advantage of generating a quasi 3D image of the specimen and was useful to observe the depth of the biofilm.

To investigate the components of the matrix and viability of the cells within biofilm, various staining methods were employed in combination with fluorescence microscopy. Calcofluor white and Congo red are two substances with high affinity to structural polysaccharides (Herth 1980). Calcofluor white is a fluorescent stain that can bind strongly to structure containing cellulose and chitin (β-1, 6 linked sugars). This dye is widely used for staining cells walls components of both algae and higher plants (Hughes and McCully 1975; Herth 1980). Here I used it as a particular sugar indicator to determine the components of the biofilm matrix. Fluorescent multichannel image (Figure 4A) highlighted an area around the bacterial aggregates where calcofluor white bounded thus, indicating the presence of the β-1, 6 linked sugars in the matrix. Congo red is a red water soluble dye that has a strong non-covalent

affinity to cellulose (mainly β-1, 4 linked sugars). In this study, the biofilm (Figure 4B) showed

negative result for Congo red staining, which indicates an absence, or at least rareness, of this

kind of polysaccharide. Dextran is a complex, branched glucan that is highly hydrophilic.

Rhodamine is a fluorescent chemical that can be used as a tracer dye within water to determine

the rate and direction of flow. I combined images of phase contrast with fluorescence

microscopy to reveal water-rich regions or possible water channels in the biofilm. I found that

biofilm formed by *F. columnare* (Figure 5) presents structured resembling mountain-valley

areas. The tracer dyes appeared at the "valley" belt around the protruding section. These

observations proved that *F. columnare* is capable of forming organized biofilms since cells were

not randomly aggregated. The part of biofilm that showed the highest degree of 3D organization

developed on the area of the slide that was placed at the air-water interface. The part of the slide

that was fully immersed in broth presented a thin biofilm of relatively flat characteristics and

with fewer cells overall (see Figure 4). These findings suggest that biofilm proliferation is

favored by oxygen, which can be justified by the fact that *F. columnare* is a strictly aerobic

bacterium. In addition, the gas-liquid inter-phase area has proved to provide additional sites for

cell and biosurfactant absorption as well as exchanges of oxygen and other gases between gas-

aqueous phases (Rockhold et al. 2002).

SYTO 9 and propidium iodide are two common fluorescent dyes that can be used to

differentiate live and dead cells. Live cells uptake the diffusible SYTO9 dye and fluoresce in

green while the cells that have a compromised membrane are permeable to propidium iodide and

appear red after staining. Biofilms were stained with both dyes and with the aid of CLSM I was

able to visualize the growing pattern of *F. columnare* biofilm. Dead or compromised cells

formed a peripheral halo that surrounded the green live cells in the center. Dead cells appeared

enwrapped by a layer of extra polysaccharides which were stained by calcofluor white. However, it is possible that sample preparation methods and handling (e.g. exposure to laser radiation) injure the most outside layers of the microcolonies. Therefore, my results should be taken in the context of the limitations imposed by the methods used for biofilm observation.

Light (i.e. DIC) and confocal microscopy (i.e. CLSM) complemented each other for the structural analysis of biofilms, which provide us a complete view of the complexity of their ultrastructure. Specifically, DIC gave us an original and intact surface overview on support surface while CLSM demonstrated the distribution of compromised and viable cells in the 3-dimension level analysis. The tiled view showed the third dimensional structure of the biofilm, and indicated that cells on the top layer were more metabolically active and "healthy" than those at bottom layer, which support out fist assumption that aged cells lye on the peripheral area and the bottom section

In recent years, SEM and CLSM have become a critical technology to study biofilm structures due to their powerful magnification and 3-D structure reconstruction ability. However, images of SEM may differ significantly due to different sample preparation methods (Fratesi et al. 2004). In this study, I tried different SEM preparation procedures to eliminate possible bias and to obtain a more comprehensive picture of the biofilm morphology. Samples fixed with formalin and dehydrated with ethanol showed less extracellular organic excretions and more detailed individual cell morphology. When biofilm was fixed with osmium, a thicker layer of extracellular organic materials was observed. It appears as if formalin/ethanol maintained the hydrophilic nature of the EPS as the matrix was observed in a very thin layer that still remained attached to the glass. Conversely, osmium seemed to compact EPS, perhaps by creating a hydrophobic environment. Fratesi et al. investigated the effect of SEM preparation methods on

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the appearance of bacteria and biofilm and suggested that the ethanol incorporated dehydration preparation technique would result in stranded mucilaginous secretions and even cylindrical cell morphology, which could give a dramatically different overview compared to other none-ethanol incorporated methods (Fratesi et al. 2004).

SEM requires fixation samples in either glutaraldehyde or osmium tetroxide, followed by dehydration using either an alcohol series or critical point drying with liquid CO_2, and coated with conductive metallic material, such as gold or carbon. All these extensive sample treatments are considered to cause considerable distortion of the specimen (Evans 2000). The dehydration step can produce a significant shrinking effect due to the destruction of highly hydrated extracellular polymeric matrix, resulting less residues than the original sample (Richards and Turner 1984; Fisher et al. 1988). Thus, I utilized a complementary procedure that includes osmium tetroxide fixation and air drying. This method did not underwent any liquid fixation or dehydration step and preserved significantly more hydrophobic loose-embedded extracellular residues such as lipid, proteins and nucleic acid, which can be otherwise dissolved and removed in ethanol and water.

Richards (1984) reported that process of critical point drying can remove most of the 'slime' covering the bacterial cells, thus allowing for a close-up on the cell surface. I got similar results to those described by Richard. When I used a none-critical point drying method the cells were covered with a thick layer of 'slime'. However, after critical point drying I was able to observe more details on the individual bacterial cells, including blebbing vesicles.

It has been reported that the dehydration processes during SEM preparations has a shrinking effect on the cells, which could condense the hydrated components of the glycocalyx to 1% of their original volume (Fisher et al. 1988). I observed clusters of what appeared to be

vesicles that measured approximately 1 µm in diameter. These clusters looked like vesicles that were secreted from the outer membrane of the bacteria (image not shown). These vesicle-like structures were observed in both biofilm cells as well as planktonic control cells. Some of the cells clustered together resembling a "grape" cluster that have also been reported in other species of bacteria (Fratesi et al. 2004). I speculate that these vesicles contain metabolic products that are either secreted or excreted into the environment. In Gram-negative bacteria such as *F. columnare,* membrane vesicles are formed by the outer membrane of cells and are used to secrete extracellular enzymes and nucleic acids. These vesicles may function as mobile elements in the EPS matrix (Flemming and Wingender 2010).

The microfluidic chamber provided a unique platform to observe biofilm formation in real time under controlled dynamic fluid conditions, which mimic water flows in the environment. I observed that *F. columnare* exhibited weak adhesion under those conditions. Only a small portion of the injected bacteria were successfully attached to the substratum. This could be due to the lack of cell anchor mechanisms such as pili or flagella (De La Fuente et al. 2007). *F. columnare* is motile by gliding but lacks other typical appendages involved in cell motility such as pili or flagella.

Table 1. Formulation of Modified Shieh broth medium. MS agar was prepared by adding 12 g/L of agar to the formula below.

Components	Concentration
Tryptone	5 g/L
Yeast extract	2 g/L
K$_2$HPO$_4$	1.08 mM
MgSO$_4$·7H$_2$O	1.2 mM
FeSO$_4$·7H$_2$O	4.1 mM
CaCl·2H$_2$O	45.6 mM
Adjust pH to 7.2—7.4	

Figure 1. Bacteria attachment to glass slides under microcosm conditions. Panel A) microcosm. Panel B) example of a microcolony formed on slides (light microscopy, 400 X magnifications).
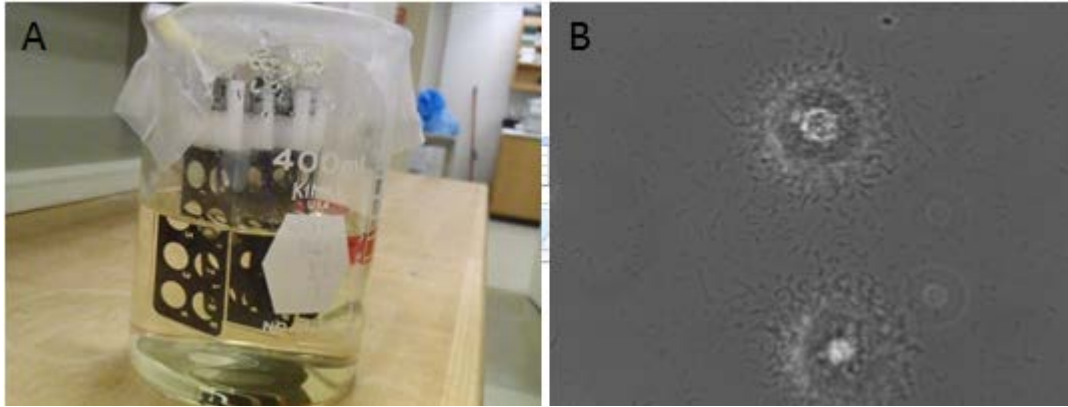
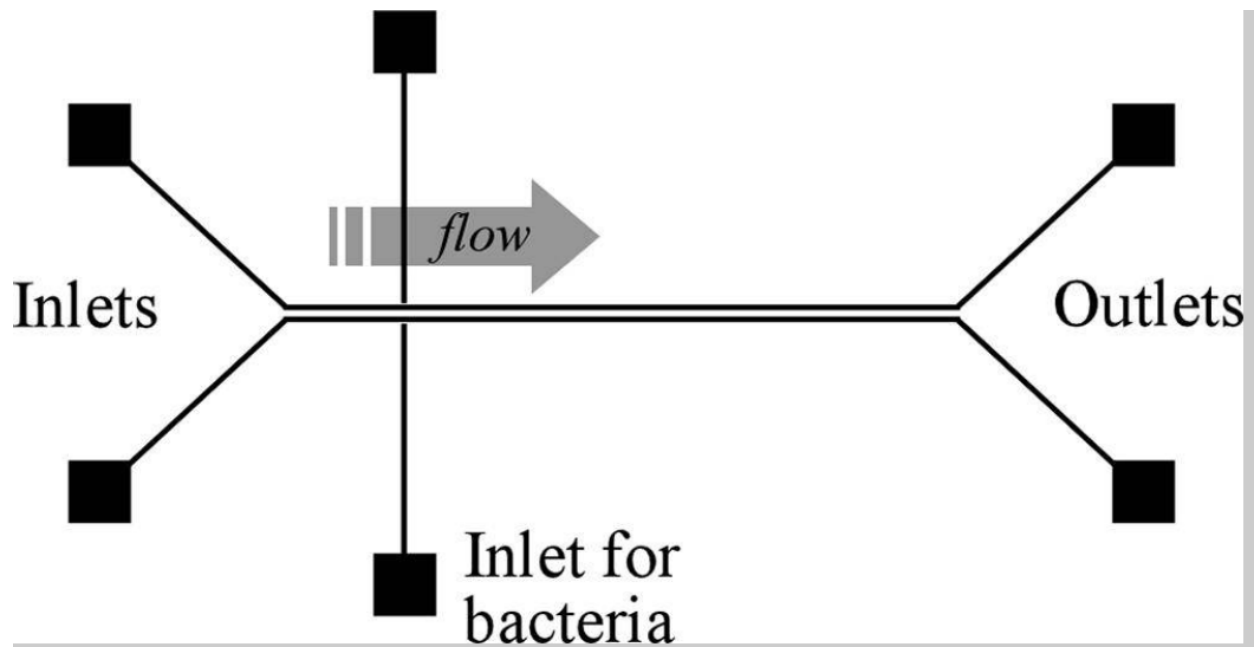Figure 2. Microfluidic chamber with two parallel channels (by De La Fuente, 2007).

Figure 3. Bacteria attachment on glass slides under light microscope. Panels A, B, C, and D display cell attachment and biofilm formation at 6 h, 12 h, 24 h, and 48 h post-inoculation (bright field, 400 X magnifications).
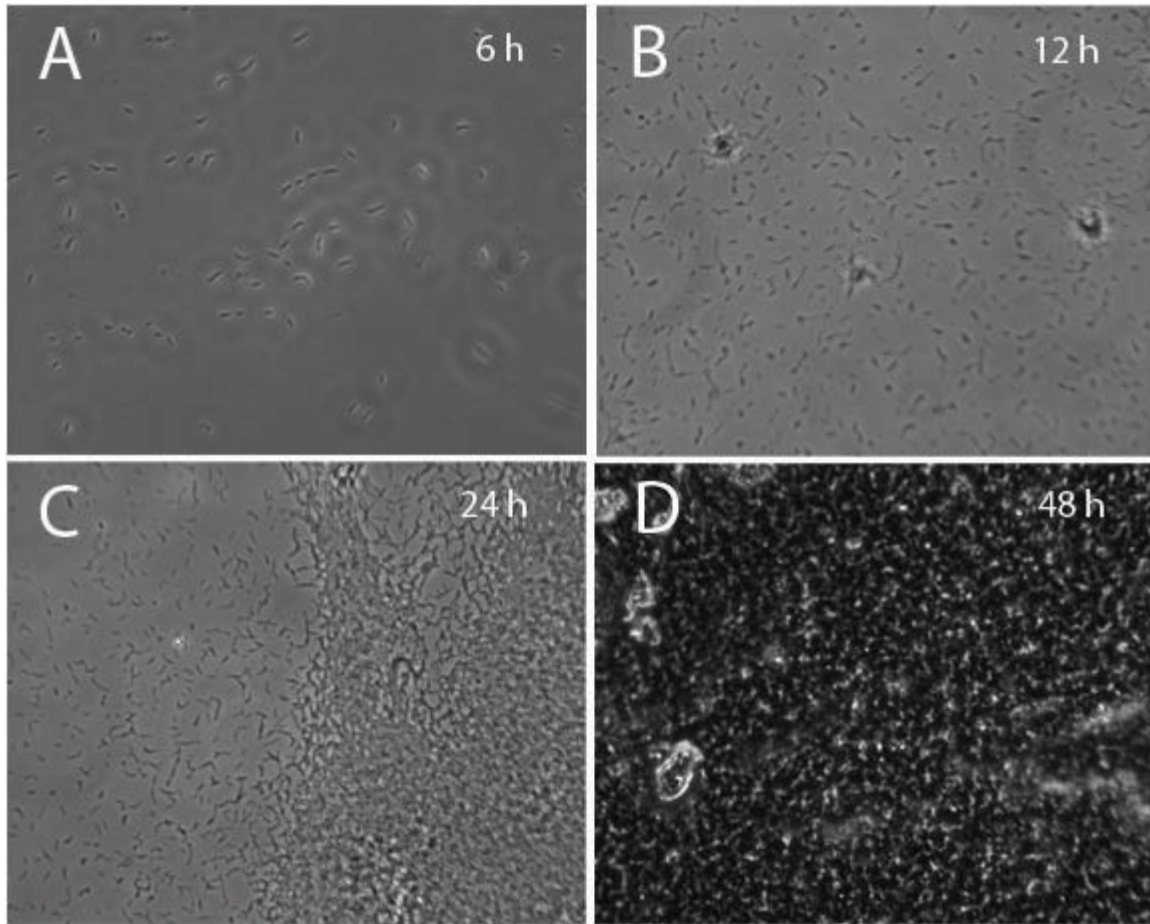
Figure 4. Biofilm stained with calcofluor white (Panel A: positive result) and Congo red (Panel B: negative result) using differential interference contrast microscopy at 600 X magnification.
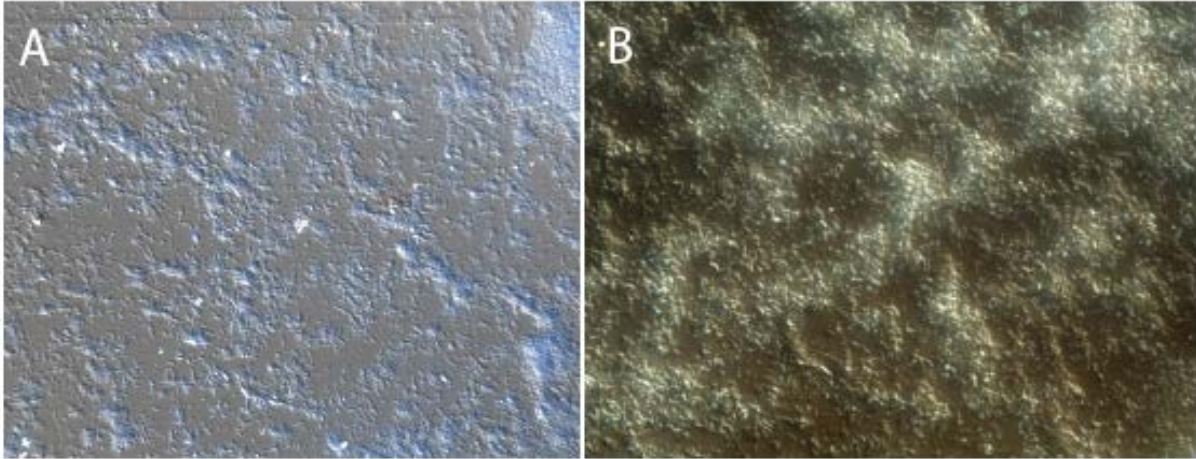
Figure 5. Water channels of Biofilm stained with Dextran/Rhodamine using phase contrast microscopy (at 600 X magnification).
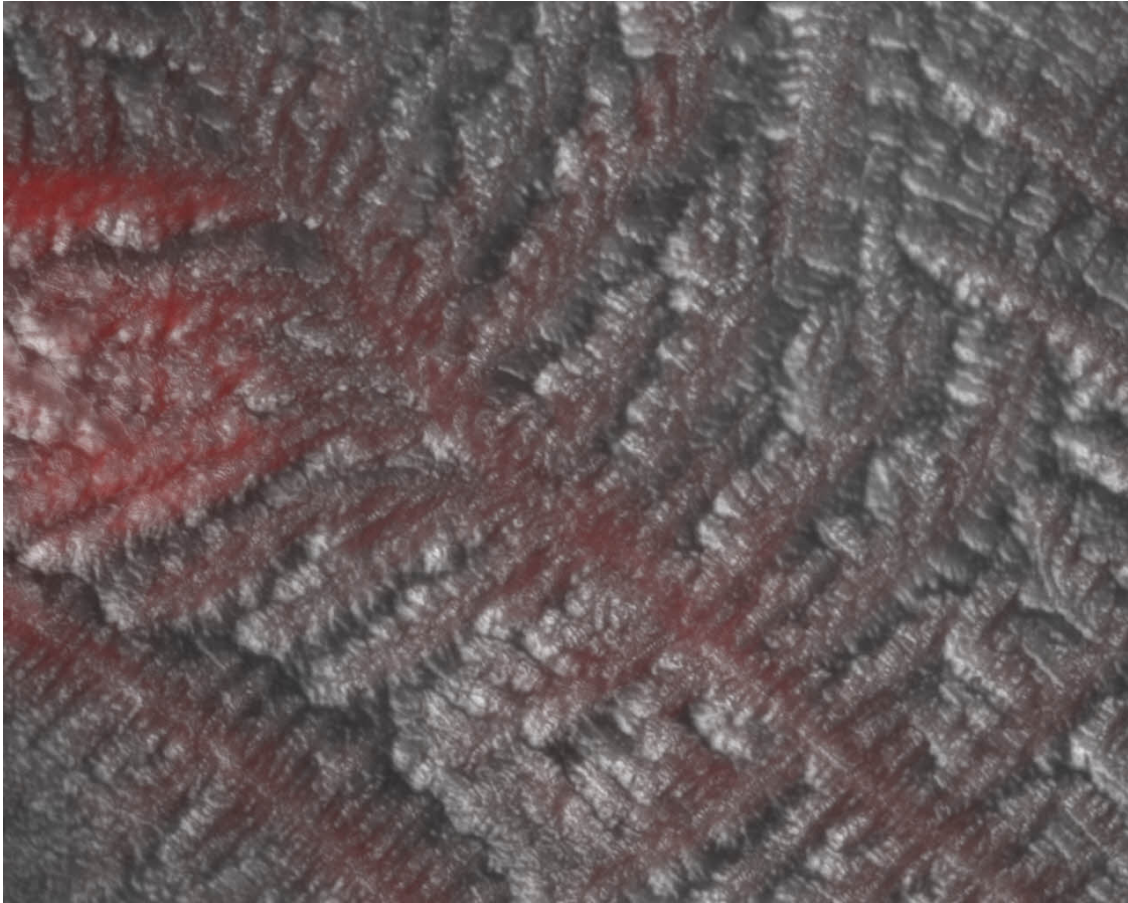
Figure 6. SEM images of biofilm at 3 days post-inoculation. Panel A: diversity of cell morphology on the outside areas of biofilm (scale bar 30 µl). Panels B and C: details on cell aggregation (scale bar 20 µm). D: the rod-shaped bacteria with polysachharide secretions linking the bacteria to each other and surface. Arrows indicate bacrterial cell (BC), surface blebbing (SB), and extracellular polysaccharide (EPS). Scale bar represent 1 µm.
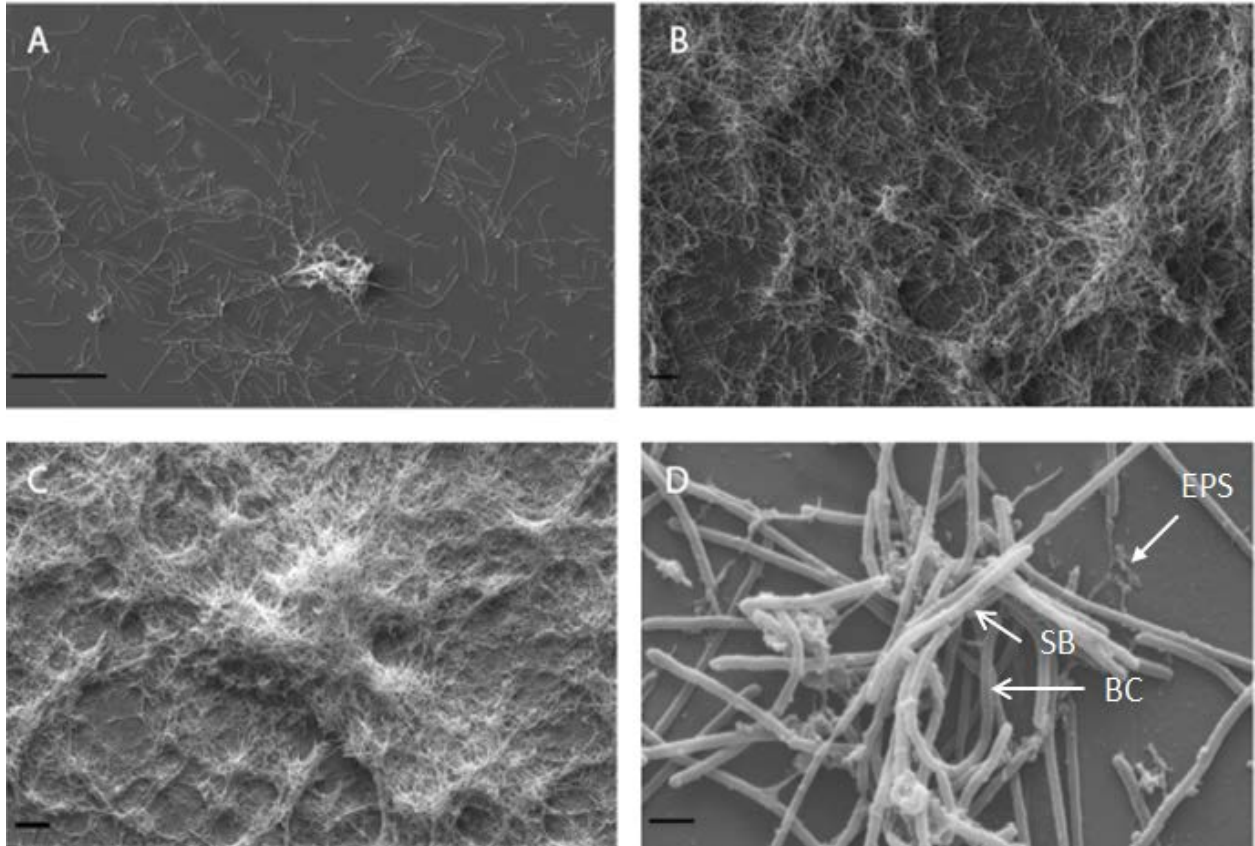
Figure 7. Cell aggregations of *F. columare* cells fixed with HMDS (Panel A) and osmium tetroxide (Panel B): Arrows indicate extracellular pollysaccharide (EPS) and bacterial cells (BC). Scale bar represent 2 µm.
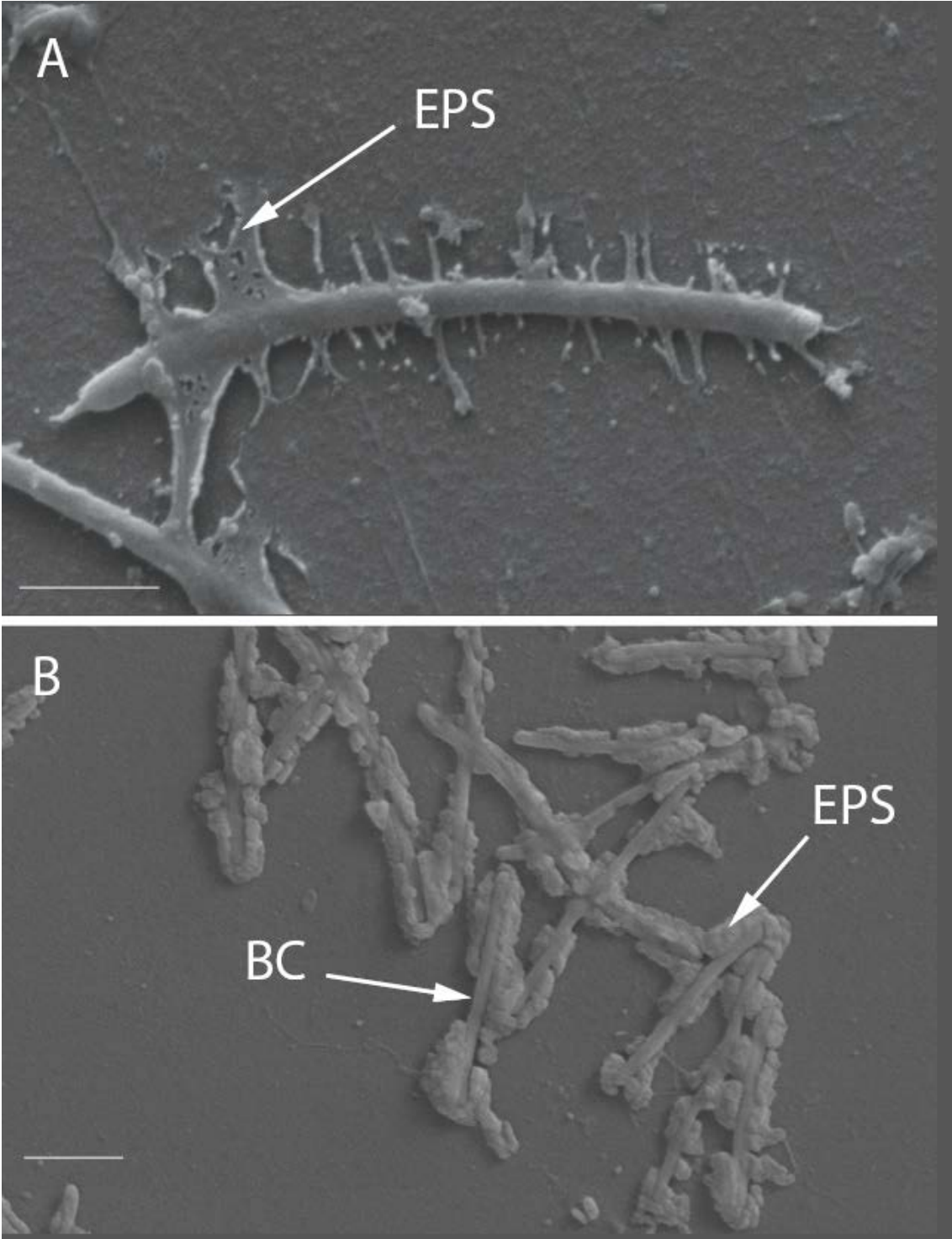
Figure 8. Viability of cells in biofilm using the Live/Dead kit under CLSM at 3 days post-inoculation. Red indicates dead cells, green indicates live cells, and blue reflects the extracellular polysaccharides.
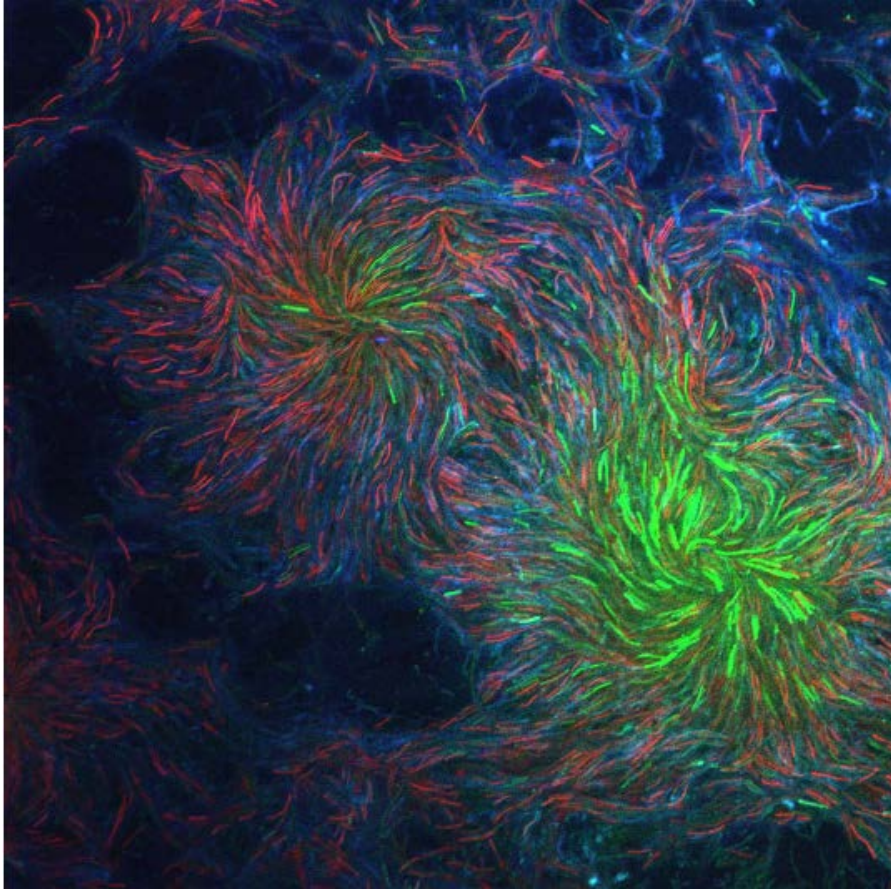
Figure 9. Tiled view of biofilm using the Live/Dead kit under CLSM at 3 days post-inoculation. Each panel represents one layer of biofilm at 0.9 µm increments from top to bottom (A to J).
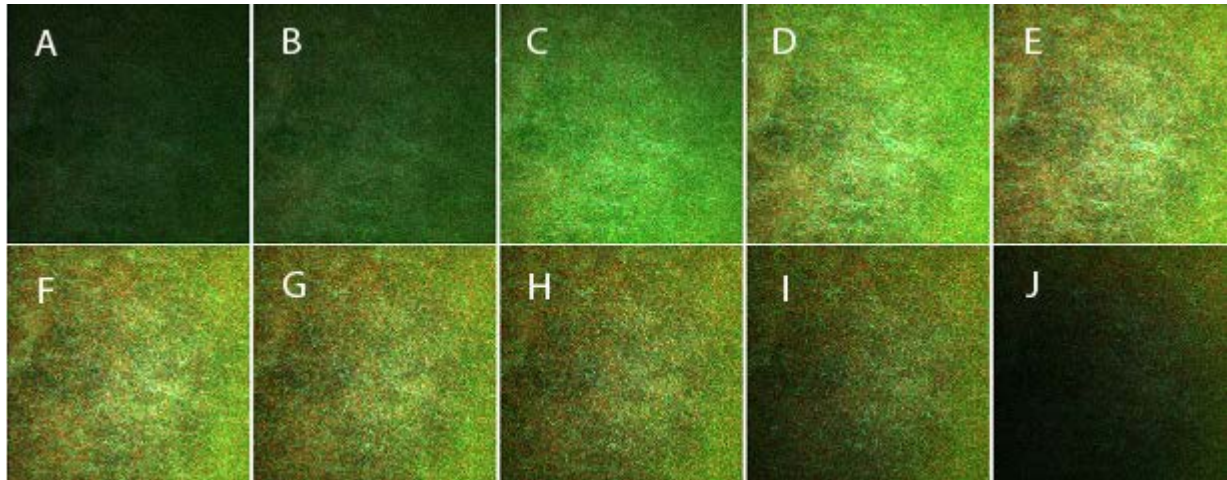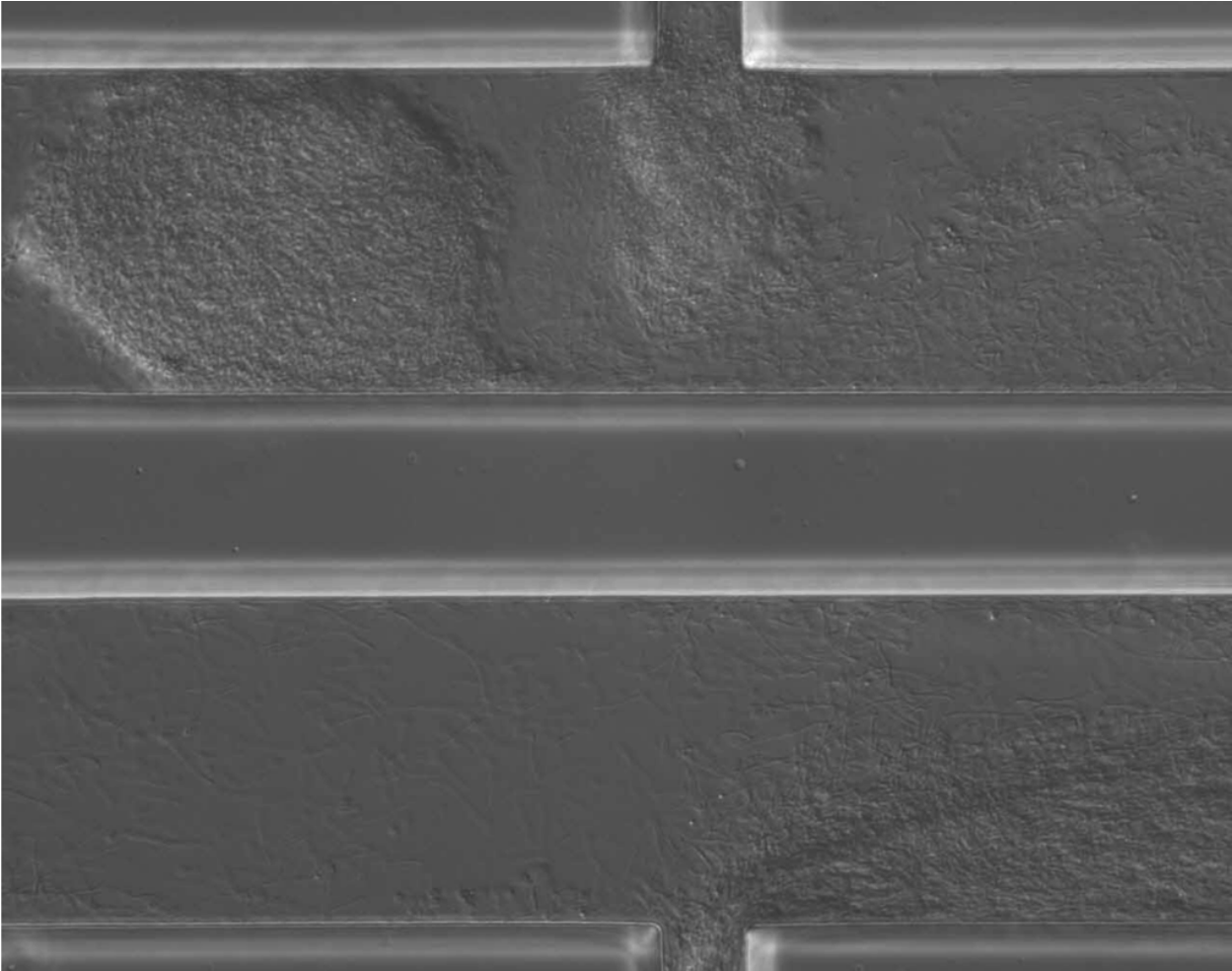
Figure 10. Biofilm formed on microchannels under dynamic fluid.

Chapter 2. Quantitative evaluation of variables affecting biofilm formation by *F. columnare*

2.1. Introduction

Flavobacterium columnare is a genetically heterogeneous species, which is divided into 3 distinct genomic groups or genomovars (Tryanto and Wakabayashi 1999). Differences in virulence are known to exist among *F. columnare* isolates (Pacha and Ordal 1970; Olivares-Fuster et al. 2010) with genomovar II being more virulent towards catfishes than genomovar I strains (Shoemaker et al. 2008; Arias et al. 2012a). The exact mechanism by which genomovar II is more virulent towards catfishes than genomovar I is unknown but previous studies pointed to the superior ability of genomovar II to recognize and attach to the host. Klesius et al. (2008) reported that genomovar II isolates were more chemotactic to channel catfish mucus than genomovar I isolates. Similarly, Shoemaker et al. (2008) showed a positive correlation between virulence and adherence in channel catfish fry when challenged with *F. columnare*. These previous studies lead us to hypothesize that significant differences in cell attachment exist between genomovars of *F. columnare*.

Attachment is a crucial step to both bacterial pathogenesis and biofilm formation. The attachment of bacteria to a surface is affected by physical and chemical properties of the surrounding environment, such as temperature, pH, hardness, etc… (Mai 2006). Bacterial cells, as most inert surfaces, are negatively charged due to an excess of carboxyl and phosphate groups (Fletcher 1977; van Loosdrecht et al. 1989; Mai 2006). Electrostatic repulsion between surfaces of same charge prevents surfaces from coming in close contact to each other. However, the physiochemical characteristics of the environment (including cation concentration, pH, and temperature) affect those interactions and can modulate how bacterial cells approach and attach to inert surfaces. Previous studies have attempted to characterize the effect that ionic strength

plays on bacterial attachment to hydrophobic and hydrophilic polystyrene but conclusive results were not obtained (McEldowney and Fletcher 1986; Mai 2006).

Since *F. columnare* is able to attach to and colonize inert surfaces (as shown in Chapter II), my objective for the following studies was to evaluate the effect that some physiochemical variables have on biofilm formation. I chose temperature, pH, salinity, hardness, and the presence of specific carbohydrates as my variables since those play an significant role in fish stress and disease incidence in aquaculture settings (Verma et al. 2011). To assess if genomovar ascription influences attachment and biofilm proliferation, I included a collection of *F. columnare* strains with representatives of all three genomovars.

2.2. Materials and Methods

2.2.1. Bacterial Culture. Nineteen *F. columnare* stains isolated from different sources were used in this study (Table 2). All the strains had been previously identified as *F. columnare* based on standard biochemical methods (Bernardet et al. 1996), and they exhibited distinct genetic profiles according to previous genotyping analysis (Olivares-Fuster et al. 2007). Representatives of all three genomovars were included in the study although only two genomovar III strains could be obtained. Bacteria were routinely grown and maintained in MS (Shieh 1980) broth for 48 h at 28°C with gentle shaking. Stock suspensions of all isolates were stored in MS broth supplemented with 20% glycerol at -80°C.

2.2.2. Quantification of biofilm. The biofilm formation by *F. columnare* was assessed according to methods described previously (Alvarez et al. 2006), with some modifications to accommodate for *F. columnare* growth. Briefly, a 28-h old inoculum was diluted 100 times in the corresponding medium, and then 100 µl of each dilution was inoculated in 96-well microtiter polystyrene plate (NuncImmuno MaxiSorp, Nunc, Rochester, NY) in quadruplicate to allow for

bacterial attachment and biofilm formation. The microtiter plates were incubated for 48 h at 28°C unless otherwise stated. Bacterial growth was estimated by measuring the optical density (OD) of the wells at 600 nm. OD quantified both the planktonic as well as cells attached to the bottom of the wells (Altinok and Grizzle (2001)).

After 48 h of incubation, the supernatants were discarded and the wells were washed four times with 300 µl distilled water. After washing, wells were stained with 150 µl of 1% (wt/vol) crystal violet. After 20 min, the crystal violet solution was removed and the wells were washed four times with 300 µl distilled water. Finally, 200 µl of 96% (vol/vol) ethanol was added to dissolve the dye and the biofilm formation was quantified by measuring the OD at 595 nm.

2.2.3. Physicochemical variables tested. The effects of five variables (i.e. temperature, pH, salinity, hardness and carbohydrates) on biofilm formation were investigated as follows. Temperature effect was compared by incubating the plates at 21°C, 28°C, and 35°C. Five different pH were assessed by adjusting the pH of MS broth to 5.8, 6.2, 6.6, 7.3 and 7.9 with 5 M NaOH or 1 M HCl and the aid of a pH meter (SevenEasy, Mettler Tolello, Switzerland). Salinity was tested by adjusting the MS broth to 0, 5.0, 7.5, 10.5, 14.0 ppt of NaCl. Final MS salinity was confirmed by using a salinity meter (Vital Sine[TM] Model SR-6, Aquatic Eco-Systems, Inc.). Hardness of MS broth was adjusted to 12, 64, 120, 300 and 360 ppm with $CaCl_2 \cdot 2H_2O$. Finally, for carbohydrates test, MS broth was supplemented with 50 mM of mannose, fucose, galactosamine hydrochloride, glucose and N-acetyl-D-glucosamine. All these modified culture broths were inoculated with bacteria according to method described above.

2.2.4. Statistical analysis. Data on biofilm formation on polystyrene plates were analyzed by one-way analysis of variance (ANOVA) using SAS software for Windows (Version

9.2; SAS Inc.). Salinity, hardness, pH, carbohydrate were tested using Tukey ANOVA, and temperature was analyzed with LSD ANOVA. Significant difference was set at $p < 0.05$.

2.3. Results

2.3.1. Effect of temperature on bacterial growth and biofilm formation. Overall, I observed a great variation in the ability of different stains to adhere to polystyrene plates (Figure 11). Under standard conditions, 28°C in MS broth, the four strains that exhibited the highest adhesion ability were: BZ-1-02, ALM-05-202, Grizzle and BioMed. To simplify the effect of the different variables, the following figures show the mean of crystal violet absorbance when all strains and all replicates were taken into account. Temperature exerted a significant effect in biofilm formation. Cells incubated at 28°C showed significantly higher biofilm formation ability than those grown at 35°C. It is important to point out that the growth observed in planktonic cells did not differ between 28°C and 35°C. Growth under 21°C was limited compared growth under 28°C for planktonic cells but their biofilm formation was not significantly different (Figure 12).

2.3.2. Effect of pH on bacterial growth and biofilm formation. Variability of bacterial growth and biofilm proliferation among the strains was also observed. Cells grew well on a broad range of pH from 6.2 to 7.9 and did not show a significant difference among the different treatments. However, pH 5.8 did in fact limit bacterial growth. I did not observe a significant difference on cell colonization and biofilm formation at any of the tested pH (Figure 13).

2.3.3. Effect of salinity on bacterial growth and biofilm formation. Salinity played a critical role in biofilm formation. Planktonic cells actively grew at salinities of 5 to 14 ppt but cell grow was inhibit by absence of NaCl. Biofilm formation significantly decreased with an increasing salinity from 5 ppt to 14 ppt (Figure 14).

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2.3.4. Effect of hardness on bacterial growth and biofilm formation. The broad range of hardness tested (from 12 to 360 ppm) did not produce dramatic differences on bacterial growth although a few significant differences among treatments were recorded. However, hardness did play a significant role in biofilm proliferation (Figure 15). Bacteria incubated at 200 ppm $CaCl_2·2H_2O$ or lower produce little biofilm while 360 ppm dramatically increased biofilm formation.

2.3.5. Effect of carbohydrates on bacterial growth and biofilm formation. Culture media supplemented with galactosamine hydrochloride and glucose significantly inhibited the growth of *F. columnare* and subsequently impeded biofilm formation. Mannose did not promote general bacteria growth but significantly enhanced biofilm formation. The other two polysaccharides tested (fucose and N-acetyl-D-glucosamine) did not have a significant effect on biofilm formation or growth (Figure 16).

2.3.6. Biofilm formation and genomovar. Contrary to my initial hypothesis, no correlation between genomovar ascription and the ability to attach to and colonize polystyrene plates was detected in this study (Figure 17). The variability observed at the strain level clearly overshadowed the genomovar effect, if any.

2.4. Discussion

Maintaining optimal water quality is a must in aquaculture. Poor water quality disrupts host homeostasis and favors the onset of infectious diseases. In the US, catfish are produced in earthen ponds that cannot be maintained under control conditions as in other aquaculture industries. In general, the optimal temperature range for in-pond catfish aquaculture is between 27°C to 30 °C, although fish growth occurs as long as the temperature is above 13°C (Jiang 2005). The recommended values for alkalinity and total hardness are >50 mg/L (Chapman 2000)

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although they can fluctuate from 47 to 90 mg/L depending on the geographic locality and source of water (Boyd et al. 2000; Jiang 2005). Wurts reported that the acceptable range of free calcium is 63-250 mg/L $CaCO_3$ but a desirable range would be between 75-200 mg/L $CaCO_3$ (Wurts and Durborow 1992). The hardness values tested in this study were therefore in range with what is considered normal in US catfish aquaculture. Salinity can also vary with water source and, in fact, there are catfish farms in West Alabama that use near brackish water concentrations (Perry and Avault 1968; Remane and Schlieper 1971; Boyd et al. 2000). The range of values used in this study was selected to encompass the water quality parameters typically found in catfish farms.

In our latitude, columnaris disease often occurs in late spring and early fall (Austin and Austin 1999), which typically coincides with the optimal temperature for the pathogen (24-28°C). The temperature range in which *F. columnare* can grow extends approximately from 15°C to 35°C. However, this may only apply to planktonic cells as no studies regarding temperature and biofilm proliferation exist to date on *F. columnare.* Due to the protection function of the biofilm, it is possible that biofilm formation could enhance the potential for survival of these bacteria. Kunttu al. (2011) found adhesion can be significantly enhanced in *F. columnare* cells derived from rhizoid colonies from 5 to 20°C. In our study, we also found that growth was influenced by temperature. At 21°C, planktonic cells and biofilm growth was lower than those observed at 28 °C, probably due to a lower metabolic rate at this temperature. However, at 35°C planktonic cell growth was not significantly different than at 28°C but biofilm proliferation was significantly reduced indicating that at higher temperatures, cells prefer to remain in the water column.

Verma et al. (2011) studied the effect of pH and salinity in the pathogenicity of *F. columnare* in Indian catfish. They found that the effect of pH was strain-specific since some strains were more tolerant to low pH than others. In our experiment, the pH of the medium had little effect on planktonic cell growth and biofilm formation, which agrees with previous studies that reported a broad pH tolerance in *F. columnare* (Groff and Lapatra 2000). By contrast, salinity is known to prevent columnaris disease (Chowdhury and Wakabayashi 1990; Altinok and Grizzle 2001). Altinok and Grizzle studied the effect of salinity on bacterial growth and on *in vitro* adhesion. They found that bacterial growth in 1.0 or 3.0 ppt salinity was significantly higher than in control medium (0.3 ppt), but *in vitro* adhesion of bacteria was reduced with increasing salinity. My findings are in agreement with theirs as I found that that absence of NaCl significantly inhibited both planktonic multiplication and biofilm proliferation. However, planktonic cells were able to maintain similar growth patterns under different NaCl concentrations while biofilm formation was significantly inhibited with the increased salinity. These results agree with those presented by Chowdhury and Wakabayashi in where they reported that survival of *F. columnare* in water was improved by the addition of calcium, magnesium, potassium and sodium ions (Chowdhury and Wakabayashi 1988). Several studies has reported that the growth of *F. columnare* was completely inhibited by 10 ppt NaCl (Pacha and Porter 1968; Bernardet 1989) but in my study I observed growth in salinities as high as 14 ppt. Salt baths (30 second dip in 80 g/L NaCl) is an approved method to treat columnaris disease (Austin and Austin 1999). Based on my data and others, it seems that salt baths will prevent bacterial attachment more than inhibit bacterial growth.

F. columnare can survive for long periods in lake water and distilled water (Kunttu 2010; Arias et al. 2012b). Shieh reported that addition of calcium, magnesium and potassium ions into

synthetic medium could stimulate the growth of *F. columnare* (Shieh 1980). Later, Chowdhuyry

and Wakabayashi reported (1988) that *F. columnare* cells survival was optimal with a calcium

carbonate hardness of approximately 70 ppm. Culture conditions with calcium carbonate

hardness lower than 50 ppm decreased the survival of the bacteria (Fijan 1968). Our study

showed that planktonic cells had an optimum growth at 64 ppm, whereas biofilm formation was

greatly promoted at higher hardness (360 ppm). Cruz et al. (2012) studied the effect that

supplementation of metals had on biofilm formation by the plant pathogen *Xylella fastidiosa*, and

pointed out that calcium was able to increase surface attachment, biofilm formation and

twitching motility of the cells (Cruz et al. 2012). It is suggested that divalent cations such as

calcium may be important for the maintenance of biofilm structure by acting as bridging agents

within the three-dimensional EPS matrix (Branda et al. 2005).

Decostere et al. (1999) studied the adhesion of *F. columnare* to gill tissue and found that

D-glucose exhibited the strongest inhibitory activity followed by D-galactose. They proposed

that *F. columnare* cells attached to glucose-like substance on host tissues and had possibly more

than one lectin type and thus could recognize more than one type of sugar residue (Decostere et

al. 1999b). Klesius et al. (2008) evaluated the effect of several carbohydrates on *F. columnare*

(stain ALG-00-530) chemotaxis, and found mannose significantly inhibited the chemotactic

response of *F. columnare* towards catfish mucus. In the Klesius' study, significant inhibition was

also observed following treatment with D-glucose and N-acetyl-D-glucosamine, but not D-

galactosamine and L-fucose. They concluded that carbohydrate-binding receptors of capsule

were involved in the chemotactic response and these receptors recognized and bound to the D-

mannose, D-glucose and N-acetyl-D-glucosamine structure of the chemoattractants associated

with fish mucus. Similarly, I found that mannose could dramatically promote cell attachment and

colonization on inert surfaces of polystyrene plates. However, I did not observe a significant stimulation of biofilm by the addition of sugars like glucose, N-acetyl-D-glucosamine, and fucose. Interestingly, D-galactosamine has been reported to significantly inhibit the adhesion of *F. columnare* to gill tissue (Decostere et al. 1999b). This effect was not found in the present study although D-galactosamine significantly inhibited the growth of *F. columnare*.

A significant difference of adhesion ability to inert surfaces among different genomovars was not observed in this study. Although virulence and *in vivo* attachment to the host have been proved to be influenced by genomovar in previous studies (Shoemaker et al. 2008; Olivares-Fuster et al. 2011), *in vitro* adhesion was not a factor of genomovar ascription.

In summary, I was able to quantify biofilm formation by *F. columnare* under *in vitro* conditions. Among all variables tested, pH did not seem to play a major role in either growth of planktonic cells or biofilm proliferation. Temperature and salinity showed significant influence both in cell growth and attachment and addition of mannose enhanced attachment to polystyrene surface. Finally, hardness (at 360 ppm) was the tested variable that most influenced biofilm formation. Based on my data, aquaculture facilities in where columnaris is a recurrent problem and in where water quality can be modified (i.e. hatcheries) should increase their salinity (>10 ppt) and lower hardness (~360 ppm) in order to prevent biofilm formation by this pathogen.

Table 2. *Flavobacterium columnare* strains used in the study.

Strain	Genomovar	Geographic origin	Source	Date
ARS-1	I	Alabama, USA	Channel catfish	1996
ALM-05-122	I	Alabama, USA	Blue catfish	2005
GA-02 -14	I	Georgia, USA	Rainbow trout	2002
Grizzle	I	Alabama, USA	Channel catfish	Unknown
ALM-05-114	I	Alabama, USA	Threadfin shed	2005
ALG-03-063	I	Alabama, USA	Channel catfish	2003
BioMed	I	Alabama, USA	Channel catfish	1996
ALG-00-530	II	Alabama, USA	Channel catfish	2000
BZ-1-02	II	Brazil	Nile Tilapia	2002
ALM-05-202	II	Alabama, USA	Blue catfish	2005
LT-ulcer	II	Alabama, USA	Nile Tilapia	2011
ALM-05-173	II	Alabama, USA	Blue catfish	2005
BGFS-27	II	Alabama, USA	Channel catfish	2005
ALG-00-527	II	Alabama, USA	Channel catfish	Unknown
ALM-05-121	II	Alabama, USA	Channel catfish	2005
CL-gill	II	Alabama, USA	Nile Tilapia	2011
BGFS-25	II	Alabama, USA	Channel catfish	2005
AU-98-24	III	Alabama, USA	Channel catfish	1998
Dickson-1	III	Unknown	Channel catfish	1995

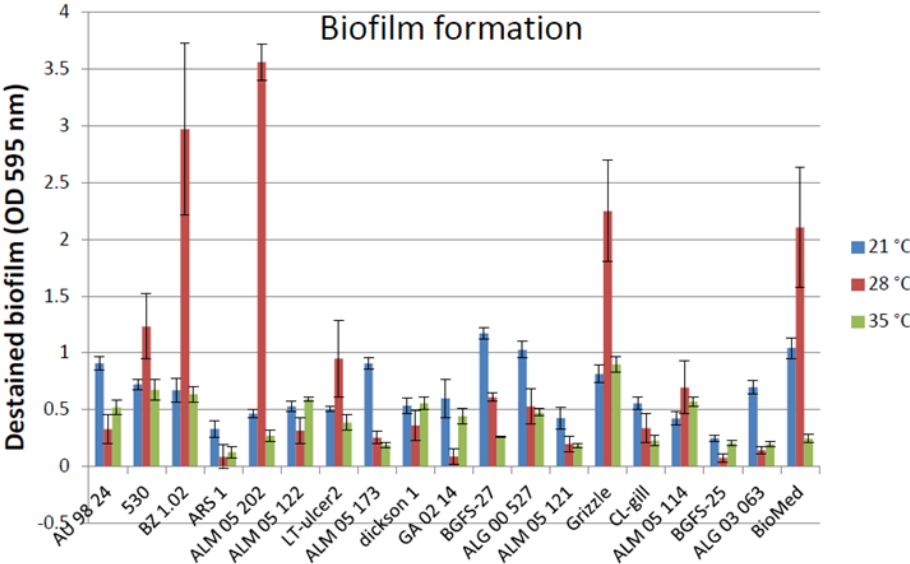Figure 11. Biofilm formation at 21°C, 28°C and 35°C (mean absorbance ± SE).

Figure 12. Bacterial growth (mean absorbance ± SE) (blue) and adhesion (mean absorbance ± SE) (red) of all strains of *F. columnare* after 48 h of incubation at different temperatures. Different letter indicate significant difference ($p < 0.05$).
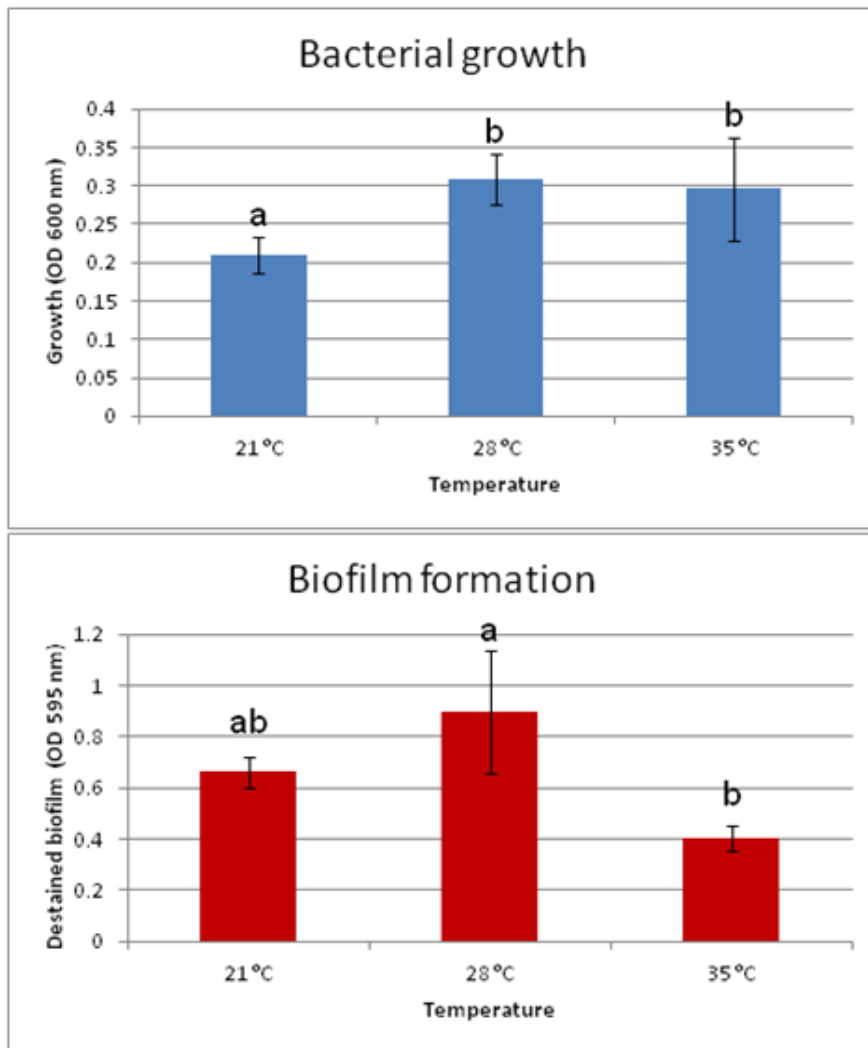
Figure 13. Bacterial growth (mean absorbance ± SE) (blue) and adhesion (mean absorbance ± SE) (red) of all strains of *F. columnare* after 48 h of incubation at different pH. Different letter indicate significant difference ($p < 0.05$).

Figure 14. Bacterial growth (mean absorbance ± SE) (blue) and adhesion (mean absorbance ± SE) (red) of all strains of *F. columnare* after 48 h of incubation at different salinity. Different letter indicate significant difference ($p < 0.05$).
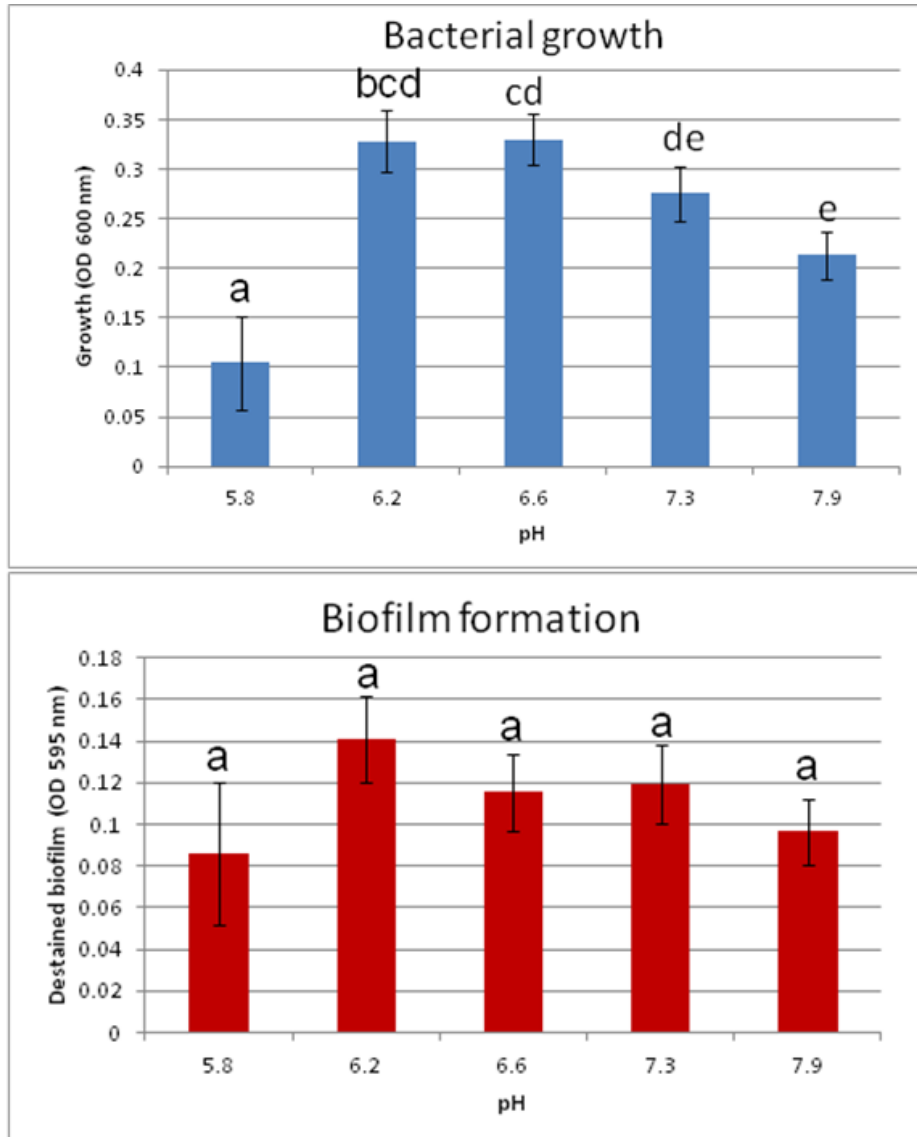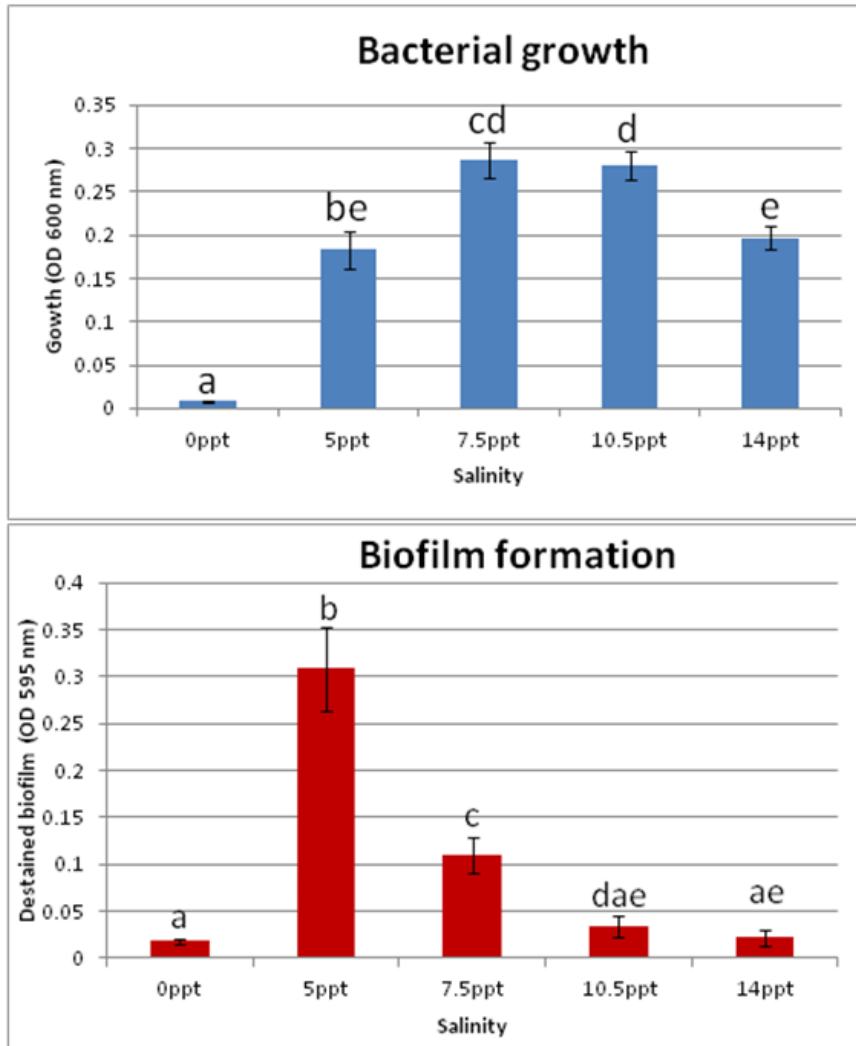
Figure 15. Bacterial growth (mean absorbance ± SE) (blue) and adhesion (mean absorbance ±

SE) (red) of all strains of *F. columnare* after 48 h of incubation at different hardness. Different

letter indicate significant difference ($p < 0.05$).

Figure 16. Bacterial growth (mean absorbance ± SE) (blue) and adhesion (mean absorbance ± SE) (red) of all strains of *F. columnare* after 48 h of incubation at different carbohydrates. Different letter indicate significant difference ($p < 0.05$). (NAD stands for N-acetyl-D-glucosamine).
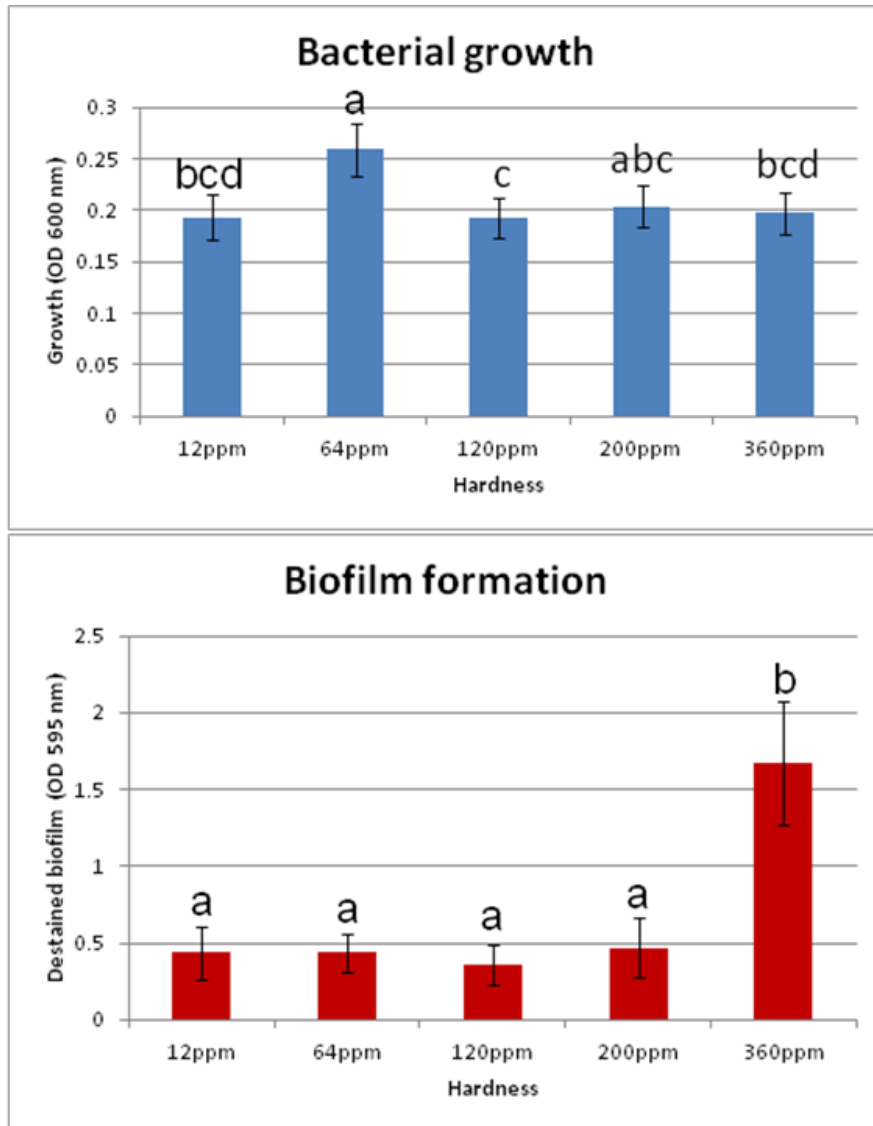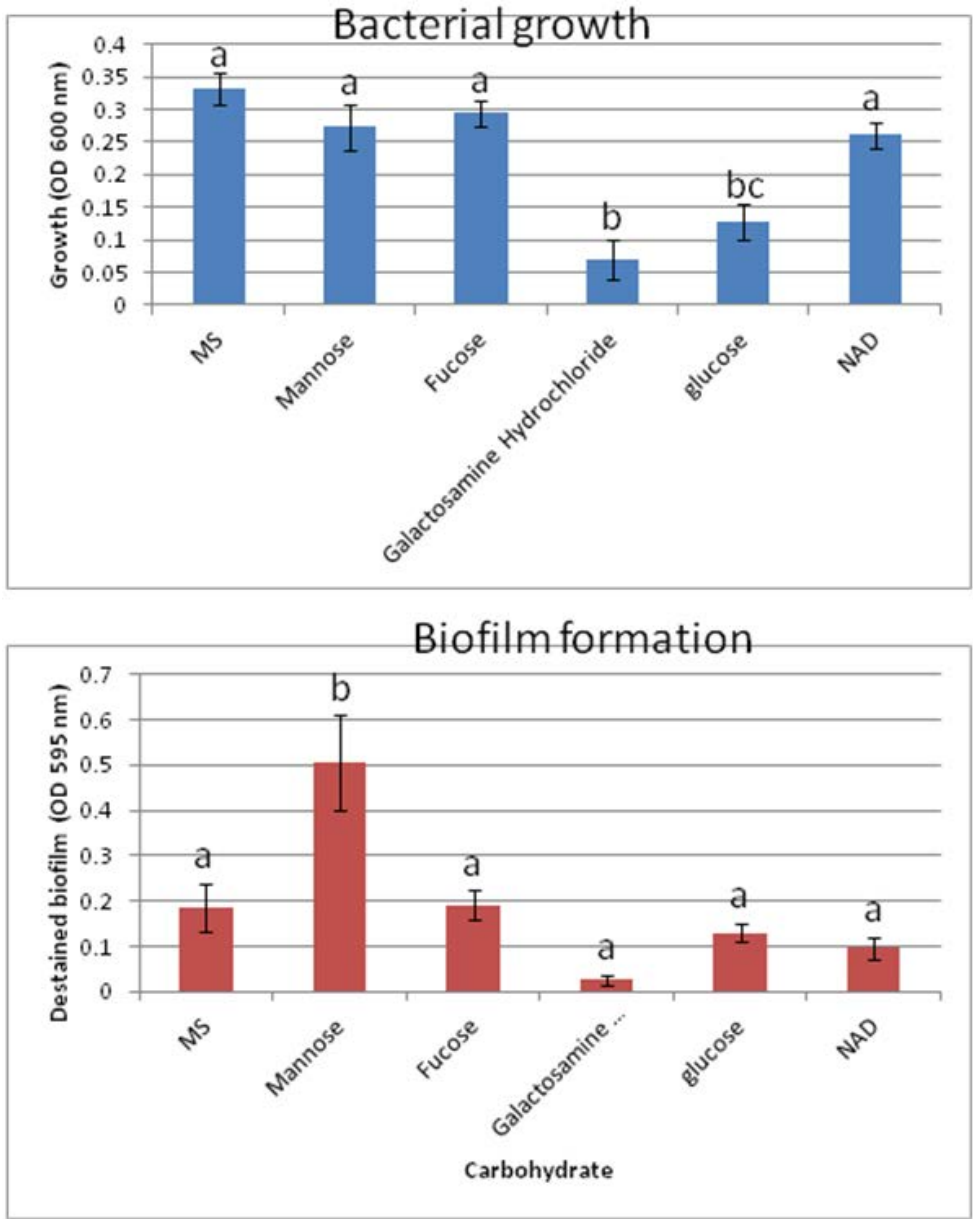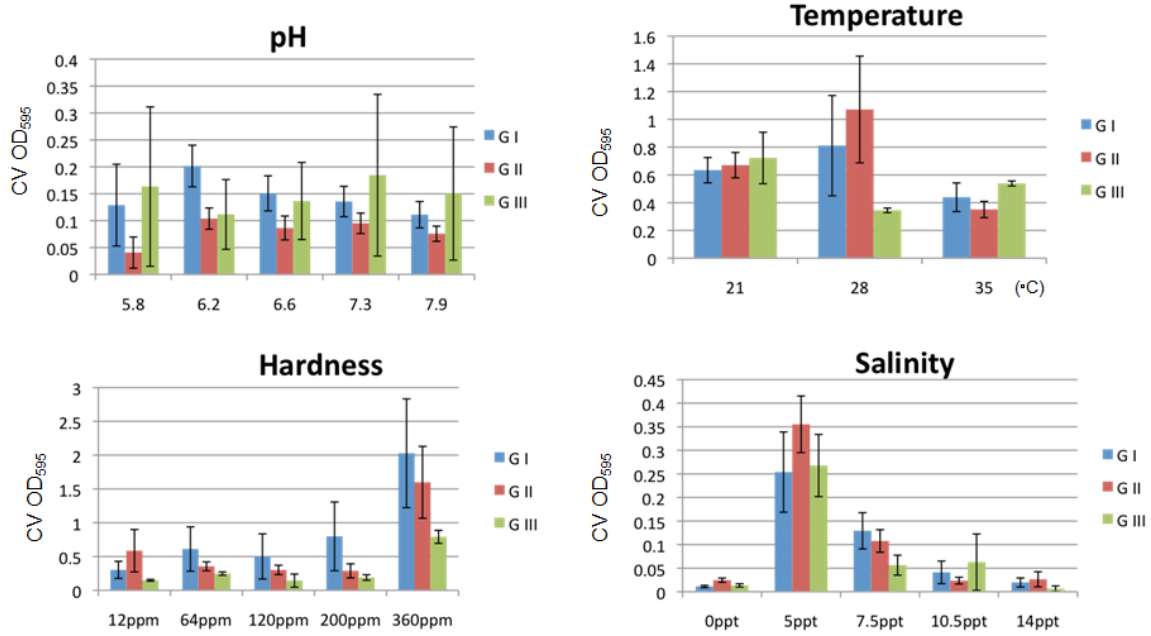
Figure 17. Summary of results on biofilm formation when strains were grouped based on genomovar ascription (G I, G II, and G III stand for genomovars I, II, and III, respectively).

CONCLUSIONS

Biofilm formation of fish pathogen *F. columnare* was characterized using qualitative and quantitative approaches in this work. The main conclusions of my thesis are:

1. *F. columnare* was able to colonize inert surfaces under still and flow liquid conditions.

2. Under static conditions, *F. columnare* attached to glass in 6 h and formed dense biofilms within 48 h in a nutrient-rich microcosm.

3. Water channels and EPS were present in mature biofilms.

4. Metabolically active cells were predominantly located at the center and top of the microcolonies.

5. Fixation protocols affect SEM observation. Osmium tetroxide fixation preserved most of the extracellular organic residues while formalin and ethanol fixation better retained the outer membrane structure of cells.

6. *F. columnare* in biofilms retains its virulence towards channel catfish.

7. Temperature and pH had little effect on biofilm formation, while salinity and, mainly, hardness greatly influenced biofilm formation.

8. Great variability in cell attachment was observed among strains.

9. Genomovar ascription did not significantly impact *in vitro* attachment.

10. Biofilm can serve as reservoir for virulent forms of *F. columnare* and should be minimized in aquaculture facilities.

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