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Flavobacterium columnare in carp (*Cyprinus carpio* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum): antimicrobial susceptibility and interplay with its host

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FLAVOBACTERIUM COLUMNARE IN CARP (*CYPRINUS CARPIO* L.) AND RAINBOW TROUT (*ONCORHYNCHUS MYKISS* WALBAUM): ANTIMICROBIAL SUSCEPTIBILITY AND INTERPLAY WITH ITS HOST

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LIST OF ABBREVIATIONS

ACP	Alterative complement pathway
AHL	*N*-acylhomoserine lactone
AI-2	Auto-inducer 2
$Al_2SI_2O_5(OH)_4$	Kaolin
ALP	Alkaline phosphatase
APES	3-aminopropyl-triethoxysilane
AST	Aspartate aminotransferase
BUN	Blood urea nitrogen
CAMHB	Cation-adjusted Mueller Hinton broth
CIA	Critically important antimicrobials (for veterinary health)
CK	Creatine kinase
CLSI	Clinical and Laboratory Standards Institute
CPM	Cumulative percent mortalities
$CuSO_4$	Copper sulphate
CxB	Female channel catfish crossbred with male blue catfish
dUTP	Deoxyuridine triphosphate
EGC	Eosinophilic granular cells
EMA	Ethidium bromide monoazide
EPS	Extracellular polymeric substances
FAO	Food Agriculture Organization of the United Nations
F. columnare	*Flavobacterium columnare*
GC-MS	Gas chromatography-mass spectrometry
H&E	Hematoxylin and eosin
HV	Highly virulent
iNOS	Inducible nitric oxide synthases
$KMnO_4$	Potassium permanganate
LAMP	Loop-mediated isothermal amplification method
LC-MS	Liquid chromatography-mass spectrometry
LDH	Lactate dehydrogenase
LH-PCR	Length heterogeneity polymerase chain reaction

LPS	Lipopolysaccharide
LSD	Least significant difference
LV	Low virulent
MIC	Minimum inhibitory concentration
OD	Optical density
OIE	World Organisation for Animal Health
OMV	Outer membrane vesicles
PAS/AB	Periodic acid Schiff/alcian blue
PCV	Packed cell volume
PGRP	Peptidoglycan recognition protein
PI	Post inoculation
(q)PCR	(quantitative) Polymerase chain reaction
QS	Quorum sensing
RBL	Rhamnose binding lectin
RFLP	Restriction fragment length polymorphism
R-M system	Restriction-modification system
rpm	Revolutions per minute
RPS	Relative percent survival
RT-PCR	Real-time polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SP	Sampling point
SSCP	Single strand conformation polymorphism
TdT	Terminal deoxynucleotidyl transferase
TEM	Transmission electron microscopy
TSA	Trypticase soy agar
USA	Unites States of America

CHAPTER 1: COLUMNARIS DISEASE IN FISH: A REVIEW WITH EMPHASIS ON BACTERIUM-HOST INTERACTIONS

This chapter is adapted from:

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CHAPTER 1: COLUMNARIS DISEASE IN FISH: A REVIEW WITH EMPHASIS ON BACTERIUM-HOST INTERACTIONS

1.1. THE AGENT

1.1.1. History and taxonomy

Flavobacterium columnare (*F. columnare*), the causative agent of columnaris disease, belongs to the family Flavobacteriaceae (Bernardet et al., 1996; Bernardet et al., 2002; Bernardet and Bowman, 2006). Columnaris disease was first described by Davis (1922) among warm water fishes from the Mississippi River. Although unsuccessful in cultivating the etiological agent, Davis described the disease and reported large numbers of slender, motile bacteria present in the lesions. Upon examining a wet mount preparation of these lesions, column-like structures formed by these bacteria were evident. The organism was hence named *Bacillus columnaris* and the disease elicited columnaris disease. Over the decades the taxonomic status of the pathogen has changed several times since the pioneering work of Davis (1922). In 1944, Ordal and Rucker were the first to isolate the bacterium from a natural outbreak of columnaris disease among sockeye salmon (*Onchorhynchus nerka*). A diluted culture medium was used to grow the bacterium. Based on cellular morphology, they identified the bacterium as a myxobacterium. Organisms classified in the order Myxobacteria are long, thin Gram-negative rods that are motile on agar media by a creeping or flexing motion. They have a life cycle composed of vegetative cells, microcysts (resting cells), and fruiting bodies, or only vegetative cells and microcysts (Bullock et al., 1986). Ordal and Rucker (1944) reported that the myxobacterium from columnaris disease produced both fruiting bodies and microcysts and named the organism *Chondrococcus columnaris*. Garnjobst (1945) assigned the bacterium to the family Cytophagaceae as *Cytophaga columnaris* after isolating a pathogenic bacterium resembling *Chondrococcus columnaris* morphologically, but not producing microcysts. Bernardet and Grimont (1989) reclassified the organism and placed it in the family *Cytophagaceae* and the genus *Flexibacter*, assigning it as *Flexibacter columnaris*. Finally, in 1996, the bacterium received its current name, *Flavobacterium columnare*, based on DNA-rRNA hybridization data and protein and fatty acid profiles (Bernardet et al., 1996). In 1999, the *F. columnare* cluster was subdivided in three genomovars based on differences in 16S rRNA sequences, restriction fragment length polymorphism (RFLP) and DNA-DNA-hybridization (Triyanto and Wakabayashi, 1999). Very recently, five genomovars were described : I, I/II, II, II-B and III (LaFrentz et al., 2014).

1.1.2. Morphology and biochemical characteristics

The morphological and biochemical characteristics of *F. columnare* are summarized in Table 1. For a full biochemical profile of *F. columnare*, the reader is referred to Bernardet and Bowman (2006).

Table 1. Morphological and biochemical characteristics of *F. columnare* (adapted from [Bernardet and Bowman, 2006; Bernardet et al., 2002; Decostere et al., 1998; Decostere et al., 1999; Ourth and Bachinski, 1987])

Characteristic	Description
Growth condition	Strictly aerobic
Gram-stain	Gram-negative
Morphology	Long, slender gliding rods of 4 to 10 μm and 0.3 to 0.5 μm wide. In aging cultures spheroplasts may occur
Capsule	Described to be absent or present depending on the adopted strain
Congo red absorption	Present due to an extracellular galactosamine glycan in the mucus and the production of flexirubin-type pigments
H_2S-production	Present
Degradation of crystalline cellulose	Absent
Degradation of complex acidic polysaccharides of connective tissue	Present

1.1.3. Epidemiology

F. columnare is distributed worldwide in fresh water sources and may infect many different wild and cultured freshwater fish species, such as (but not limited to) catla, carp, channel catfish, goldfish, eels, perch, salmonids and tilapia (Bernardet and Bowman, 2006; Decostere et al., 1998; Figueiredo et al., 2005; Morley and Lewis, 2010; Post 1987; Řehulka and Minařík, 2007; Soto et al., 2008; Suomalainen et al., 2009; Verma and Rathore, 2014). This disease also assails many tropical freshwater aquarium fish (Post, 1987; Decostere et al., 1998). In the channel catfish (*Ictalurus punctatus*) industry in the United States, *F. columnare* is the second most prevalent bacterium, after *Edwardsiella ictaluri*, to cause disease and mortality (Hawke and Thune, 1992; Wagner et al., 2002), with yearly losses estimated at 30 million dollars (Shoemaker et al., 2011). This organism can also be part of the bacterial microbiota of freshwater fish, eggs and the rearing waters the fish live in (Flaherty et al., 1984; Liebert et al., 1984; Barker et al., 1989). Fish may reside in a clinically healthy carrier status harbouring an isolate remaining from a previous outbreak of columnaris disease and in this way act as an infection source for other fish (Fujihara and Nakatani, 1971; Suomalainen et al., 2005). Fujihara and Nakatani (1971) reported that rainbow trout surviving a

F. columnare infection can release up to $5x10^3$ colony forming units/ml/h of viable bacteria into tankwater. The gills were shown to be the major release site of this pathogen. Dead fish would be able to spread the disease at a higher transmission rate compared to living fish (Kunttu et al., 2009).

Several studies have indicated the potential for *F. columnare* to survive for extended periods in water. Survival was demonstrated to be influenced by physical and chemical characteristics of the surrounding water. Fijan (1968) indicated that *F. columnare* can survive up to 16 days at 25°C in hard, alkaline water with a high organic load. Soft water with 10ppm $CaCO_3$, especially when acid or with a low organic content, does not provide a favorable environment for the organism (Fijan, 1968). Chowdhury and Wakabayashi (1990) determined that calcium, magnesium, potassium and sodium ions all are important for long-term survival of *F. columnare* in water. Collins (1970) reported a relationship between eutrophication and the numbers of *F. columnare* in lake water. Ross and Smith (1974) found that survival of *F. columnare* in static, sterile, river water was directly related to temperature, with a higher survival percentage at lower temperatures. The bacterium can keep its infectivity in lake water in laboratory conditions for at least five months (Kunttu et al., 2012). *F. columnare* is also capable of surviving in sterile river mud (Bullock et al., 1986). Apparently, mud slurry often contains sufficient nutrients to maintain viability of *F. columnare* longer than sterile river water. In this case, however, the percentage survival of *F. columnare* seeded into mud seems to be higher at 25°C than at 5°C. Temperatures below 5°C are even detrimental to *F. columnare* cells in mud. *F. columnare* also grows well on particulate fish feed (Wakabayashi, 1991). When surviving outside the host, *F. columnare* can change from a virulent to a less virulent form with an altered colony morphology, probably to save energy (Kunttu et al., 2012). It is suggested that *F. columnare* strains at fish farms originate from environmental waters and that the farm environment and practices may select for virulent strains that cause outbreaks in the farm (Pulkkinen et al., 2010; Kunttu et al., 2012).

1.2. THE DISEASE

1.2.1. Clinical signs, histopathology, ultrastructural features and haematology

F. columnare causes acute to chronic infections and typically affects the gills, the skin and fins. The clinical manifestation of columnaris disease amongst others is dependent on the virulence of the eliciting strain. In a study of Rucker et al. (1953), the strains of low virulence induced slow progressive infections at water temperatures above 21°C and caused massive tissue damage before death occurred. Strains of high virulence caused fulminating infections and killed young salmon (*Salmo salar*) in 12 to 24 h at 20°C. Ordinarily, these fish did not show gross tissue damage at the

time of death (Rucker et al., 1953). The same was found in a study of Pacha and Ordal (1967) and Foscarini (1989). The gross pathology observed in the fish experimentally infected with strains of *F. columnare* of high virulence was usually very limited. Apparently, death occurred before gross external manifestations of the disease appeared. However, some of the last fish to die did show macroscopically visible signs (Pacha and Ordal, 1970). Besides the virulence of the strain being a determinant factor, in coldwater and temperate fish, age also seems to have an important impact on the severity of the clinical signs. In young fish, the disease develops acutely and mostly damages the gills (Figure 1).

Figure 1. *F. columnare* elicited gill lesions (arrow) in rainbow trout (*Oncorhynchus mykiss*) fry (operculum removed). In young fish, the disease is mostly acute and the gill is the major site of damage. The lesions are exhibited by pale necrotic areas. Bar = 1 cm.

In adults, the disease may adopt an acute, subacute or chronic course. When the disease course is acute or subacute in adult fish, yellowish areas of necrotic tissue can appear in the gills ultimately resulting in complete gill destruction (Figure 2) (Bernardet and Bowman, 2006; Decostere, 2002; Pacha and Ordal, 1967).

Figure 2. Gill lesions in a shubunkin (*Carassius auratus*) (operculum removed) caused by *F. columnare*. Yellowish-white areas of degeneration are visible in the ventral part of the filaments of the first gill arch (arrow). When the *F. columnare* infection spreads rapidly throughout the gill lamellae, the fish may die in a short period of time without any other apparent lesions. Respiratory distress, caused by damage to the gills, appears to be the cause of death. Bar = 1 cm.

In chronic cases, it takes longer before gill damage appears and skin lesions may develop as well (Bernardet and Bowman, 2006; Decostere, 2002; Pacha and Ordal, 1967). On the body, small lesions start as areas of pale discolorations of the skin, which usually are surrounded by a zone with a distinct reddish tinge. This mostly begins at the base of the dorsal fin. Fin deterioration then occurs, starting from the lesion at the base of the fin and progressing to the outer edge, the opposite to normal fin rot. The lesions then begin extending laterally from their common location at the base of the dorsal fin to encircle the fish resembling a "saddleback". The disease therefore is referred to as "saddleback disease" (Figure 3) (Bernardet and Bowman, 2006; Morrison et al., 1981; Pacha and Ordal, 1967). Finrot is also often present (Decostere and Haesebrouck, 1999; Bernardet and Bowman, 2006).

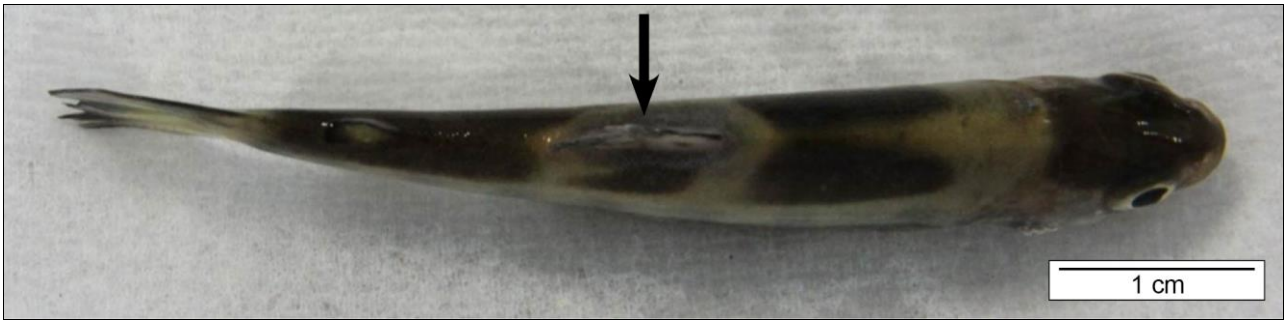

Figure 3. *F. columnare* induced a saddleback lesion (arrow) in rainbow trout fry. The lesion is visible as a discolouration starting around its common location at the base of the dorsal fin and extending laterally to encircle the fish resembling a saddle. Hence, the descriptive term "saddleback" is often used and the disease is denoted as "saddleback disease". Bar = 1 cm.

In rainbow trout, the area around the adipose fin may become dark and show erosions. These lesions expand to the peduncle, hence the name "peduncle disease" (Bernardet and Bowman, 2006). The lesions may progress cranially and caudally and even into the deeper skin layers, exposing the musculature leading to deep ulcers (Figure 4, 5) (Bernardet and Bowman, 2006; Decostere et al., 2002). The lesions typically are covered with yellowish-white mucus (Bernardet and Bowman, 2006).

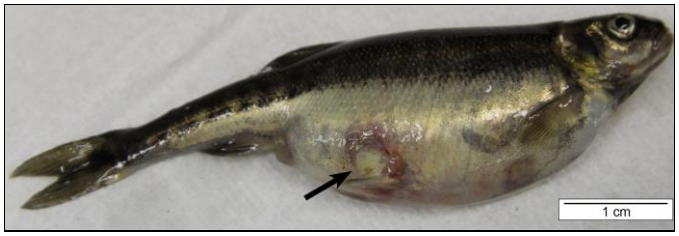

Figure 4. Skin ulceration (arrow) in a minnow (*Phoxinus phoxinus*) caused by *F. columnare*. The lesion has progressed into deeper skin layers, exposing the musculature. The edge of the ulceration displays a distinct reddish tinge and its centre is covered with yellowish-white mucus. Bar = 1 cm.

Figure 5. Tail fin erosion in a minnow caused by *F. columnare*. Columnaris disease led to complete disappearance of the upper half of the fin and exposing the underlying musculature (arrow). Bar = 500 µm.

Ulceration of the oral mucosa also occurs, resulting in mouth rot. These mouth lesions are more lethal than are the skin lesions, since the painful oral lesions render the fish anorectic and lead to death due to starvation. Moreover, the disease spreads easily to the mandible and the maxilla. Secondary infections with fungi or other bacteria may deteriorate the situation and can be seen together with the filamentous bacteria (Ferguson, 2006). In tropical fish, this clinical sign led to the disease being termed "cotton wool disease" or "mouth fungus" (Bernardet and Bowman, 2006). Finrot can also be present in tropical fish (Decostere and Haesebrouck, 1999). Lesions can be restricted to local skin discoloration, with or without ulceration, and degeneration of underlying muscle fibers (Bernardet and Bowman, 2006; Decostere et al., 1998; Michel et al., 2002).

When the skin or gills are abraded, bacteria can enter the bloodstream and cause systemic infections (Wood, 1968). However, Hawke and Thune (1992) have isolated *F. columnare* from internal organs without any external lesions appearing. Foscarini (1989) described that the pathological changes to the gill structure caused by columnaris disease go hand in hand with cardiac alterations. The first day after infection, bradycardia was noticed with the formation of hyperplastic gill lesions. Degenerative processes of the lamellae in the following days resulted in a compensatory tachycardia. This research suggests that the interaction between the impaired gill vascular blood circulation and the cardiac changes could result in the death of the fish (Foscarini, 1989).

Light microscopical examination of the affected gill tissue reveals the disappearance of the normal structure of primary and secondary filaments (Decostere et al., 1997; Ferguson, 2006; Foscarini, 1989; Pacha and Ordal, 1967). In the initial phase, proliferation of epithelial cells of the gill filaments may be accompanied by an increase of mucous cells (Foscarini, 1989). The proliferating tissue can occlude the space between adjacent gill lamellae. In more advanced stages, the occlusion can be total causing the gill lamellae to be completely surrounded by the propagating tissue. Congestion of gill lamellae occurs due to accumulation of blood masses and inflammatory cell infiltration can be noticed. Edema causes lifting of the surface epithelium of gill lamellae from the underlying capillary bed. In more advanced stages of the disease, fusion of gill lamellae and/ or gill filaments appears (Decostere et al., 1997; Ferguson, 2006; Foscarini, 1989; Pacha and Ordal, 1967). Complete clubbing of gill filaments can finally result in circulatory failure and extensive internal hemorrhage (Foscarini, 1989). However, a detailed description of the chronological changes following a *F. columnare* infection in terms of type and extensiveness of the gill lesions and histological alterations in the various gill cell types, viz. epithelial, goblet and chloride cells, is lacking.

Columnaris disease may cause acute ulcerative dermatitis extending into the hypodermis and the muscle. Waterlogging can be present. The latter appears when the osmotic barrier is broken and thus forces water into the tissues, leading to severe dermal edema. Rupture of pigment cells with the loss of melanocytes can also be seen. Columns or hay-stack-like aggregates of bacteria can be gathered between dermal and collagen fibers. The typical long and slender bacterial cells can easily be noted upon inspecting hematoxylin and eosin (H&E) or Giemsa stained sections from affected tissue where they appear bluish-purple and blue, respectively (Ferguson, 2006). When all these changes occur rapidly, they may proceed to severe necrosis and sloughing of the epidermis (Ferguson, 2006; Pacha and Ordal, 1967).

Scanning electron microscopical (SEM) pictures of affected gill tissue reveal the presence of rod-shaped bacterial cells, approximately 0.3-0.5 µm wide and 3-10 µm long. These long, thin bacteria adhere on the surface of the gills and appear to be aggregated rather than evenly distributed across the gill epithelium (Decostere et al., 1999a; Olivares-Fuster et al., 2011). Transmission electron microscopic (TEM) examination of gill tissue shows long, slender bacteria in close contact with the gill tissue (Decostere et al., 1999a). Bullard et al. (2011) were the first to describe the ultrastructural features of saddleback lesions associated with experimental infections of *F. columnare* in channel catfish and zebrafish (*Danio rerio*) using SEM. Channel catfish skin lesion samples had margins typified by shed epidermal cells and lesion centers that exhibited a multitude of rod-shaped bacterial cells, approximately 3-10 µm long and 0.3-0.5 µm wide, intermixed with cellular debris. Zebrafish skin lesion samples displayed a multitude of rod-shaped bacterial cells and exhibited comparable ultrastructural changes. Scales were missing or, when present, denuded of epidermis. However, detailed descriptions of the bacterial cell organization and the evolvement and severity of lesions in the gill tissue using ultrastructural examination are still missing and could further elucidate possible virulence factors involved in the pathogenesis of *F. columnare* infections.

In a cutaneous columnaris challenge model in koi carp, significant changes in blood parameters were observed in the infected fish. For the haematologic parameters, a significant decrease was noted in Packed Cell Volume (PCV), hemoglobin concentration, red blood cell count, mean corpuscular volume and absolute lymphocyte counts. As for the biochemical parameters, marked hyponatremia, hypochloridemia and hyperglycemia were observed. Calcium and magnesium levels dropped only slightly and total serum protein and albumin-like protein concentrations decreased mildly. Alkaline phosphatase (ALP), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and creatine kinase (CK) secretions were significantly increased (Tripathi et al. 2005). Blood parameters from brook trout suffering from an acute, natural outbreak of *F. columnare* also revealed anemia, but levels for mean corpuscular volume and mean corpuscular haemoglobin were higher. Total protein levels fell much below physiological parameters in infected fish. Calcium concentrations reduced significantly. Blood Urea Nitrogen (BUN) measurements were much higher compared to normal levels. The catalytic activity of AST, alanine aminotransferases and LDH reached multiples of normal values. Contrary, ALP concentrations decreased. Hypoglycemia was noted. No data were shown on sodium or chlorine (Řehulka and Minařík, 2007).

1.2.2. Diagnosis

Timely detection of this pathogenic agent is important to prevent its spreading and to reduce the economic loss to fish farmers.

The isolation of *F. columnare* is possible from external lesions, provided that the samples are taken from the edge of recent lesions (Bernardet and Bowman, 2006). *F. columnare* requires low nutrient media. No growth of *F. columnare* is reported on trypticase soy agar (TSA), nutrient agar or Marine 2216 agar (Bernardet and Bowman, 2006). In 1944, *F. columnare* was first cultured on Cytophaga agar, a nutrient poor medium (Ordal and Rucker, 1944). Since then, several media, including Shieh (Shieh, 1980), modified Shieh (Song et al., 1988) and TYES (Holt, 1988) have been developed in an attempt to improve growth of the bacterium. Growth does not occur in media that contain NaCl concentrations of over 0.5% or that have a pH lower than 6 (Bernardet and Bowman, 2006). Depending on the strain, the bacterium grows between 15 and 37°C (Decostere et al., 1998; Triyanto and Wakabayashi, 1999), with an optimal growth occurring between 25°C and 30°C. Colonies appear after 24 to 48 hours of incubation (Decostere et al., 1998). Glucose does not improve growth. Considering that *F. columnare* is easily overgrown by contaminating bacteria, selective media have been developed based on inherent resistance of *F. columnare* to different antimicrobial agents, including polymyxin and neomycin (Fijan, 1968; Bullock et al., 1986; Bernardet and Grimont, 1989). Decostere et al. (1997) added 1µg/ml tobramycin to modified Shieh medium, demonstrating this to be an effective selective supplement for isolating *F. columnare.*

F. columnare largely displays two colony types on solid media; smooth and rhizoid (Bader et al., 2005). Kunttu et al. (2011) characterized a third, rough colony morphology variant. The rhizoid colony variants were assigned virulent and moderately adherent, the non-rhizoid rough colony variants non-virulent and highly adherent, and the smooth colony variants non-virulent and poorly adherent (Kunttu et al., 2011). Colonies of *F. columnare* are notorious for their sometimes strong adherence to the agar. This trait is also exhibited in broth cultures where yellow, filamentous clumps of bacterial cells can form a thick ring at the surface of a glass recipient (Bernardet, 1989; Decostere et al., 1998). Adherence may be lost after several subcultures. Colonies can be recognized by their distinctive yellow pigmentation. The yellow color is due to the production of flexirubin pigments (Bernardet and Bowman, 2006). A method to identify the typical protein profile of *F. columnare* is whole-cell protein analysis (Bernardet et al., 1996). Genomovar ascription has been performed using 16S-RFLP to divide *F. columnare* into genomovars (Triyanto and Wakabayashi, 1999). Compared to RFLP, single strand conformation polymorphism (SSCP) would have an improved resolution power in the study of intraspecies diversity in *F. columnare* (Olivares-Fuster et al., 2007a). Polymerase chain reaction (PCR) has gained interest for definitive identification *F. columnare* based

on the selective amplification of the 16S ribosomal RNA gene using species-specific primers (Darwish et al., 2004; Welker et al., 2005). This technique gives a conclusive identification of the organism within a few hours and eliminates the need for biochemical testing which is laborious and sometimes inconclusive (Darwish et al., 2004).

Besides isolation, other methods may be used for detecting this pathogen. These include serological methods such as the enzyme-linked immunosorbent assay (Shoemaker et al., 2003b) and the fluorescent antibody test (Huh and Wakabayashi, 1987, Panangala et al., 2006; Speare et al., 1995). Both have proven to be efficient and rapid for diagnosing columnaris disease. A loop-mediated isothermal amplification method (LAMP) for rapid detection of *F. columnare* from infected fish organs (gills, skin and head kidneys) was established in channel catfish (Yeh et al., 2006). PCR may also be adopted, having the advantage of being able to detect very low levels of *F. columnare* (Darwish et al., 2004; Welker et al., 2005). In addition, Panangala et al. (2007) have developed a TaqMan-based real-time PCR targeting a 113 bp nucleotide region of the chondroitin AC lyase gene of *F. columnare*. This PCR is specific, sensitive and reproducible for the detection and quantitation of *F. columnare* in tissues (blood, gills and kidney) of infected fish. Moreover, real-time PCR-based methods are distinctively more advantageous than conventional PCR in that they eliminate the need for detection of amplified products by gel electrophoresis thus reducing costs, time and labour (Panangala et al., 2007). Length heterogeneity PCR (LH-PCR) is based on comparing naturally varying lengths of 16S rDNA PCR products between bacterial groups (Suzuki et al., 1998; Tiirola et al., 2003). LH-PCR has also proven effective in detecting *F. columnare* in fish tissue (Suomalainen et al., 2005a).

1.3. PATHOGENESIS

Various reports have noted the difference in virulence among *F. columnare* strains (Bernardet and Bowman, 2006; Dalsgaard, 1993; Decostere et al., 1998, 1999a, b, c; Klesius et al., 2008, 2010; Kunttu et al., 2010; Pacha and Ordal, 1967, Suomalainen et al., 2006). In the last decade, various studies have attempted to elucidate the pathogenesis of columnaris disease. Although significant progress has been made, still many questions remain unanswered on how this pathogenic organism elicits disease and information concerning the bacteriological events preceding disease and death is scarce. Li et al. (2012) related this lack of knowledge to the absence of an efficient molecular manipulation system for *F. columnare*, especially a plasmid-based inframe knockout system, despite two recent reports on the establishment of genetic manipulation systems for the bacterium (Staroscik et al., 2008; Zhang et al., 2012). Li et al. (2012) identified the type I restriction-modification system

(R-M system) in *F. columnare* to improve electroporation efficiency and suggested that it would be of significant interest to examine the composition and diversity of R-M systems in strains of *F. columnare* in order to set up a suitable genetic manipulation systems for the bacterium. Only very recently, the full genome of *F. columnare* ATCC 49512 was sequenced (Tekedar et al., 2012).

1.3.1. Colonization

The colonization of the fish's tissue is to be regarded as a complex multistep process which can be subdivided into the stages of attraction, adhesion and aggregation, requiring a step-by-step analysis. The exact factors mediating colonization have however not yet received the full attention they merit and to date remain largely unidentified.

The research group of Klesius et al. (2008) demonstrated by means of the traditional capillary tube method that the mucus from the skin and gills of catfish promotes chemotaxis of *F. columnare* (Klesius et al., 2008). The gliding motility of this bacterium is well known (Bernardet and Bowman, 2006; Decostere et al., 1997, 1998). Indeed, the observation of a drop of bacteria grown in broth under a phase contrast microscope shows the slowly forward and backward gliding of *F. columnare*. Pate and Ordal (1967) described that the location of fibrillar structures spanning the gap between the outer membrane and the mucopeptide layer, might play a role in the gliding motility of the *F. columnare* bacterial cells. Although Klesius et al. (2008) acknowledged that they were not able to fully define the role that chemotaxis plays in the virulence of *F. columnare*, the chemotactic response of the more virulent genomovar ll isolates suggested that chemotaxis could be associated with virulence. LaFrentz and Klesius (2009) developed a culture independent method to quantify the chemotactic response of *F. columnare* to skin mucus using blind-well chemotaxis chambers generating similar results as stated above (Klesius et al., 2008). At least three carbohydrate-binding receptors (D-mannose, D-glucose and *N*-acetyl-D-glucosamine) associated with the capsule of *F. columnare* might be involved in chemotactic responses (Klesius et al., 2010). Gliding motility gene *gld*H was found to be significantly ($p < 0.001$) upregulated in *F. columnare* as soon as five minutes post-exposure to catfish mucus. When pretreated with D-mannose, there was no upregulation of gliding motility genes (Klesius et al., 2010). Sebastião et al. (2014) very recently stated that the carbohydrate composition of extracellular polymeric substances (EPS, forming an important part of the biofilm), predominantly consisted of glucose and glucuronic acid, with traces of galactose, mannose and rhamnose. Polysaccharides isolated from *F. columnare* bacterial culture filtrates were all heteropolysaccharides, with varying proportions of neutral sugars and uronic acids.

The occurrence of capsules on *F. columnare* cells was not directly related to biofilm formation according to this latter research group (Sebastião et al., 2014)

The ability to adhere is a prerequisite for the successful colonization of the host tissue. Decostere et al. (1998, 1999a, c) performed a series of both *in vivo* and *in vitro* experiments and found that a high virulence strain adhered more readily to the gill tissue of black mollies (*Poecilia reticulata*) than did the low virulence strain. This research group underscored that the adhesion of *F. columnare* to the gill tissue constitutes an important step in the pathogenesis of columnaris disease. Bader et al. (2005) adopted an adhesion defective mutant of *F. columnare* in immersion challenge trials and found that the mortality was reduced with 75% and occurred 24 h later compared to the strains that still possessed the adhesion capacities, confirming the findings of the former research group. In channel catfish genomovar II is considered to be more virulent than genomovar I (Olivares-Fuster et al., 2007a; Shoemaker et al., 2008). Challenge of rainbow trout with genomovar I and II isolates of *F. columnare* demonstrated a difference in the cumulative percent mortalities (CPM), with the genomovar II isolates inducing significantly higher CPM (LaFrentz et al., 2012). The correlation between virulence and genomovar in *F. columnare* is not always present though, as recently shown by Shoemaker and LaFrentz (2014) in tilapia and Evenhuis et al. (2014) in rainbow trout fry. Olivares-Fuster et al. (2011) compared adhesion of *F. columnare* genomovar I and II strains to the skin and gill of channel catfish and the gill of zebrafish (*Danio rerio*). At 0.5 h post-challenge, both strains adhered to the gill of channel catfish at comparable levels, but significant differences in adhesion were found in later datapoints over time. They concluded that particular strains of *F. columnare* exhibit different levels of adhesion to their fish hosts and that adhesion to fish tissues is not sufficient to cause columnaris disease. The same statement was previously made by Klesius et al. (2008).

Kunttu et al. (2009a, 2011) have shown that colony morphology affects the adhesion capacity of *F. columnare* to polystyrene and put forward the hypothesis that there is a link between virulence and rhizoid colony morphology. The formation of different colony morphologies could be caused by changes in the cell surface components of the bacteria. It was also found that *F. columnare* changes colony morphology during experimental long-term storage in lake water, indicating the importance of different colony morphologies to bacterial survival (Kunttu et al., 2009a; Kunttu et al., 2010; Arias et al., 2012a). The rhizoid colony morphotype would be associated with virulence, the rough one with starvation and phages (Sundberg et al., 2014; Zhang et al., 2014). Arias et al. (2012a) noted that long-term *in vitro* starvation appears to decrease cell fitness and resulted in loss of virulence. *F. columnare* bacteria developed a unique cell configuration resulting in coiling of the bacteria. The authors suggested that this state should not be regarded as degenerative, but rather as an active

adaptation to the lack of nutrients allowing the bacteria to remain viable in the water. However, whether adhesion impairment was the cause of the observed virulence loss was not investigated. In the same *in vitro* research, viable bacteria were observed to form outer membrane vesicles (OMV) (Arias et al., 2012a). Laanto et al. (2014) very recently isolated OmpA, a virulence factor often linked with adhesion and invasion, and SprF, a protein connected with gliding motility and the protein secretion of flavobacteria, from these OMV retrieved from *in vitro F. columnare* cultures, strongly suggesting their role in adhesion and hence as virulence factor aiding in *F. columnare* colonization. Whether these OMV are also encountered *in vivo* in proximity to host tissue and how and to what extent these may be regarded as a virulence marker, remains to be demonstrated.

F. columnare produces two types of slime. The first one is an acidic polysaccharide and is made visible by ruthenium red staining. Another type of slime is a basic, partially acetylated polygalactosamine, which cannot be stained with ruthenium red (Johnson and Chilton, 1966). Pate and Ordal (1967) described a capsular material which coated the surface of the bacterial cell that could be stained with ruthenium red. They stated that the ruthenium red-positive material was probably an acid mucopolysaccharide that might be involved in the adhesive properties of the cells. The exact role of the mucopolysaccharides was however not further illuminated in this study. Decostere et al. (1999c) demonstrated that the adherence capabilities of a highly virulent *F. columnare* strain to the gill were significantly reduced following treatment of the bacteria with sodium metaperiodate or incubating them with D-glucose, *N*-actetyl-D-glucosamine, D-galactose and D-sucrose. Treatment with pronase or trypsin did not cause any significant inhibition of adhesion (Decostere et al., 1999c). The same research group noted that the high virulence strain had a thick capsule with a regular and dense appearance, whereas the capsule of the low virulent strain was much thinner. This made them to speculate that a lectin-like carbohydrate substance incorporated in the capsule might be partially responsible for the adhesion to the gill tissue (Decostere et al., 1999c).

Sun et al. (2012) conducted the first transcriptomic profiling of host responses to columnaris disease following an experimental immersion challenge by using illumina-based RNA-sequencing expression profiling. A rhamnose-binding lectin (RBL) was detected as by far the most highly up-regulated gene observed in their differentially expressed set, with expression increasing 105-fold by 4 h following infection. This upregulation dramatically decreased at the later measured timepoints (24 h and 48 h), suggesting the importance of this gene during early infection events (Sun et al., 2012). Peatman et al. (2013) confirm susceptible fish to show high expression levels of RBL. 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 (Tateno et

al., 2002). This expression pattern is consistent with the finding of *F. columnare* bacterial cell aggregates around goblet cells in common carp (Decostere et al., 1999c) and catfish (Olivares-Fuster et al., 2011) following experimental challenge with a highly virulent strain (Sun et al., 2012). Beck et al. (2012) identified two distinct catfish families with differential susceptibilities to columnaris disease, with the one family completely resistant and the other susceptible. In the susceptible family, an acute and robust upregulation in catfish RBL was observed following challenge that persisted for at least 24 h. After exposure of the catfish 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. Moreover, RBL expression was upregulated in fish fasted for 7 d (as compared to fish fed to satiation daily), yet expression levels returned to those of satiated fish within 4 h after re-feeding. These findings highlight putative roles for RBL in the context of columnaris disease and reveal new aspects linking RBL regulation to feed availability (Beck et al., 2012). Further studies are needed to further pinpoint the (various) factor(s) responsible for mediating adhesion to the fish tissue.

The adhesion to the fish tissue was shown to be impacted by various environmental parameters. Using a gill perfusion model (Decostere et al., 2002), Decostere et al. (1999b) noted that the adhesion of a highly virulent strain to the gill tissue was enhanced by a number of factors. These were immersion of the gill in divalent ion water, presence of nitrite or organic matter and high temperatures. The positive effect of high temperatures on the adhesion was also demonstrated by Kunttu et al. (2011). Adhesion seems to decrease *in vitro* as salinity goes up (Altinok and Grizzle, 2001). Expression of adhesins by bacteria is regulated on two different levels. One is directed by environmental sensing and transcription of specific regulatory elements and the other is a random switching on and off of adhesion genes by submission of the bacterial population to unpredictable environmental conditions (Ofek et al, 2003a). If and how both phenomena are established in *F. columnare* remains to be elucidated.

An aggregative adhesion pattern of a highly virulent *F. columnare* strain onto gill tissue is a distinct feature in both *in vivo* and organ culture experiments (Decostere et al., 2002; Decostere et al., 1999a, 1999b; Foscarini, 1989; Olivares-Fuster et al., 2011; Pacha and Ordal, 1967). This results in an irregular gill surface covered by a thick mat consisting of numerous clumps of *F. columnare* bacterial cells, most likely impeding oxygen uptake and causing death of the fish. These microcolonies are not observed when a low virulence strain is used. Biofilm formation capacity was demonstrated before *in vitro* for *F. columnare* (Cai et al., 2013; Staroscik and Nelson, 2008). Cai et al. (2013) investigated biofilm formation using light microscopy, confocal laser

scanning microscopy, and scanning electron microscopy. They showed that *F. columnare* was able to attach to and colonize inert surfaces by producing biofilm. Surface colonization started within 6 h postinoculation, and microcolonies were observed within 24 h. Extracellular polysaccharide substances and water channels were observed in mature biofilms (24 to 48 h). The importance of biofilm in virulence was moreover confirmed by cutaneous inoculation of channel catfish fingerlings with mature biofilm (Cai et al., 2013). These features point towards biofilm formation being an important stage in the pathogenesis of *columnaris* disease, warranting further investigation especially at the level of the gills, a major target organ for *F. columnare* for which data on the interplay largely are unavailable.

The developmental switch to the biofilm state is commonly regulated by quorum sensing (QS). Quorum sensing is a mechanism of gene regulation in which bacteria coordinate the expression of certain genes in response to the presence or absence of small signal molecules. In many Gram-negative bacteria, the signal molecule is an *N*-acylhomoserine lactone (AHL) (Romero et al., 2009). Additionally, a signaling molecule known as autoinducer-2 (AI-2) may also be employed (Camilli and Bassler, 2006). Wagner-Döbler et al. (2005) found short-chain AHL-type activity in *Flavobacterium* sp., but no AHL-presence could be confirmed using gas chromatography-mass spectrometry (GC-MS). Using liquid chromatography-mass spectrometry (LC-MS), Romero et al. (2009) described the presence of short-type AHL activity in the culture media of nine *Tenacibaculum maritimum* strains, biofilm-forming members of the phylum Bacteroidetes, formerly referred to as "Cytophaga-Flavobacterium-Bacteroides*"* group. To our knowledge, so far, QS by AHL or AI-2 has not yet been demonstrated in *F. columnare*.

1.3.2. Exotoxins, bacteriocins and endotoxins

It is known that polysaccharide degradation in combination with the secretion of various extracellular enzymes participate in the destruction of skin, muscle and gill tissue (Bertolini and Rehovec, 1992; Nomura and Ohara, 1994; Bernardet and Bowman, 2006), enhancing pathogenicity. In culture, *F. columnare* produces an enzyme that degrades chondroitin sulfates A and C and hyaluronic acid, the complex polysaccharides of connective tissue. This so-called chondroitin AC lyase acts specifically on a group of acidic mucopolysaccharides found primarily in animal connective tissue (Dalsgaard, 1993). No correlations were found between the host origin, geographic distribution, and amount of enzyme produced by different isolates (Griffin, 1991). AC lyase is alleged to play a role in the virulence of *F. columnare* (Suomalainen et al., 2006; Kunttu et al., 2011). Though high AC lyase activity solely would not be enough to induce virulence in

F. columnare strains, both high AC lyase activity and gliding motility of the bacteria would be needed for *F. columnare* to be virulent (Kunttu et al., 2011). Proteases also contribute to damaging the tissue or enhancing invasive processes (Dalsgaard, 1993). Newton et al. (1997) isolated and partially characterized proteases of 23 isolates of *F. columnare* derived primarily from channel catfish raised in the southeastern United States. The bacterial isolates were divided into two groups according to the apparent molecular masses of proteases after zymographic resolution by non-reducing, non-denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with gelatin as the protease substrate. Isolates of group one produced two proteases with apparent molecular masses of 53.5 and 58 kilodaltons. The isolates of group two revealed three proteases with apparent molecular masses of 44.5, 48 and 59.5 kilodaltons. All isolates degraded gelatin and casein. Seven out of 23 isolates degraded elastin. More protease was produced in a medium with low nutrients and salts than in media with higher concentrations of nutrients. Moreover, a sharp increase was seen in protease production during the first 24 h of incubation and these levels dropped only slightly in the remaining days of the experiment (Newton et al., 1997).

Bacteria not only need to enter the host tissue, they also need to eliminate competitive bacteria. 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 (Anacker and Ordal, 1959). It was postulated that cells of *F. columnare* also possess multiple specific receptors for the bacteriocins, and, consequently, the cells are sensitive only to those bacteriocins for which the cell possesses receptors (Anacker and Ordal, 1959). Antagonism of *Pseudomonas sp.* MT5 against *F. columnare* bacteria was found to be very strong in agar assays (Tiirola et al., 2002). However, antagonistic baths of the *Pseudomonas* bacterial strain could not yet prevent nor treat an *F. columnare* infection following experimental challenge (Suomalainen et al., 2005).

Differences in lipopolysaccharide (LPS) composition between highly and low virulent strains of *F. columnare* retrieved from channel catfish have been detected (Zhang et al., 2006). Analysis of LPS by immunoblotting revealed that an avirulent mutant of a *F. columnare* isolate lacked the high molecular weight components of LPS present in virulent isolates. Based on these differences of LPS and total protein profiles, the research group was able to discriminate the attenuated mutant from other *F. columnare* strains (Zhang et al. 2006). Kunttu et al. (2011) determined LPS-profiles of different colony morphology variants of *F. columnare* in rainbow trout. Colony morphology variants of the same strain produced a similar single LPS band. However, there were size differences between different strains. Both research groups used different LPS extraction and detection methods, rendering comparison of the obtained results impossible (Kunttu et al., 2011).

1.3.3. Interaction with the fish immune system

When encountering an infection, the first system activated is the innate immune system. It is assumed that the surface mucus layer, as a first physical-immunological barrier, plays an important adhesive role and that it is part of the innate host resistance of fish to disease (Olivares-Fuster et al., 2011). Antibacterial characteristics of the fish slime against *F. columnare* have been demonstrated. In an experimental challenge trial of cutaneous columnaris disease in koi carp, lesions were noted on locations where mucus had been removed. After incubating *F. columnare* inoculated agar plates, a lower number of colonies was counted on plates where mucus was added. Fluorescent microscopy of a stain-based bacterial viability assay also revealed a higher number of dead bacteria in *F. columnare* cultured with mucus (Tripathi et al., 2005). Staroscik and Nelson (2008) compared growth, biofilm formation, extracellular protease production and changes in protein expression of a highly virulent *F. columnare* strain cultured in media supplemented with juvenile Atlantic salmon (*Salmo salar* L.) skin mucus with the same media without mucus. Interestingly and in contrast to the reasoning resulting from the above mentioned studies, salmon surface mucus promoted the growth of *F. columnare*, induced cells to grow as a biofilm and increased extracellular protease activity (Staroscik and Nelson, 2008). This might indicate that skin mucus of different fish species responds differently to the bacteria, or that it is the *F. columnare* strain which is critical in determining the antibacterial capacities of the mucus.

The fish skin in itself also forms a barrier against pathogens. Aranishi et al. (1998) immersed Japanese eel (*Anguilla japonica*) in a *F. columnare* suspension to monitor dermal nonspecific stress responses. They found that cathepsins B and L activities in the infected fishes increased more than 1.5-fold over their initial values over a 48 h period, along with a 4.5-fold increase in bacteriolytic activity. These cathepsins likely participate in bacteriolysis associated with Japanese eel skin and their activities may represent an important nonspecific response of eels (Aranishi *et al.*, 1998).

Some studies reveal that *F. columnare* might be able to avoid parts of the immune system. In a study by Ourth and Bachinski (1987), the catfish alternative complement pathway (ACP) was inhibited by large amounts of sialic acid contained by Gram-negative bacterial pathogens, including *F. columnare*. Sialic acid seemed to be the determining factor for the pathogenicity of *F. columnare*, as very little or no bactericidal activity was produced against this bacterium by the catfish ACP. Furthermore, the latter greatly increased after removal of sialic acid with neuraminidase (Ourth and Bachinski, 1987). Another recurrent feature in *F. columnare* infections, is the lack of an inflammatory response as observed upon inspecting affected tissues microscopically. This resulted in the hypothesis that *F. columnare* triggers the endogenous programmed cell death machinery of immune cells to evade the immune system. Do Vale et al. (2003) have already proven that apoptosis

can be a very powerful pathogenic strategy by inducing lysis of phagocytic cells. Sun et al. (2012) speculated that negative regulation of one of the central innate immune signaling pathways NF-κB, may be the result of immune evasion or manipulation by *F. columnare* via secreted toxins. Furthermore, high levels of inducible nitric oxide synthases (iNOS), apoptotic-promoting interferon (IFN) and other members of oxidative stress responses and apoptotic pathways, like caspase were observed following a *F. columnare* infection (Sun et al., 2012). This could explain why hardly any inflammatory cells appear in early infections with *F. columnare* as was described by Morrison et al. (1981). Peatman et al. (2013) also found that a number of critical innate immune components, including iNOS2b, IL-8 and TNF-alpha to be constitutively higher in resistant catfish gill. Sun et al. (2014) very recently suggested peptidoglycan recognition proteins (PGRPs) to be involved in the innate immune response of catfish after infection with *F. columnare*, as shown by their upregulation in the gills after experimental infection. PGRPs could recognize bacterial cell walls (peptidoglycan) and hence activate the innate immune response (Sun et al., 2014).

Ourth and Wilson (1982) demonstrated that *F. columnare* is resistant to the bactericidal action of serum of non-immunized catfish via the ACP. Ourth and Bachinski (1987a) confirmed this finding, but also demonstrated that the classical, antibody-mediated complement pathway is highly effective in killing *F. columnare*. The virulence of *F. columnare* can also be influenced by transferrin (Kuo et al., 1981). The survival time of fish intraperitoneally challenged with *F. columnare* and iron-free human transferrin (Sigma) was reduced when iron was injected prior to exposure. The effect of iron was only evident in one of the two strains examined when the challenge was delivered via immersion. These results indicate that iron depletion may limit the virulence of *F. columnare* more in systemic infections than in external infections. This hypothesis is supported by data indicating that administration of transferrin prior to challenge increased survival after challenge by injection but had little or no effect on bath-challenged fish (Kuo et al., 1981). However, the results found were not consistent for all strains tested and no statistical data were presented.

Several immunization experiments adopting different administration routes, have proven that fish can be protected from subsequent *F. columnare* infections by activating the adaptive immune system (Bebak et al., 2009; Becker and Fujihara, 1978; Bernardet, 1997; Fujihara and Nakatani, 1971; Grabowski et al., 2004; Moore et al., 1990; Shoemaker et al., 2011). High agglutinin titers and good protection were obtained in trout following subcutaneous or intraperitoneal injection with heat-killed *F. columnare* cells (Fujihara and Nakatani, 1971). Schachte and Mora (1973) obtained a high agglutinin titer in channel catfish by intramuscular injection of heat-inactivated cells of the pathogen, but the actual level of protection was not examined. Becker and Fujihara (1978) reported that rainbow trout injected with heat-killed cells produced an agglutinating titer of 1:5120, and that

60-70% of the trout later survived an injection of 10^6 live *F. columnare* cells. Tilapia could mount a significant humoral response in plasma and cutaneous mucus to *F. columnare* after intraperitoneal immunization with formalin-killed sonicated cells in Freund's complete adjuvant (Grabowski et al., 2004). Protection levels were not investigated in the latter study. Protection was obtained after oral immunization with heat-killed or formalin-killed cells of *F. columnare* in the fish feed of three-month-old coho salmon (*Oncorhynchus kisutch*) (Fujihara and Nakatani, 1971; Ransom, 1975). However, the protection as observed in these studies did not go hand-in-hand with high agglutinin titers. Bath-immunization with a bacterin was shown to protect carp against experimental challenge but antibodies against *F. columnare* were not detected in sera from immunized fish (Liewes and Van Dam, 1982). Song's (1986) work with bacterins demonstrated unequivocally that immersion vaccination could result in high levels of protection, but field test results were inconsistent. Song was able to demonstrate that there was cross-protection between isolates and that there may be a common protective antigen among the strains tested. Polyvalent vaccines have also been tested. Using intraperitoneal injections of a combination of formalin-killed *F. columnare*, *Aeromonas salmonicida* and *Yersinia ruckeri* antigens, interference from *A. salmonicida* antigen was shown to suppress responses to two other antigens (Ransom, 1975). Commercially available oral and bathing vaccines have been successfully tested in largemouth bass (*Micropterus salmoides*) fry and salmon, respectively (Bebak et al.,2009; AFS-FCS, 2011). Vaccination trials are further elaborated on below.

1.4. IMPORTANCE OF ENVIRONMENTAL FACTORS

Karvonen et al. (2010) described the effect of global warming on the prevalence of different parasites and bacteria. *F. columnare* could be one of the many taking advantage of this phenomenon. Indeed, transmission of columnaris disease is more efficient in higher temperatures (Suomalainen et al. 2005b; Pulkkinen et al. 2010). Holt et al. (1975) found that when steelhead trout (*Oncorhynchus mykiss*) or coho salmon (*0ncorhynchus kisutch*) experimentally infected with *F. columnare* were held in water at 12 to 20°C, mortality increased with temperature. As stated above, adhesion to gill tissue of a highly virulent *F. columnare* strain is enhanced at increased temperature (Decostere et al., 1999b) and chondroitin AC lyase activity of this pathogen increases along with the temperature (Suomalainen et al., 2006a). The influence of rearing density and water temperature in rainbow trout was studied by Suomalaien et al. (2005b). Normal rearing densities with high temperatures (23°C) proved to increase both transmission rate of columnaris disease and mortality in the fish. Normal densities at low temperatures (18°C) did not affect mortality, but increased the transmission rate of columnaris disease.

Columnaris disease is furthermore intensified by low dissolved oxygen and elevated ammonia levels (Chen et al., 1982). The addition of an organic material such as formulated feed in Japanese eels (*Anguilla japonica*) would also intensify the disease (Sugimoto et al., 1981). Decostere et al. (1999b) observed significantly higher bacterial titers on the gills when organic matter or nitrite were added to an organ bath when performing *ex vivo* trials with *F. columnare*. They discussed that organic matter could concentrate nutrients to feed the bacteria and that degradative enzymes could be kept in close contact with the host tissue. Bacterial titers were furthermore markedly lower in gills placed in an organ bath with distilled water with or without 0.03% NaCl compared to the titers of gills suspended in Ringer solution or in formulated water containing especially divalent ions (magnesium and calcium) (Decostere et al., 1999b). With regard to ammonia levels, in contrast to the findings of Chen et al. (1982), Morris et al. (2006) noted that the survival of the fish exposed to *F. columnare* significantly increased as unionized ammonia concentrations increased. These results suggest that complex interactions can complicate prediction of the responses of fish to concurrent chemical stressors and bacterial pathogens (Morris et al., 2006). Bandilla et al. (2006) described that co-infections of ectoparasites with *F. columnare* increased the susceptibility of rainbow trout to the bacterial pathogen. Compared with single infections, the mortality was significantly higher and the onset of disease condition was earlier among fish, which were concomitantly infected by the parasite *Argulus coregoni* and *F. columnare* (Bandilla et al., 2006).

1.5. EXPERIMENTAL TRIALS/ PATHOGENICITY TESTS

A reliable and reproducible experimental infection model is crucial for studying bacterium-host interactions and evaluating the efficacy of both curative and preventive measures. Most researchers recognize, in experimental challenges with *F. columnare*, the fine and delicate balance and complex interplay between the bacterial cells, fish and the environment in the successful reproduction of columnaris disease.

Hitherto, columnaris disease has been reproduced experimentally in black mollies (*Poecilia sphenops*) (Decostere et al., 1998), channel catfish (Bullard et al., 2011; Darwish et al., 2011; Figueiredo et al., 2005; Olivares-Fuster et al., 2011; Shoemaker et al., 2008; Thomas-Jinu and Goodwin, 2004), eel (*Anguilla japonica*) (Kuo et al., 1981), koi carp (*Cyprinus carpio*) (Tripathi et al., 2005), rainbow trout (*Oncorhynchus mykiss*) (Bandilla et al., 2006; Kunttu et al., 2009b, 2011; Suomalainen et al., 2005b, 2006), tilapia (Kuo et al., 1981) and zebrafish (*Danio rerio*) (Bullard et al., 2011; Olivares-Fuster et al., 2011). However, no gill lesions were generated in these infection models, making it difficult to investigate the series of events occurring at the site of the gill tissue.

In the various experimental infection trials, several ways to both cultivate and harvest the bacterial cells for inoculation were adopted. Different media, including Hsu-Shotts or modified Hsu-Shotts medium (Altinok and Grizzle, 2001; Bader et al., 2003), Ordal's medium (Darwish et al., 2012), Anacker and Ordal's medium also referred to as "Cytophaga medium" (Kuo et al., 1981; Soltani et al., 1996; Stringer-Roth et al., 2002), *F. columnare* growth medium broth (Darwish et al., 2012) and Shieh or modified Shieh medium (Bandilla et al., 2006; Bullard et al., 2011: Decostere et al., 1998, 1999; Kunttu et al., 2009, 2009b; Olivares-Fuster et al., 2011; Panangala et al., 2007; Shoemaker et al., 2008; Suomalainen et al., 2005, 2005a, 2005b; Tripathi et al., 2005) were used to cultivate the bacteria. The bacteria were mostly grown on a shaker (Arias et al 2012a, Bandilla et al., 2006; Bullard et al., 2011; Darwish et al., 2011, 2012; Kunttu et al., 2009, 2009b; Olivares-Fuster et al., 2011; Panangala et al., 2007; Shoemaker et al., 2011; Shoemaker et al., 2008; Soltani et al., 1996; Suomalainen et al., 2005a, b; Thomas-Jinu and Goodwin, 2004; Tripathi et al., 2005). Incubation times and temperatures of the bacteria varied from 21°C to up to 30°C and from 24 to 48 h, respectively (Altinok and Grizzle, 2001; Bader et al., 2003; Bandilla et al., 2006; Bullard et al., 2011; Darwish et al., 2011, 2012; Decostere et al., 1998; 1999a; Figueiredo et al., 2005; Foscarini, 1989; Kunttu et al, 2009, 2009b; Kuo et al., 1981; Bader et al., 2003; Olivares-Fuster et al., 2011; Panangala et al., 2007; Shoemaker et al., 2008; Suomalainen et al., 2005b, 2006; Thomas-Jinu and Goodwin, 2004; Tripathi et al., 2005).

To reproduce columnaris disease experimentally, largely two inoculation routes were adopted, bath (immersion) and injection. With regard to immersion challenges, mostly full-grown broth cultures were added to the water (Altinok and Grizzle, 2001; Bandilla et al., 2006; Bullard et al., 2011; Darwish et al., 2011, 2012; Decostere et al., 1998, 1999a; Figueiredo et al., 2005; Kunttu et al, 2009, 2009b; Kuo et al., 1981; Olivares-Fuster et al., 2011; Shoemaker et al., 2008; Suomalainen et al., 2005b, 2006; Thomas-Jinu and Goodwin, 2004) with water temperatures of the immersion water varying in between 18°C to 30°C (Altinok and Grizzle, 2001; Bader et al., 2003; Bandilla et al., 2006; Bullard et al., 2011; Darwish et al., 2011, 2012; Decostere et al., 1998; 1999a; Figueiredo et al., 2005; Kunttu et al, 2009, 2009b; Foscarini, 1989; Kuo et al., 1981; Olivares-Fuster et al., 2011; Shoemaker et al., 2008; Suomalainen et al., 2005b, 2006; Thomas-Jinu and Goodwin, 2004; Tripathi et al., 2005). Only in a minority of cases were the bacterial cells first harvested through centrifugation and consequently resuspended before being added to the inoculation bath (Bader et al., 2003; Foscarini, 1989; Tripathi et al., 2005). Times during which the fish are bath exposed varied from 15 minutes (Figueiredo et al., 2005; Shoemaker et al., 2008; Suomalainen et al., 2005b) over 30 minutes (Bandilla et al., 2006; Bullard et al., 2011; Decostere et al., 1998; Olivares-Fuster et al., 2011; Suomalainen et al., 2005, 2006) to 45 minutes (Decostere et al., 1999a), one hour (Altinok

and Grizzle, 2001; Kuo et al., 1981; Tripathi et al., 2005) and longer (Darwish et al., 2011; Thomas-Jinu and Goodwin, 2004). When the bacterial cells were administered through injection, then these were retrieved from centrifuged broth cultures following discard of the supernatant (Decostere et al., 1998) or immediately taken from a full-grown broth culture without primary centrifugation (Figueiredo et al., 2005; Kuo et al., 1981; Panangala et al., 2007). A third inoculation route consisted of adding dead fish that were first injected intraperitoneally with a virulent *F. columnare* strain, to the aquarium water wherein fish were immersed (Shoemaker et al., 2011). In some researches, the successful reproduction of columnaris disease depended on or the severity of the elicited disease increased by abrading the skin or gills of the fish (Kuo et al., 1981; Suomalainen et al., 2005b; Tripathi et al., 2005) while in another study, clipping the fin had no effect on success after immersion challenge with *F. columnare* (Decostere et al., 1998).

Not only has the infection route played a determining role in the way the bacterial cells were grown/collected, it also has defined the disease producing capacity of the adopted strains and the disease picture they elicited. Indeed, especially for low virulent strains it was noted that injecting the fish resulted in higher morbidity and mortality rates compared to when inoculation through immersion was adopted (Decostere et al., 1998). Pacha and Ordal (1970) reported a similar finding. They stated that contact with highly virulent strains induced infection and disease contraction more than intramuscular injection, but injection of low virulent bacteria more readily induced infection and disease than did contact.

Environmental conditions can also highly influence morbidity and mortality rates during a challenge trial. To illustrate the dramatic effects of water temperature on the level of mortalities, the investigation of Holt et al. (1975) especially is relevant. This team challenged steelhead trout (*O. mykiss*), Chinook salmon (*O. tshawytscha*) and coho salmon with *F. columnare*, via the water-borne route. At a water temperature of 9.4°C, there were no mortalities attributable to *F. columnare*. By increasing the temperature to 12.2°C, mortality became as high as 4-20%; at 20.5°C, all the steelhead trout and coho salmon and 70% of the Chinook salmon died (Holt et al., 1975). High (23°C) rearing temperatures also increased mortality significantly in rainbow trout compared to lower (18°C) temperatures (Suomalainen et al., 2005b). Fish density at fish farms is also a key player in influencing mortality rate in an outbreak of columnaris disease as was demonstrated under laboratory circumstances. Indeed, mortality rates started earlier and remained higher when fish were stocked at high densities (Suomalainen et al., 2005b). Water flow is another important factor since it acts as a determining factor with regard to the contact time between the possibly present bacteria and the host tissue. High mortality rates were observed in elvers (*Anguilla japonica*) kept in standing

water, while in aquaria with running water, mortality in elvers was reduced by half (Kuo et al., 1981).

1.6. CONTROL

1.6.1. Preventive measures

Management plays a key role in the prevention of the disease. Cunningham et al. (2012) showed that some commonly recorded production variables (feed consumption, pond depth, ammonia levels and stocking events) were associated with columnaris disease outbreaks and, if monitored, could help identify "at risk" ponds prior to disease outbreaks. Suomalainen et al. (2005b) pointed out that reduction of fish density could be used in the prevention of columnaris disease especially if water temperature is high. As lower rearing density can also decrease the transmission of ectoparasites and penetrating endoparasites, it could be an efficient tool in ecological disease management as a whole (Suomalainen et al., 2005b). As high nitrite levels and organic load can stimulate the adherence capacity of *F. columnare* (Decostere et al., 1999b), it is important to control these parameters as well. Furthermore, water treatment could aid in averting a bacterial outbreak. Conrad et al. (1975) reported that ozone treatment of water significantly reduced the numbers of added *F. columnare*, which could be a practical method of prevention. Salt and acidic bath treatments could be used to disinfect water contaminated by *F. columnare* (Suomalainen et al., 2005). An *in vivo* immersion challenge of *F. columnare* in channel catfish and goldfish (*Carassius auratus* L.) revealed decreasing mortality as salinity goes up, with significantly lower and no mortalities when salinity reaches values of 1.0‰ and between 3 and 9‰, respectively (Altinok and Grizzle, 2001). If the fish can be adapted to salt levels of at least 1.0‰, this method could be used as a possible preventive measure in columnaris disease. Shoemaker et al. (2003a) suggested that in the absence of natural food, juvenile channel catfish should be fed at least once every other day to apparent satiation to maintain normal physiological function and improve resistance to *F. columnare* since deprivation reduced innate resistance of catfish to columnaris disease.

Besides optimizing and adjusting management practices, chemical agents can also be adopted as a preventive approach. Davis (1922, 1953) concluded that the development or intensification of columnaris disease could be prevented by treating the fish for 20 min in a copper sulfate ($CuSO_4$) bath at 37 mg/L (1:30 000) or by adding copper sulfate to pond water at 0.5 mg/L. Dipping the fish one at a time in a 1:2000 copper-sulfate for one to two minutes was also proven to be effective in the prevention of the disease. Rogers (1971) suggested the addition of potassium permanganate ($KMnO_4$) to the water at 2 mg/L. Darwish et al. (2009) also confirmed the prophylactic value of

$KMnO_4$ at doses around 2 mg/L. 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% (Riley, 2000). Thomas-Jinu and Goodwin (2004) demonstrated the efficacy of prophylactically given oxytetracycline against mortality in channel catfish and also reported zero mortality for the combination of sulphadimethoxine and ormetoprim in feed prior to bacterial challenge with four highly virulent strains of *F. columnare*.

Another method to prevent columnaris disease is through vaccination. Vaccination trials have not always been successful, as was demonstrated by Fujihara (1969) and Schachte (1978). However, success rates have increased as knowledge on fish immunity and its role in the defense against bacterial diseases continues to expand. Bath immunization with a bacterin was shown to protect carp against experimental challenge, but no agglutinin could be found in sera from immunized fish (Liewes and Van Dam, 1982). Immersion of channel catfish in a bacterin performed each year induced a significant decrease in mortality compared to unvaccinated fish (Moore et al., 1990). Fujihara and Nakatani (1971) obtained protection against columnaris disease in 3-month-old coho salmon by oral immunization with heat-killed cells of *F. columnare* incorporated into fish feed. Ransom (1975) proved that prolonged feeding (over three months) of formalin-killed bacteria provided high levels of protection. Ourth and Bachinski (1987) proposed that strains containing sialic acid could serve as potential vaccine strains for columnaris disease. As stated above, immunization with formalin-killed sonicated cells in Freund's complete adjuvant injected intraperitoneally in tilapia resulted in a significant systemic humoral response within two weeks and antibody levels almost tripled following secondary immunization (Grabowski et al., 2004). At 10 weeks postimmunization, the mean antibody titer remained significantly elevated. Antibodies were also observed in cutaneous mucus of these fish at six and eight weeks postimmunization (Grabowski et al., 2004). An attenuated immersion vaccine currently is registered for the use in channel catfish in the USA (Shoemaker et al., 2011). Fry between 10 to 48 days post hatch that were administered the vaccine through immersion achieved a relative percent survival (RPS) between 57 and 94% following *F. columnare* challenge. This vaccine was also proven to be efficient in largemouth bass fry resulting in RPS values between 74 and 94%, depending on the vaccine dose (Shoemaker et al., 2011). Bebak et al. (2009) tested a commercial oral vaccine in largemouth bass fry. Vaccinated fish had a 43% lower risk of death from *F. columnare* during the field trial (Bebak et al., 2009). An immersion vaccine consisting of a bacterin of *F. columnare* was also brought to the market in the USA as an aid in the prevention of columnaris disease in healthy salmonids of over 3 g (AFS-FCS, 2011).

Probiotics appear to be a promising way in the prevention of different bacterial diseases in aquaculture (Merrifield et al., 2010). Boutin et al. (2011) isolated different strains of commensal bacteria from the skin mucus of unstressed brook charr (*Salvelinus fontinalis*) which *in vitro* revealed antagonistic effects against *F. columnare*. The strains were mixed and used to treat columnaris disease in an *in vivo* experiment. This resulted in a significant decrease of mortality indicating the potential use of these probiotic candidates in the efficient and durable management of columnaris disease. The immunostimulants β-glucan and β-hydroxy-β-methylbutyrate raised the levels of immune function parameters, but did not improve survival in challenge trials with *F. columnare* at any concentration of the stimulants used (Kunttu et al., 2009b).

Recent studies have demonstrated genetic variation in resistance towards *F. columnare* (Arias et al., 2012; Beck et al., 2012; LaFrentz et al., 2012). Arias et al. (2012) presented experimental data on the susceptibility to columnaris disease of hybrid catfish (female channel catfish × male blue catfish (*I. furcatus*)) (C×B). Under experimental conditions, C×B hybrids were significantly more resistant to columnaris disease caused by a highly virulent strain of *F. columnare* belonging to genomovar II compared to channel catfish and blue catfish. Beck et al. (2012) also found one of the two investigated catfish families to be completely resistant towards *F. columnare* resulting in no mortality after inoculation with the bacteria. These *F. columnare* resistant strains could be of great financial importance in the catfish industry. Interestingly, LaFrentz et al. (2012) found that the two families that exhibited the highest CPM after *F. columnare* challenges, had the lowest CPM following *E. ictaluri* challenge, the latter being the most prevalent bacterium to cause disease and mortality in the catfish industry. Further research on larger numbers of families is needed to determine whether there is any genetic correlation between resistance to *E. ictaluri* and susceptibility to *F. columnare*, the two leading bacterial diseases in the catfish industry (LaFrentz et al., 2012a). Recently, the MH-*DAB* gene polymorphism was suggested to be used as a potential genetic marker for disease resistance breeding of grass carp in the future as expression of this gene was up-regulated after infection with *F. columnare* (Yu et al., 2013).

1.6.2. Curative approach

The treatment of columnaris disease using antimicrobial agents has known different success rates. It must be remembered that external treatments are possible only in early stages of the disease, when the infection is still superficial (Bullock et al., 1986; Snieszko and Bullock, 1976). Drugs which have been used effectively in bath therapies are chloramphenicol (Snieszko, 1958), nifurpirinol (Amend, 1972; Amend and Ross, 1970; Ross, 1972; Williams, 1973), nifurprazine

(Shiraki et al., 1970; Deufel, 1974) and oxolinic acid (Endo et al. 1973, Soltani et al., 1995). If the disease is in an advanced stage and/or signs of septicemia are observed, it is necessary to administer antimicrobials in the feed. Oxytetracycline given orally for up to 10 days proved effective in early as well as advanced outbreaks of columnaris disease in Pacific salmon (Bullock et al., 1986; Ferguson, 1977; Wood, 1968, 1974). Lack of success of orally administered oxytetracycline has also been reported (Koski et al., 1993). Sulfonamides, such as sulfamerazine and sulfamethazine, can be used orally but would be less effective than other drugs (Bullock, Hsu and Shotts, 1986; Snieszko and Bullock, 1976; Wolf and Snieszko, 1963). Nitrofuran can also be administered orally for 3 to 5 days (Becker and Fujihara, 1978; Bullock et al., 1986; Deufel, 1974). Gaunt et al. (2010) demonstrated the efficacy of florfenicol in the feed against columnaris disease in channel catfish. Darwish et al. (2012) also illustrated the clear benefit of florfenicol against a mixed infection of *A. hydrophila* and *F. columnare* in sunshine bass (hybrid striped bass, *Morone chrysops* female x *Morone saxatilis* male). The excessive use of antimicrobial agents to withstand *F. columnare* has its negative attributes though (Shoemaker et al., 2011). These include possible allergic reactions elicited in the user after food contact (Serrano, 2005). Potential impacts on human health resulting from the emergence of drug-resistant bacteria and the associated risk of transfer of these resistant traits to the environment and human-associated bacteria are also a major concern (Serrano, 2005). However, data on the antimicrobial susceptibility pattern of *F. columnare* are lacking, rendering it very difficult to fully grasp the above pitfalls. In addition, such information is needed to be able to choose effective antimicrobial agents and to monitor antimicrobial resistance development.

Besides resorting to antimicrobial agents, chemicals have also been adopted in the curative treatment of columnaris disease. In a study by Thomas-Jinu and Goodwin (2004), Diquat® (herbicide) was shown to significantly reduce channel catfish mortalities to zero percent after challenge with *F. columnare*. Diquat® has also proven to be effective in the treatment of columnaris disease in salmonids (Bullock et al., 1986; Darwish and Mitchell, 2009). Copper sulfate (Davis, 1922, 1953) and potassium permanganate (Rogers, 1971; Jee and Plumb 1981) are among the older chemicals used for treatment and prevention of columnaris disease in pond fishes. The organic load in water affects the efficacy of potassium permanganate, but methods are available to estimate that organic load and compensate by adjusting the level of the chemical (Bullock et al., 1986). Darwish et al. (2011) suggested that copper sulfate has clear therapeutic value against *F. columnare* infections in channel catfish when treated in an ultra-low flow-through system during 4 hours. Thomas-Jinu and Goodwin (2004) on the other hand proved the inefficacy of this same chemical

against columnaris disease, which might be due to the advanced stage of the experimental infection at the time of treatment. An *in vitro* assay of commercial products containing peracetic acid was proven to be effective against *F. columnare* infection (Marchand et al., 2011). The potential of kaolin ($Al_2Si_2O_5(OH)_4$), a type of clay, has also been evaluated recently by Beck et al. (2014). Kaolin could improve survival, reduce gill pathologies and reduce bacterial attachment to key tissues associated with columnaris disease in channel catfish by binding to *F. columnare* (Beck et al., 2014). Furthermore, strong antibacterial activity of betaine-type alkaloid ungeremine, obtained from the plant *Pancratium maritimum* L., and derivatives were found to have strong antibacterial activity against *F. columnare* (Schrader et al., 2013). Laanto et al. (2011) reported some *Flavobacterium* sp. phage lysates to inhibit growth or lyse the bacterial cultures. The authors recommended that the causative agent of this strong inhibition or lysis should be studied further for the possibility of developing antimicrobial agents. Prasad et al. (2011) also described the successful use of *F. columnare* phage FCP1 to combat columnaris disease in walking catfish (*Claries batrachus*). Phage treatment led to disappearance of gross symptoms, resulted in a negative bacteriological test and a detectable phage and 100% survival in experimentally infected *C. batrachus*. The result of this study opens new perspectives for the treatment of columnaris disease elicited by antimicrobial resistant *F. columnare* strains (Prasad et al., 2011).

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CHAPTER 2: AIMS OF THE STUDY

CHAPTER 2: AIMS OF THE STUDY

The general aims of this thesis are to determine the antimicrobial susceptibility of *F. columnare* and to obtain insights into the pathogenesis of infections with this agent in carp and rainbow trout.

The specific aims of the present studies may be defined as follows.

1) Data on the antimicrobial susceptibility pattern of *F. columnare* are lacking, rendering it very difficult to understand possible pitfalls in the use of antimicrobial agents to withstand columnaris disease and possible antimicrobial resistance occurring. In addition, such information is needed to be able to choose effective antimicrobial agents and to monitor antimicrobial resistance development. Hence, the first specific aim was to determine the antimicrobial susceptibility pattern of *F. columnare* by using reliable screening methods on a representative number of *F. columnare* isolates collected worldwide (study **Chapter 3**).
2) Hitherto, skin lesions caused by columnaris disease have been reproduced experimentally in different fish species. However, no gill lesions were generated in these infection models, making it difficult to understand and try to prevent the process of gill lesions elicited in (naturally) infected fish. Hence, the second specific aim of this thesis was to develop an infection model generating gill lesions typical for columnaris disease in carp and rainbow trout and to classify *F. columnare* field isolates regarding virulence (study **Chapter 4**).
3) Better insights in the interaction of *F. columnare* isolates of differential virulence with the gills of carp and rainbow trout need to be obtained, as well as the sequence of events taking place at the level of the gill after challenge with *F. columnare* mapped. Hence, the third specific aim was to study the development of elicited gill lesions, alterations in the various gill cell types and the interaction of *F. columnare* isolates with the gill tissue in carp and rainbow trout using the *in vivo* model as described in Chapter 4, adopting both highly and low virulent isolates (study **Chapter 5**).

CHAPTER 3: ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF *FLAVOBACTERIUM COLUMNARE* COLLECTED WORLDWIDE FROM 17 FISH SPECIES

This chapter is adapted from:

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CHAPTER 3: ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF *FLAVOBACTERIUM COLUMNARE* COLLECTED WORLDWIDE FROM 17 FISH SPECIES

3.1 ABSTRACT

Flavobacterium columnare is the causative agent of columnaris disease in diverse fish species worldwide. Although columnaris is an important disease, the antimicrobial susceptibility pattern of *F. columnare* is not studied well. Thus, the purpose of this study was to test the *in vitro* antimicrobial susceptibility of 97 *F. columnare* isolates collected worldwide between 1987 and 2011 from 17 fish species. The broth microdilution technique was utilized for reliable testing of these fastidious organisms. None of the isolates displayed acquired resistance to florfenicol, gentamicin, ormetoprim-sulfadimethoxin and trimethoprim-sulfametoxazole. Acquired resistance to chloramphenicol was detected in 1%, to nitrofuran in 5%, to oxytetracycline in 11% and to enrofloxacin, flumequin and oxolinic acid in 10%, 16% and 16% of the isolates, respectively, as reflected by a bimodal or trimodal distribution of their minimum inhibitory concentrations (MICs). One isolate showed acquired resistance towards several antimicrobial agents including erythromycin. Another isolate revealed acquired resistance towards – amongst others – ampicillin. The isolates displaying acquired resistance originated from ornamental fish species or Vietnamese catfish, except for two isolates coming from wild channel catfish in which acquired resistance was encountered towards oxytetracycline only. Fifty percent of the resistant isolates from ornamental fish were shown to have acquired resistance against three classes of antimicrobial agents, assigning these isolates as multiple resistant. These data might indicate less prudent use of antimicrobials especially in ornamental fish species.

Keywords : *Flavobacterium columnare*, columnaris, antimicrobial susceptibility testing, multiple resistance, broth microdilution

3.2 INTRODUCTION

Columnaris disease is a predominant bacterial disease of both cultured and wild freshwater fish. Many commercially important fish species are susceptible to columnaris disease, such as (but not limited to) salmonids, eels, carp, goldfish, tilapia and channel catfish (Řehulka and Minařík, 2007; Soto et al., 2008; Suomalainen et al., 2009). This disease also poses a problem for many freshwater tropical aquariumfish (Post, 1987; Decostere et al., 1998). *F. columnare* infections may result in skin lesions, fin erosion and gill necrosis, with a high degree of mortality, leading to severe economic losses (Decostere et al., 1998). In the United States of America (USA), *F. columnare* is the second most prevalent bacteria, after *Edwardsiella ictaluri*, to cause disease and mortality (Hawke and Thune, 1992; Wagner et al., 2002), with yearly losses estimated at 30 million dollars (Shoemaker et al., 2011).

Up until now, only a limited number of effective preventive measures against columnaris disease are available, including an attenuated immersion vaccine registered for use in channel catfish in the USA only (Shoemaker et al., 2011). A *F. columnare* bacterin was also brought to market in the USA as an aid in the prevention of columnaris disease in healthy salmonids of over 3g (AFS-FCS, 2011). Awaiting further research aimed at developing and validating more precautionary measures, there is still an excessive use of antimicrobial agents in the contemporary treatment of *F. columnare* (Shoemaker et al., 2011). It should be noted that significant negative attributes are associated with the use of antimicrobials including the extensive expenditure on these substances and possible allergic reactions elicited in the user after food contact. Potential impacts on human health resulting from the emergence of drug-resistant bacteria and the associated risk of transfer of these resistant traits to the environment and human-associated bacteria are also a major concern (Serrano, 2005).

To seize on the aforementioned risks associated with antimicrobial use and to inform the veterinary practitioners on the therapeutic value of antimicrobial agents enabling them to make an informed choice, antimicrobial susceptibility monitoring is a necessary prerequisite. Hitherto, only limited information is available on the antimicrobial susceptibility pattern of *F. columnare*. Antimicrobial susceptibility studies of Thomas-Jinu & Goodwin (2004) using agar disk diffusion revealed susceptibility of two out of the four tested strains towards a combination of sulphadimethoxine and ormetoprim and acquired resistance of two isolates towards oxytetracycline. The same assay was adopted to assess the antimicrobial susceptibility of eight Finnish *F. columnare* strains by Suomalainen et al. (2006). All strains were susceptible to ampicillin, erythromycin, gentamicin, nitrofuran, streptomycin, tetracycline, trimethoprim-sulpha and florfenicol,

but displayed acquired resistance to neomycin and polymyxin B. However, agar disk diffusion tests are challenging because of the rhizoid growth of the organism, which tends to make the zones of inhibition ill defined. Moreover, no specific breakpoints are available for *F. columnare*. Additionally, the apparent zones of inhibition may be the result of delayed growth (Hesami et al., 2010; Miller et al., 2005). The Clinical and Laboratory Standards Institute (CLSI) therefore proposed the use of the broth microdilution technique in 1:7 diluted cation adjusted Mueller Hinton broth (CAMHB) for fastidious growing organisms like *F. columnare* (CLSI 2006). Darwish et al. (2008) used 1:5 dilution of CAMHB as an improved method to the one proposed by CLSI. They determined the minimum inhibitory concentration (MIC) of 23 *F. columnare* isolates to eight antimicrobial agents. All isolates came from channel catfish except for three isolates originating from a common carp (*Cyprinus carpio*) and two type strains isolated from a Chinook salmon (*Oncorhynchus tshawytscha*) and a brown trout (*Salmo trutta*). All isolates were procured in the USA except for one (type strain) which was isolated in France. The authors underscored the noted resistance against the combination ormetoprim/sulfadimethoxin but did not interpret the other encountered MIC values and acknowledged the relatively low number of tested isolates.

The purpose of this study was to address the above described lack of information on the antimicrobial susceptibility pattern of *F. columnare*. Therefore, 97 isolates of *F. columnare* collected worldwide from 17 different fish species were tested for their susceptibility to 12 antimicrobial agents by means of the broth microdilution technique.

3.3 MATERIALS AND METHODS

3.1.1 Bacterial strains

Ninety-seven *F. columnare* isolates originating from 17 fish species collected worldwide between 1987 and 2011 from both cultured and wild fish populations were included in this study (Table 1). The identity of *F. columnare* was confirmed by polymerase chain reaction (PCR). Briefly, genomic DNA was extracted by suspending one colony of a pure bacterial culture in 20 ml lysis buffer (0.25% SDS, 0.05N NaOH). This suspension was heated at 95°C for five minutes and centrifuged for ten seconds at 16,100 G for vapor deposition. One hundred and eighty ml sterile distilled water was added and centrifugation was done at 16,100 G for five minutes. Specific primers were synthesized at the Integrated DNA Technologies (Leuven, Belgium). PCR mixtures and cycle conditions were the same as described before (Panangala et al., 2007). *Escherichia coli* (ATCC 25922) and *Aeromonas salmonicida* subsp. *salmonicida* (ATCC 33658) were included as reference strains in the broth dilution tests.

Table 1. *F. columnare* isolates included in the antimicrobial susceptibility testing

Isolate of *Flavobacterium columnare*	**Fish host**	**Origin**	**Year of isolation**	**Provided by**
JIP 44/87 (ATCC* 49512)	Brown trout *(Salmo trutta)*	France	1987	Dr. J.F. Bernardet
JIP 39/87 (ATCC 49513)	Black Bullhead *(Ictalurus melas)*	France	1987	Dr. J.F. Bernardet
LVDL 3414/89	European eel *(Anguila anguilla)*	France	1989	Dr. J.F. Bernardet
P06/90	Black Bullhead	Unknown	1990	Dr. J.F. Bernardet
90-106	Channel catfish *(Ictalurus punctatus)*	USA	1990	Dr. A. Karsi
L90-629	Channel catfish	USA	1990	Dr. A. Karsi
P 11/91	Rainbow trout *(Oncorhynchus mykiss)*	France	1991	Dr. J.F. Bernardet
C91-20	Channel catfish	USA	1991	Dr. A. Karsi
LVDI 39/I	Unknown	France	1992	Dr. J.F. Bernardet
92-002	Channel catfish	USA	1992	Dr. A. Karsi
Al 94	Channel catfish	USA	1994	Dr. A.E. Goodwin
LVDJ (D7461)	Rainbow trout	France	1994	Dr. J.F. Bernardet
94-078	Channel catfish	USA	1994	Dr. A. Karsi
BioMed	Channel catfish	USA	1996	Prof. C. Arias
LDA 39 H4927	Black Bullhead	France	1998	Dr. J.F. Bernardet
Au 98-24	Channel catfish	USA	1998	Prof. C. Arias
AJS-6	Koi carp *(Cyprinus carpio)*	Belgium	1999	Unknown
JIP14/00	Neon tetra *(Paracheirodon innesi)*	France	2000	Dr. J.F. Bernardet

JIP 13/00	Neon tetra	France	2000	Dr. J.F. Bernardet
ALG-00-530	Channel catfish	USA	2000	Prof. C. Arias
Grizzle	Channel catfish	USA	2000	Prof. C. Arias
JIP 17/01	Koi carp	France	2001	Dr. J.F. Bernardet
VB2	Guppy *(Pycnostachys reticulata)*	France	2001	Dr. J.F. Bernardet
VB1	Guppy	France	2001	Dr. J.F. Bernardet
JIP 07/02	Koi carp	France	2002	Dr. J.F. Bernardet
#27	Channel catfish	USA	2002	Prof. C. Arias
MS-02-475	Channel catfish	USA	2002	Prof. C. Arias
H2	Rainbow trout	Finland	2003	Prof. L.R. Sundberg
ALG-03-57	Channel catfish	USA	2003	Prof. C. Arias
04017018	Koi carp	Netherlands	2004	Dr. O.L.M. Haenen
ALM-05-26	Blue catfish *(Ictalurus furcatus)*	USA	2005	Prof. C. Arias
ALM-05-28	Blue catfish	USA	2005	Prof. C. Arias
ALM-05-29	Blue catfish	USA	2005	Prof. C. Arias
ALM-05-30	Channel catfish	USA	2005	Prof. C. Arias
ALM-05-35	Freshwater drum *(Aplodinotus grunniens)*	USA	2005	Prof. C. Arias
ALM-05-36	Threadfin shad *(Dorosoma petenense)*	USA	2005	Prof. C. Arias
ALM-05-39	Threadfin shad	USA	2005	Prof. C. Arias
ALM-05-43	Threadfin shad	USA	2005	Prof. C. Arias
ALM-05-53	Channel catfish	USA	2005	Prof. C. Arias
ALM-05-58	Blue catfish	USA	2005	Prof. C. Arias
ALM-05-105	Threadfin shad	USA	2005	Prof. C. Arias
ALM-05-106	Threadfin shad	USA	2005	Prof. C. Arias
ALM-05-107	Threadfin shad	USA	2005	Prof. C. Arias
ALM-05-111	Threadfin shad	USA	2005	Prof. C. Arias
BGFS-08	Blue catfish	USA	2005	Prof. C. Arias
BGFS-28	Channel catfish	USA	2005	Prof. C. Arias
BGFS-29	Channel catfish	USA	2005	Prof. C. Arias
S03-579	Channel catfish	USA	2005	Dr. A. Karsi
S05-79	Channel catfish	USA	2005	Prof. C. Arias
PB 06-113#1	Largemouth bass *(Micropterus salmoides)*	USA	2006	Dr. A.E. Goodwin
JIP 02/06	Siamese Fighting Fish *(Betta splendens)*	France	2006	Dr. J.F. Bernardet
C5	Chinese high fin banded shark *(Myxocyprinus asiaticus)*	China	2007	Prof. P. Nie
LD 40 07/2489	Siberian Sturgeon *(Acipenser baeri)*	France	2007	Dr. J.F. Bernardet
B259	Rainbow trout	Finland	2009	Prof. L.R. Sundberg
S09-108	Channel catfish	USA	2009	Dr. A. Karsi
S09-157	Channel catfish	USA	2009	Dr. A. Karsi
S09-162	Channel catfish	USA	2009	Dr. A. Karsi

S09-177	Channel catfish	USA	2009	Dr. A. Karsi
S09-194	Channel catfish	USA	2009	Dr. A. Karsi
S09-378	Channel catfish	USA	2009	Dr. A. Karsi
S09-382	Channel catfish	USA	2009	Dr. A. Karsi
09013931	Koi carp	Netherlands	2009	Dr. O.L.M. Haenen
FCVK2 8T2	Rainbow trout	Finland	2010	Prof. L.R. Sundberg
10012573-2	Koi carp	Netherlands	2010	Dr. O.L.M. Haenen
10009061-1	Koi carp	Netherlands	2010	Dr. O.L.M. Haenen
10012931	Koi carp	Netherlands	2010	Dr. O.L.M. Haenen
S10-025	Channel catfish	USA	2010	Dr. A. Karsi
S10-239	Channel catfish	USA	2010	Dr. A. Karsi
C-066	Channel catfish	USA	2010	Dr. A. Karsi
C-068	Channel catfish	USA	2010	Dr. A. Karsi
C-069	Channel catfish	USA	2010	Dr. A. Karsi
C-074	Channel catfish	USA	2010	Dr. A. Karsi
S10-302	Channel catfish	USA	2010	Dr. A. Karsi
CB10-151	Channel catfish	USA	2010	Dr. A. Karsi
DT2	Vietnamese catfish *(Pangasius hypophthalmus)*	Vietnam	2011	Dr. T.T. Dung
HG13	Vietnamese catfish	Vietnam	2011	Dr. T.T. Dung
CT1	Vietnamese catfish	Vietnam	2011	Dr. T.T. Dung
CT4	Vietnamese catfish	Vietnam	2011	Dr. T.T. Dung
DT2	Vietnamese catfish	Vietnam	2011	Dr. T.T. Dung
DT4	Vietnamese catfish	Vietnam	2011	Dr. T.T. Dung
HG1	Vietnamese catfish	Vietnam	2011	Dr. T.T. Dung
HG9	Vietnamese catfish	Vietnam	2011	Dr. T.T. Dung
HG12	Vietnamese catfish	Vietnam	2011	Dr. T.T. Dung
HG10	Vietnamese catfish	Vietnam	2011	Dr. T.T. Dung
ALU A	Koi carp	Belgium	2011	Prof. A. Decostere
1191-B	Channel catfish	USA	Unknown	Dr. A. Karsi
97-01	Unknown	USA	Unknown	Prof. C. Arias
ALM-05-32	Unknown	USA	Unknown	Prof. C. Arias
ALM-05-37	Unknown	USA	Unknown	Prof. C. Arias
ALM-05-51	Unknown	USA	Unknown	Prof. C. Arias
C56	Unknown	USA	Unknown	Dr. A. Karsi
CDI-A	Koi carp	Netherlands	Unknown	Unknown
Coho92	Rainbow trout	USA	Unknown	Dr. L. Caslake
Fathead minnow	Fathead minnow *(Pimephales promelas)*	USA	Unknown	Dr. L. Caslake
IC(B)E	Unknown	USA	Unknown	Unknown
JIF E	Unknown	Unknown	Unknown	Unknown

RP	Unknown	Unknown	Unknown	Unknown
TAC	Unknown	Unknown	Unknown	Unknown

*ATTC: American Type Culture Collection

3.1.2 Antimicrobial agents

The following 12 antimicrobial agents were included: ampicillin, chloramphenicol, enrofloxacin, erythromycin, florfenicol, flumequin, gentamicin, nitrofuran, ormetoprim-sulfadimethoxin, oxolinic acid, oxytetracycline and trimethoprim-sulfadimetoxazole. These antimicrobials were obtained as laboratory standard powders from Sigma Aldrich N.V. (Bornem, Belgium), except for enrofloxacin and ormetoprim which were obtained from Medini (Oostkamp, Belgium) and from Alpharma (Wilrijk, Belgium), respectively. They were dissolved in sterile distilled water to make stock solutions of at least ten times the highest concentration to be tested.

Afterwards they were further diluted in twofold dilution series in appropriate solvents according to the methods recommended by the CLSI (2006) with the following ranges of twofold serial dilutions: ampicillin 0.016-16 µg/ml, chloramphenicol 0.03-32 µg/ml, enrofloxacin 0.002-2 µg/ml, erythromycin 0.06-64 µg/ml, florfenicol 0.016-16 µg/ml, flumequin 0.008-8 µg/ml, gentamicin 0.02-16 µg/ml, nitrofuran 0.03-32 µg/ml, ormetoprim-sulfadimethoxin 0.03/0.6-32/608 µg/ml, oxolinic acid 0.025-25 µg/ml, oxytetracycline 0.008-64 µg/ml and trimethoprim-sulfadimetoxazole 0.03/0.6-32/608 µg/ml. Fifty microliter of these dilutions were added into the wells of a plastic standard 96 well-plate (Greiner-Bio One GmbH, Frickenhausen, Germany). Each well of the last column of the tray was filled with 1:7 diluted (3g/l) CAMBH (Becton Dickinson, Erembodegem, Belgium) without antimicrobials added and served as a positive control for growth of the isolate tested.

3.1.3 Susceptibility testing

All strains, stored at -70°C, were defrosted and grown overnight at 28°C in 1:7 (3g/l) diluted CAMHB (CLSI, 2006). The method proposed by Darwish, Farmer & Hawke, being the use of 1:5 dilution of CAMHB was tested on 7 isolates (results not shown). Consequently, the broth culture was adjusted to a 0.5 McFarland suspension equivalent to 1 x 10^8 colony-forming units (CFU) per ml and then further diluted 1:100 in 1:7 (3 g/l) CAMHB. Subsequently, 50 µl of this dilution was added to each well of the 96 well plates. Bacterial growth was assessed after 44-48 h aerobic incubation at 28°C. The MIC was defined as the lowest concentration of the antimicrobial agent without any visible bacterial growth (CLSI, 2006). Following incubation, the positive control of

each isolate was inoculated on Columbia agar with 5% sheep blood (Oxoid, Erembodegem, Belgium), which was incubated at 28°C for 72 h and checked after 24, 36 and 72 h to check purity. *E. coli* (ATCC 25922) and *A. salmonicida* subsp. *salmonicida* (ATCC 33658) were included in the assays.

3.4 RESULTS

The alternative method proposed by Darwish et al. (2008) gave better growth in three out of seven isolates after 24 h of growth at 28°C. After 44-48 h of incubation at 28°C, this difference vanished though. For this reason, the method proposed by CLSI (2006) of using 1:7 dilution was adopted in this study.

For the antimicrobial agents included in the CLSI document M49-A, the MIC values of the *E. coli* and *A. salmonicida* subsp. *salmonicida* reference strains fell within acceptible quality ranges (CLSI, 2006) (Table 2). The MIC values for chloramphenicol, erythromycin and nitrofuran for *E. coli* were 4-8, 16-32 and 8-32 µg/ml, respectively. For *A. salmonicida* subsp. *salmonicida*, the MIC value for chloramphenicol was 0.5-1 and for nitrofuran 2-8 µg/ml. An overview of the MIC values for the 97 tested *F. columnare* isolates is shown in Table 2.

Table 2. Distribution of minimum inhibitory concentration (MIC) of 12 antimicrobial agents on 97 *Flavobacterium columnare* isolates from 17 different fish species collected worldwide between 1987 and 2011

	Number of isolates with MIC (µg/ml)																		
Antimicrobial agent	≤0.004	0.008	0.016	0.032	≤0.064	0.064	0.125	0.25	0.5	1	2	4	8	>8	16	32	>32	>64	Total number of resistant isolates out of 97
Ampicillin				1		22	6	67				**1***							1
Chloramphenicol										5	60	31				**1**			1
Enrofloxacin		4	72	4		2	5**		**2**	**5**	**3**								10 (+5 tailing)
Erythromycin					1	2	14	17	47	15		**1**							1
Florfenicol								14	29	49	5								0
Flumequin						15	62	4	**1**	7	**1**	**1**	**1**	**5**					16
Gentamicin						1	4	10	33	37	12								0
Nitrofuran							1	1	10	38	6	34	2			**1**	**4**		5
Oxytetracycline				4		64	19				**1**	**3**				**5**		**1**	10
Ormetoprim-sulfadimethoxin (1/19)							1	10	8	39	26	10	1		2				0
Trimethoprim-sulfametoxazole (1/19)						1	5	8	30	27	14	12							0

	Number of isolates with MIC (µg/ml)												
Antimicrobial agent	≤0.025	0.05	0.1	0.2	0.4	0.8	1.6	3.2	6.3	12.5	25	>25	Total number of resistant isolates out of 97
Oxolinic acid		2	32	47			**1**	7	**2**	**1**		5	16

* *F. columnare* isolates considered to have acquired resistance are represented in bold

** extended frequency distribution (tailing)

Grey background: value not considered for this antimicrobial agent

For florfenicol, gentamicin and for the combinations ormetoprim-sulfadimethoxin and trimethoprim-sulfametoxazole, a monomodal distribution of MICs was noted, indicating absence of acquired resistance. In contrast, for ampicillin, chloramphenicol, erythromycin and nitrofuran, the MICs showed a bimodal distribution. This was also the case for enrofloxacin, but in addition, an extended frequency distribution of MICs (tailing) was present here for isolates belonging to the *F. columnare* population with the lower MIC values. The MIC values for flumequin, oxolinic acid and oxytetracycline displayed a trimodal distribution. According to the microbiological criterion, isolates in the higher range of MICs should be considered to have acquired resistance (Turnidge and Paterson, 2007).

Resistance phenotypes of the *F. columnare* isolates are shown in Table 3. Sixty percent of the resistant isolates procured from ornamental fish had acquired resistance towards two and 50% towards three classes of antimicrobial agents, respectively, assigning these latter isolates as multiple resistant (Schwarz et al., 2010). As for the isolates originating from cultured Vietnamese catfish, 100% showed acquired resistance towards at least one of the tested quinolone antimicrobials and one isolate was multiple resistant.

Table 3. Resistant phenotypes of *Flavobacterium columnare* isolates displaying acquired resistance

Isolate	Fish host	Origin	Year of isolation	Resistance phenotypes*
CDI-A	Koi carp	Netherlands	Unknown	Enro, flum, oxol
AJS-3	Black molly	Belgium	1997	Chlor
JIP 13/00	Neon tetra	France	2000	Enro, flum, nf, oxol, oxyt
JIP14/00	Neon tetra	France	2000	Enro, flum, nf, oxol, oxyt
VB1	Guppy	France	2001	Enro, flum, nf, oxol, oxyt
VB2	Guppy	France	2001	Ampi, chlor, nf, oxyt
04017018	Koi carp	Netherlands	2004	Oxyt
ALM-05-28	Blue catfish	USA	2005	Oxyt
BGFS-28	Channel catfish	USA	2005	Oxyt
09013931	Koi carp	Netherlands	2009	Enro, flum, oxol
10009061-1	Koi carp	Netherlands	2010	Enro, flum, nf, oxol, oxyt
10012931	Koi carp	Netherlands	2010	Enro, flum, oxol, oxyt
CT1	Vietnamese catfish	Vietnam	2011	Flum, oxol
CT4	Vietnamese catfish	Vietnam	2011	Enro, flum, oxol
DT2	Vietnamese catfish	Vietnam	2011	Enro, flum, oxol
DT4	Vietnamese catfish	Vietnam	2011	Enro, flum, oxol
HG1	Vietnamese catfish	Vietnam	2011	Flum, oxol (tailing enro)
HG9	Vietnamese catfish	Vietnam	2011	Flum, oxol (tailing enro)
HG10	Vietnamese catfish	Vietnam	2011	Flum, oxol (tailing enro)
HG12	Vietnamese catfish	Vietnam	2011	Flum, oxol (tailing enro)
HG13	Vietnamese catfish	Vietnam	2011	Erythro, flum, oxol, oxyt (tailing enro)

* ampi : ampicillin; chlor : chloramphenicol, enro : enrofloxacin; erythro : erythromycin; flum : flumequin; nf : nitrofuran; oxol : oxolinic acid; oxyt : oxytetracycline.

Upon inspecting the blood agar plates inoculated with the positive controls for growth in the MIC assays, small colonies were noted for some *F. columnare* isolates after 72 h of incubation. When these colonies were scraped off from the blood plate and inoculated onto Shieh agar plates (Shieh, 1980; Song et al., 1988), pure colonies with the typical *F. columnare* morphology appeared after 24 to 36 h incubation of the Shieh plates at 28°C. Gram staining revealed the typical long and slender *F. columnare* morphology. Upon testing the colonies from the blood agar phenotypically and using PCR, all inocula were confirmed to yield only *F. columnare*.

3.5 DISCUSSION

For the interpretation of the MIC results, the microbiological criterion was used since CLSI breakpoints for aquatic organisms are not yet available. This parameter gives a reasonable approximation to distinguish wild-type populations of bacteria from those with acquired resistance (Turnidge and Paterson, 2007). This criterion does not necessarily predict how the fish will respond to antimicrobial treatment. However, for most included antimicrobial agents, MIC values were at least ten times higher for isolates designated as acquired resistant. It is therefore highly unlikely that fish infected with these isolates will be successfully treated with these antimicrobial agents. Nonetheless, it will be necessary to carry out experimental challenges adopting isolates with varying MIC values or to have clinical data to be able to draw well-founded conclusions on the *in vivo* efficacies of the antimicrobial agents in question.

In general, in countries where antimicrobial agents are allowed for use in aquatic species, only two or three antimicrobials have been granted a marketing authorization (CLSI, 2006; FDA, 2011; Rigos and Troisi, 2005; Cizek et al., 2010). This tight control on antibiotic use applies to fish cultured for human consumption, but is not enforced on ornamental fish. The ornamental fish industry is massive and many ornamental fish are worth a fortune, exceeding values of aquaculture products for human consumption in some countries (Alderman and Hastings, 1998; Verner-Jeffreys et al., 2009; Weir et al., 2011). This, combined with intensive rearing conditions and transport stress rendering the fish more susceptible to bacterial diseases, encourages the use of antimicrobial agents in this sector. Veterinarians may, using the prescription cascade, prescribe almost any antibiotic that may cure the sick fish, since consumption is excluded for ornamental fish (Alderman and Hastings 1998; Cizek et al., 2010). Indeed, extra-label drug use occurs in many countries (Bal and Gould, 2011; CLSI, 2006; Nikaido, 2009; Rigos and Troisi, 2005; Serrano, 2005), which is why the susceptibility tests in the present study included more antimicrobial agents than specifically approved for use in aquatic species.

Nowadays, the use of chloramphenicol is limited to some life-threatening conditions in humans because of the adverse side-effects (aplastic anemia and bone-marrow suppression) and the availability of less toxic antimicrobials (Schwarz et al., 2004). Though not allowed for use in aquaculture, chloramphenicol residues have been detected in fishery products from South-East Asia, giving rise to risks for the human health (Serrano, 2005). In some studies, *Aeromonas* spp. were shown to have acquired resistance towards chloramphenicol (Weir et al., 2011). In the present study, acquired resistance of one strain towards this antimicrobial agent was found, making it the first to describe acquired resistance of *F. columnare* towards chloramphenicol.

Florfenicol is a fluorinated structural analog of chloramphenicol without the above mentioned side-effects and is increasingly popular in aquaculture (Aoki, 2000; Liao, 2000; Sapkota et al., 2008). The FDA approved this antibiotic for the treatment of enteric septicemia of catfish, for coldwater disease in salmonids, for furunculosis in freshwater-reared salmonids and for columnaris disease in catfish (FDA, 2011). It is also registered in some European countries for use in aquatic species. In this investigation, no acquired resistance was noted towards florfenicol. Nevertheless, prudent use of this antibiotic remains necessary. In Finland, florfenicol is only accepted as an alternative antibiotic in case resistance towards oxytetracycline occurs (Suomalainen et al., 2006).

No acquired resistance was found for the combinations ormetoprim/sulfadimethoxin and trimethoprim/sulfametoxazole. This is in contrast to Darwish et al. (2008) who noted acquired resistance in American *F. columnare* channel catfish isolates for ormethoprim/sulfadimethoxin. In the present study, both the combinations ormetoprim/sulfadimethoxin and trimethoprim/ sulfametoxazole were tested because in the CLSI guidelines (CLSI, 2006) it is stated that it is not yet confirmed whether the former combination can be used to predict the susceptibility to the latter at 28±2°C. Isolates showing high values for ormetoprim/sulfadimethoxin, however, also displayed higher values for trimethoprim/sulfametoxazole. In the two isolates displaying MIC-values of 16/304 for ormetoprim/sulfadimethoxin, slightly higher values were noted for trimethoprim/sulfametoxazole. These two isolates were not considered resistant though, since no clear differentiation could be made from the non-resistant isolates.

For the quinolones, enrofloxacin, flumequin and oxolinic acid were included in this study. Quinolones are mainly broad-spectrum antibacterial agents, commonly used in both human and veterinary medicine. Their extensive or unnecessary use in some countries, which can to some

degree be considered as a misuse, or the use of quinolones with poor activity, has resulted in bacteria rapidly developing resistance to these agents (Ruiz, 2003). Multiple studies demonstrated resistance of different ornamental fish pathogens (*Aeromonas* spp. and *Vibrio* spp.) towards the quinolones (Verner-Jeffreys et al., 2009; Weir et al., 2011). The FDA prohibited extra-label use of fluoroquinolones in food animals (Serrano, 2005). These second line antimicrobials are to be reserved for conditions that have responded poorly to other classes of antimicrobial agents and they should not be used for prophylaxis (European Medicines Agency, 2011). In some countries including Vietnam, flumequin and/or oxolinic acid are registered for use in consumable aquatic species. In this study, the bimodal or trimodal distribution of MICs indicated acquired resistance to enrofloxacin, flumequin and oxolinic acid in 10%, 16% and 16% of the isolates, respectively. Out of the ten isolates displaying acquired resistance to enrofloxacin, seven were sampled from ornamental fish. The remaining three isolates originated from Vietnamese catfish. This is the first time acquired resistance of *F. columnare* towards this antimicrobial class is described.

Resistance to quinolones typically arises as a result of alterations in the target enzymes (mostly gyrase) and of changes in drug entry and efflux. It can also be mediated by plasmids that produce the Qnr protein which protects the quinolone targets from inhibition (Drlica and Zhao, 1997; Jacoby, 2005). In many Gram-negative bacteria resistance develops progressively through stepwise mutations. A single mutation in the gyrase gene results in resistance to the first-generation quinolones, such as oxolinic acid and flumequin, and reduced susceptibility to other quinolones. A second mutation in the gyrase gene mediates full resistance to the quinolones (Marien et al., 2007). In this study, all isolates resistant to enrofloxacin as indicated by the bimodal distribution of MIC values, also displayed acquired resistance to flumequin and oxolinic acid. Remarkably, the MICs of enrofloxacin for isolates belonging to the *F. columnare* population with the lower MIC values, showed an extended frequency distribution, possibly indicating the presence of a mechanism providing decreased susceptibility in some isolates. Indeed, the five isolates with an enrofloxacin MIC of 0.125 µg/ml also demonstrated acquired resistance towards flumequin and oxolinic acid. One isolate displaying a MIC-value of 0.5 µg/ml for flumequin, was considered resistant for this antimicrobial agent since this same isolate clearly gave resistant MIC-values for oxolinic acid (6.2 µg/ml) and evaluated MIC-values for enrofloxacin (0.12 µg/ml).

In this study, 10 isolates displayed acquired resistance towards oxytetracycline. Oxytetracycline is one of the most commonly used tetracyclines worldwide for the treatment of bacterial fish diseases (Rigos and Troisi, 2005). Multiple studies have revealed resistance of ornamental fish pathogens *Aeromonas* spp. and *Vibrio* spp. towards tetracyclines (Cizek et al., 2010; Jongjareanjai et al., 2009; Verner-Jeffreys et al., 2009; Weir et al., 2011). Previous studies showed

no resistance of *F. columnare* to oxytetracycline (Thomas-Jinu and Goodwin, 2004; Suomalainen et al., 2006) designating this study as the first to report acquired resistance against this antimicrobial agent.

Nitrofuran is not allowed for use in aquaculture. Resistance of *Vibrio* and *Aeromonas* species towards nitrofuran in ornamental fish has been described before (Weir et al., 2011). The present study shows acquired resistance of five *F. columnare* isolates coming from ornamental fish. This study is the first to report acquired resistance of *F. columnare* towards nitrofuran.

In this study, except in one case, all isolates displaying multiple resistance originated from ornamental fish. This may reflect the differences in antimicrobial use policy in fish destined for human consumption and in ornamental fish, as was stipulated by other research groups (Verner-Jeffreys et al., 2009; Cizek et al., 2010) that came across similar findings in aeromonads from ornamentals.

3.6 CONCLUSION

The present study is the first in its kind in view of the high number and mixed origin of *F. columnare* isolates in terms of fish species, year of isolation and geographical area. Acquired resistance to chloramphenicol, oxytetracycline, flumequin, oxolinic acid, enrofloxacin and nitrofuran is reported for the first time in *F. columnare*. The results obtained in this study might indicate less prudent use of antimicrobials especially in the ornamental fish industry and therefore urges to limit their use and to focus on preventive measures.

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CHAPTER 4: GILL INFECTION MODEL FOR COLUMNARIS DISEASE IN COMMON CARP AND RAINBOW TROUT

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CHAPTER 4: GILL INFECTION MODEL FOR COLUMNARIS DISEASE IN COMMON CARP AND RAINBOW TROUT

4.1 ABSTRACT

Challenge models generating gill lesions typical for columnaris disease were developed for carp *Cyprinus carpio* L. and rainbow trout *Oncorhynchus mykiss*, Walbaum fry by means of immersion challenge. Hereby, *Flavobacterium columnare* field isolates were characterized regarding virulence. Carp inoculated with highly virulent isolates revealed diffuse, whitish discolouration of the gills affecting the filaments of all arches while in trout mostly unilateral focal lesions restricted to the filaments of the first two gill arches occurred. Light microscopic examination of the gills of carp exposed to highly virulent isolates revealed diffuse loss of branchial structures, and desquamation and necrosis of gill epithelium with fusion of filaments and lamellae. In severe cases, large parts of the filaments were replaced with necrotic debris entangled with massive clusters of *F. columnare* bacterial cells, enwrapped in an eosinophilic matrix. In trout, histopathologic lesions were similar but less extensive and much more focal, being well delineated from apparently healthy tissue. Scanning and transmission electron microscopic observations of the affected gills pictured long, slender bacterial cells attained in an extracellular matrix and in close contact with the destructed gill tissue.

This is the first study to reveal gill lesions typical for columnaris disease at a macroscopic, light microscopic and ultrastructural level in both carp and rainbow trout following challenge with *F. columnare*, opening ample research opportunities regarding pathogen-gill interaction.

4.2 INTRODUCTION

Flavobacterium columnare (*F. columnare*) is a worldwide occurring bacterial pathogen causing columnaris disease in multiple freshwater fish species, including carp *Cyprinus carpio* L. and rainbow trout *Oncorhynchus mykiss*, Walbaum (Tripathi et al., 2005; Suomalainen et al., 2009). The disease is notorious as it induces gill, fin and skin lesions resulting in massive mortalities and economic losses of millions of dollars yearly (Shoemaker et al., 2011). Columnaris disease may exhibit an acute or more chronic course, with the age and immune status of the fish as major determining factors. In young fish, the disease strikes acutely and the gill is the major site of damage. In acute cases of columnaris disease in adults, yellowish-white regions of necrotic tissue may appear in the gills, causing respiratory distress and death of the animal. In more chronic cases, it takes longer for gill pathology to develop and skin and fin lesions may also occur (Pacha and Ordal, 1967; Bernardet and Bowman, 2006; Decostere, 2002; Declercq et al., 2013b). Besides host-related parameters, the virulence of *F. columnare* isolates can also markedly influence the disease course. Highly virulent strains are able to induce acute mortality, with the vast majority of fish not displaying macroscopic lesions at the time of death due to the speed of mortality (Rucker et al., 1953; Pacha and Ordal, 1967).

Considering the pathogenesis of columnaris disease, especially with regard to the interaction of the pathogenic agent with the gill tissue, multiple domains remain to be explored and research questions to be answered. This information is crucial to be able to combat the disease without having to resort to antimicrobial agents. Recently, increasingly more data have become available on the reciprocal effects between *F. columnare* and its host. However, most of these studies focussed on the interaction between *F. columnare* and the skin tissue (Bader et al., 2003; Suomalainen et al., 2005b; Tripathi et al., 2005; Bullard et al., 2011) or linked mortality to the genomovars and genetic traits of the isolates involved (Thomas-Jinu and Goodwin, 2004; Suomalainen et al., 2006a, 2006b; LaFrentz et al., 2012), leaving the interplay between this pathogen and the gill tissue a challenging wasteland to cultivate. Indeed, only a mere handful of studies explored the interaction between *F. columnare* and the gill tissue (Sun et al., 2012; Peatman et al., 2013), mainly concentrating on mucosal actors in the teleost gill.

Currently, to our knowledge, a reproducible experimental infection model eliciting gill lesions as noted in the field (Decostere et al., 2002) is non-existing, hampering in-depth research on the interplay between this pathogen and the host gill.

In this respect, the aim of this study was to develop a reliable and reproducible experimental infection model for columnaris disease in carp and rainbow trout with a particular focus on eliciting gill lesions. A virulence profile of eleven *F. columnare* isolates was additionally assessed. Furthermore, we ambiated to provide a detailed description of the induced gill lesions depicting the end-stage of the disease, which could provide a building block to move forward in the elucidation of the pathogenesis of columnaris disease with special emphasis on the gill tissue.

4.3 MATERIALS AND METHODS

4.3.1 Fish

Two-day old carp fry were obtained from a Belgian hatchery. After transportation to the experimental facilities, the fry were grown to a mean length of five centimetres before inclusion in the experiment. Rainbow trout with an average length of five centimetres were kindly provided by Laboratoire de Pisciculture Huet (Leuven, Belgium) and acclimatized for two months. The fish were maintained in one cubic metre stocking tanks filled with 800 L of recirculating and aerated tapwater. The water temperature was 22 ± 1°C for the carp and 19 ± 1°C for the trout. Starting from two weeks before the experimental challenge, the water temperature of the stocking tanks was gradually increased by 1°C every two days until a temperature of 25 ± 1°C and 22 ± 1°C was reached for the carp and trout, respectively. This water temperature was then kept constant until the onset of the challenge. Ammonia and nitrite concentrations were monitored daily and were below detectable levels at all times. The pH of the water was 8. A photoperiod of 12 h light/ 12 h darkness was provided and the fish were fed a commercial diet (trout: Trouw Nutrition, carp: Fin Perfect Feed, Sonubaits) to satiation twice daily. Fish were deprived from food 24 h prior to the experimental challenge. Twenty-five carp and twenty-five trout were sacrificed with an overdose of benzocaine (ethylaminobenzoate; Sigma, Belgium). Gill and skin were screened for parasites and for the absence of *F. columnare* by means of bacteriological examination using cultivation onto modified Shieh agar (Shieh, 1980; Song et al., 1988) containing 1 µg/mL tobramycin (Decostere et al., 1997) and Polymerase Chain Reaction (PCR). For the latter, DNA from the tissue samples was extracted using a DNeasy blood and tissue kit (Qiagen, Venlo, the Netherlands), according to the guidelines of the manufacturer. PCR mixtures and cycle conditions were as described before (Declercq et al., 2013a).

4.3.2 Bacterial propagation

Five carp isolates (0401781, 0901393, 10009061-1, 10012573/2 and CDI-A) and six trout isolates (JIP 44/87, JIP P11/91, LVDJ (D7461), H2, B259 and Coho 92) were used for the inoculation trials. For more information concerning origin, year of isolation and provision of the isolates, the reader is referred to Declercq et al. (2013a). The identity of all *F. columnare* isolates was confirmed by PCR as described by Declercq et al. (2013a). The genomovar of the koi carp and trout isolates was determined at the Aquatic Microbiology Laboratory of Auburn University (USA) using 16S-Restriction Fragment Length Polymorphism (RFLP) according to the protocol as described by Olivares-Fuster et al. (2007b). Before inclusion in the challenge trials, each isolate was passaged once in the fish species it was retrieved from to assure all isolates were first passage isolates. This was done by experimentally inoculating fish through immersion, sampling the gills 6 h following challenge and streaking the sample onto modified Shieh agar containing 1 µg/mL tobramycin. Following incubation at 28°C during 36 h, five colonies displaying the typical *F. columnare* morphology were purified and the resulting cultures stored at -70°C in freezing medium (1% casitone Difco, 0.1% $MgSO_4 7H_2O$ and 10% sterile glycerol resolved in distilled water, pH 6.8-7). For the experimental challenge, the isolates were defrosted and grown during 36 h at 28°C on modified Shieh agar plates. Five colonies were used to inoculate plastic 15 mL Falcon tubes filled with 4 mL of modified Shieh broth. The inoculated broth was placed on a shaker for 24 h at 28°C at 100 revolutions per minute (rpm). The cultivated broth of two Falcon tubes was used to inoculate 392 mL of modified Shieh broth in 500 mL glass bottles. After 24 h of incubation at 28°C on a shaker at 100 rpm, bacterial titres were measured by making tenfold dilution series in triplicate on modified Shieh agar plates. Optical densities (OD) were measured using a spectrophotometer at 600 nm (Pharmacia LKB Ultraspec III, Biotech, Gaithersburg, USA). The grown bacterial cultures were used in the inoculation trials as described below.

4.3.3 Experimental challenge

For each koi isolate, a group of 20 arbitrarily chosen carp and for each trout isolate, a group of 20 arbitrarily chosen trout was removed from the stocking tanks and placed in a 10 L tank filled with 4.6 L of aerated water of 27 ± 1°C (carp) or 23 ± 1°C (trout). Following, 400 mL of cultivated modified Shieh broth was added to the tank water. A control group of 20 fish was included, from which the fish were immersed in a tank with 4.6 L water with 400 mL modified Shieh broth but without *F. columnare*. For the carp, after a 90 min inoculation period, each group of 20 fish was transferred to a 60 L tank filled with 48 L of recirculated aerated tapwater of 25 ± 1°C. Two litres of

the challenge water from the 10 L tank were transferred as well. The trout were inoculated during 120 min and each group of 20 fish was transferred to a one cubic metre tank filled with 350 L of aerated recirculated tapwater of 22 ± 1°C. Both the carp and the trout were monitored every three hours and mortality was recorded. Every 12 h, 75% of the water was replaced. Following challenge and during the remainder of the trial, the animals were checked every one to two hours. As soon as the predetermined humane endpoints (no reaction to stimuli, hanging at the water surface, loss of balance) were reached, the fish were euthanized with an overdose of benzocaine and sampled. The first left gill arch was removed and cut in three parts for light microscopic, scanning (SEM) and transmission electron microscopic (TEM) examination as stated below. The counterpart right gill arch served for bacterial titration of *F. columnare* as described below. The trial lasted for seven days and at the end of the experiment, all surviving animals, including the control fish, were sacrificed and the gill tissue sampled as previously stated. The gill tissue of the control fish was additionally screened for the presence of *F. columnare* using PCR as described above.

The trials as described above were executed twice at different time-points. For two rainbow trout isolates, B259 and JIP P11/91, the challenge experiments were carried out an additional third time, as discussed below. Isolates that were able to elicit 80% mortality or more within 72 h were assigned as highly virulent, whereas isolates causing 20% mortality or less were designated low virulent. The isolates giving a mortality rate in between 20 and 80% within 72 h, were assigned as moderately virulent. The experiments were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University under the number EC2011/012.

4.3.4 Bacteriological examination

The right gill arch was excised using sterile scissors and crushed in a sterile plastic tray. Half of the right gill arch was placed in a plastic 15 ml Falcon tube filled with 5 ml of a 1:7 diluted (3 g/L) Cation Adjusted Mueller-Hinton Broth (CAMHB) (Becton Dickinson, Erembodegem, Belgium). Tenfold dilution series were made in plastic 15ml Falcon tubes. Of each dilution, 50 µL was retrieved and inoculated on specific modified Shieh agar containing 1 µg/mL tobramycin. Following incubation at 28°C during 36 h, the bacterial titres were determined. In addition, special attention was paid to the viscosity of the cultivated broth.

4.3.5 Light microscopic examination

In case skin lesions were noted, the skin was excised over the full length of the lesion including the transitional zone between affected and normally looking skin. The skin tissue and one third of the first left gill arch were placed in phosphate-buffered 4% formaldehyde at room temperature for 24 h, dehydrated in an alcohol-xylene series (Microm tissue processor STP420D, Prosan, Merelbeke, Belgium) and embedded in paraffin wax (Microm embedding station EC 350-1, Prosan). All tissues were sectioned (8 µm) (Microm microtome HM 360, Prosan) and stained with haematoxylin and eosin (H&E) and Giemsa stain.

4.3.6 Electron microscopic examination

For SEM, the gill samples were preserved in a HEPES-glutaraldehyde solution. Tissue samples were postfixed in 1% buffered osmium tetroxide for 2 h and dehydrated in an increasing alcohol series followed by increasing ethanol–acetone series up to 100% acetone as described by De Spiegelaere et al. (2008). The samples were then dried to the critical point with a Balzers CPD 030 critical point drier (Sercolab bvba, Merksem, Belgium) and further mounted on metal bases and sputtered with platinum using the JEOL JFC 1300 Auto Fine Coater (Jeol Ltd, Zaventem, Belgium). The samples were examined with a JEOL JSM 5600 LV scanning electron microscope (Jeol Ltd). For TEM processing, a similar protocol as described by De Spiegelaere et al. (2008) was used. For viewing, both the JEM-1200EX II Jeol electron microscope (Jeol Ltd) operating at 60 kV and the JEM-1400 plus Jeol electron microscope (Jeol Ltd) operating at 80 kV were used. Micrographs were taken both analogous and scanned with EPSON scanner (Type V700 PHOTO) with the former microscope or were taken digitally with the latter.

4.3.7 Statistical analysis

The data for mortality were not normally distributed and therefore transformed into a binary dataset with values “moribund fish” and “fish euthanized at the end of the experiment” reclassified as “0” and “1”, respectively. Based on this classification and by means of logistic regression, the data of each isolate were compared in between trials to see whether the trials were reproducible.

Per fish species, the mortality pattern was compared for the different isolates based on the time to death. To do so, a Cox regression survival analysis was performed to compare the mortality rate between the isolates for each fish species.

For the statistical analysis of the bacterial titration counts, data of positive titres – corresponding with the ability to retrieve bacteria from the gill tissue – were log transformed and differences between the different isolates per fish species were tested using one-way ANOVA.

Statistical results were considered to be significant when p-values were lower than 0.05. All analyses were performed using SPSS version 21.0.

4.4 RESULTS

4.4.1 Mortality and bacteriologic data

Before initiation of the experiments, all screened fish were negative for parasites and *F. columnare*. All *F. columnare* isolates included in this study displayed the typical genomovar I profile. For each trial, the optical densities and bacterial titres as determined by plate count of the 400 mL inoculation broth and the mortality percentage of the various adopted isolates are given in Table 1.

Table 1. Optical densities and bacterial titres (CFU mL-1) as determined by plate count of the 400 mL cultivated Shieh broth and the associated elicited mortality per trial for the immersion challenges in carp and rainbow trout using *F. columnare*

Fish species	**Isolate**	**Cultivated broth**		**Mortality percentage per trial (1-2-(3*))**
		Optical density (trial 1-2-(3*))	**CFU mL-1 (trial 1-2-(3*))**	
Carp	0401781	0.58 - 0.69	$6x10^9$ - $2x10^8$	100 - 100
	10012573/2	0.59 - 0.65	$2x10^8$ - $8x10^9$	100 - 100
	0901393	0.56 - 0.65	$2x10^8$ - $2x10^8$	100 - 100
	CDI-A	0.51 - 0.58	$2x10^8$ - $2x10^9$	5 – 10
	10009061-1	0.54 - 0.66	$8x10^7$ - $3x10^8$	100 - 100
Trout	Coho 92	0.73 - 0.80	$4x10^8$ - $1x10^8$	20 – 5
	H2	0.77 - 0.89	$2x10^8$ - $8x10^8$	15 – 0
	B259	0.94 - 0.97 - 0.91*	$2x10^8$ - $6x10^8$ - $2x10^8$*	100 - 20 - 100*
	JIP 44/87	0.61 - 0.74	$2x10^8$ - $4x10^8$	0 – 0
	LVDJ D	0.63 - 0.36	$2x10^8$ - $2x10^7$	50 -15
	JIP P11/91	0.80 - 0.89 - 0.82*	$1x10^8$ - $4x10^8$ - $4x10^8$*	0 - 100 - 100*

* For trout isolates B259 and JIP P11/91 a third trial was performed

The optical densities of the cultivated broth varied between 0.51 and 0.97, with the lowest and highest OD not corresponding with the lowest and highest bacterial titre as determined by plate count, respectively.

In all control fish, no clinical signs nor mortality occurred and *F. columnare* could not be isolated from the gill samples.

For the carp, immersion challenge with four of the carp isolates (040178, 10012573/2, 0901393 and 10009061-1) resulted in 100% mortality within 9 to 12 h post inoculation in both trials. These isolates hence were assigned as highly virulent. The bacterial broth of these isolates was assigned viscous in both trials. Challenge with isolate CDI-A caused 5% mortality within 18 h in the first trial and 10% mortality within 11 h in the second trial, designating this isolate as low virulent. The bacterial broth of this isolate was less viscous compared to the cultured broths of the highly virulent isolates. No significant differences were found in the type of mortality (spontaneous or euthanized at the end of the experiment) between the two trials for each isolate. The survival analysis showed that the mortality rate for the low virulent isolate CDI-A differed significantly from the highly virulent isolates. The mortality rates of the highly virulent isolates did not differ significantly from each other. The average bacterial log transformed titres which could be retrieved from the carp gills and the corresponding percentage of fish from which *F. columnare* retrieval from the gills of euthanized fish after the challenge experiment was possible, are presented in Table 2. No *F. columnare* colonies could be isolated from any of the fish that were sacrificed at the end of the experiment, being the surviving fish inoculated with the low virulent isolate CDI-A and the control fish.

Table 2. Average ± standard deviation log transformed titres of *F. columnare* (in decreasing order) as determined by serial dilutions of the gill tissue of euthanized fish and the associated average % retrieval from the gill tissue after immersion challenge in carp

Carp isolate	Average log transformed titres	% retrieval
0901393	$7.73 \pm 0.647_{c*}$	100
10012573/2	$7.70 \pm 0.845_{bc}$	100
10009061-1	$7.38 \pm 0.697_{a}$	100
0401781	$7.38 \pm 0.912_{a}$	100
CDI-A	$7.13 \pm 0.977_{ab}$	100

*Values with a different subscript in the same column are significantly different ($p < 0.05$)

For the trout, in the first trial all fish challenged with isolate B259 died between 15 and 24 h post inoculation. However, in the second trial only 20% of the fish inoculated with B259 died starting 30 h until 72 h post challenge. In the third trial, mortality started 18 h post inoculation and by 54 h post challenge, all fish had died (Table 1). The bacterial broth of this isolate was assigned non-viscous in the trial in which it gave only 20% mortality, while in the other two trials in which 100% mortality was perceived, the broth was assigned viscous. The overall elicited mortality hence was 73%. In the group challenged with JIP P11/91, no mortality occurred in the first trial. In the second and third trial on the other hand, 100% mortality was perceived occurring between 15 h and 72 h in the second and within 18 h post challenge in the third trial, resulting in an overall mortality of 67%. As was the case for B259, the bacterial broth was assigned viscous in the trials in which this isolate resulted in 100% mortality compared to non-viscous in the trial in which it lead to no mortality. Since isolates B259 and JIP P11/91 were able to elicit 100% mortality in two trials, they were assigned as highly virulent. Fifty per cent of the fish inoculated with isolate LVDJ (D7461) died, of which 80% within 54 h and the other 20% within 138 h after inoculation. In the second trial, challenge with this isolate resulted in 15% mortality within 72 h. Challenge with isolate Coho 92 elicited 20% mortality between 9 and 99 h post inoculation in the first trial. In the second trial adopting this isolate, 5% mortality occurred 72 to 75 h post challenge. Inoculating the fish with isolate H2 generated 15% mortality between 15 and 51 h post challenge in the first trial but no mortality in the second trial. All fish inoculated with isolate JIP 44/87 survived. The isolate LVDJ (D7461) was designated as moderately virulent, whereas isolates Coho 92, H2 and JIP 44/87 were tagged low virulent. The trials differed statistically from one another ($p < 0.05$) as isolates JIP P11/91 and B259 gave only 0 to 20% mortality in one trial, while in the other two trials they elicited 100% mortality. For the other isolates, the mortality following challenge did not differ statistically in between the two trials. Survival analysis revealed no statistically significant differences in mortality rate between the highly virulent isolates. The survival of the fish inoculated with isolates Coho 92, LVDJ D, and H2 was significantly longer than for the highly virulent isolates.

The average bacterial log transformed titres which could be retrieved from the trout gills and the corresponding percentage of fish from which *F. columnare* retrieval from the gills of euthanized fish after the challenge experiment was possible, are presented in Table 3. *F. columnare* could not be retrieved from the gill tissue of the surviving fish at the end of the experiment, being the surviving fish inoculated with the low virulent isolates and the control fish, except for one fish inoculated with isolate H2 (average bacterial titre 6.50 E+01 CFU/100mg gill tissue).

Table 3. Average ± standard deviation log transformed titres of *F. columnare* (in decreasing order) as determined by serial dilutions of the gill tissue of euthanized fish and the associated elicited average % retrieval from the gill tissue after immersion challenge in trout

Trout isolate	Average log transformed titres	% retrieval
B259	$6.70\pm1.27_{a*}$	100
Coho 92	$6.21\pm1.49_{a}$	80
JIP P11/91	$6.21\pm1.56_{a}$	93
LVDJ D	$5.77\pm1.37_{a}$	100
H2	$4.49\pm1.90_{b}$	67
JIP 44/87	0.00_{c}	-

*Values with a different subscript in the same column are significantly different ($p < 0.05$).

4.4.2 Necropsy findings in carp

Macroscopic examination

The control fish did not reveal any abnormalities.

In the carp that died following inoculation with one of the highly virulent isolates, gill filaments of all four gill arches of both sides were affected displaying diffusely spread yet well-delineated yellowish-white discolourations (Figure 1). No skin lesions were discerned. The fish that were challenged with the low virulent isolate CDI-A did not display any macroscopic lesions.

Figure 1. Gill lesions in a carp fry 12 h post inoculation with the highly virulent *F. columnare* isolate 0901781. Diffuse yellowish-white discolorations of the gill filaments of all four gill arches are evident as the left operculum has been removed.

Light microscopic findings

In the control fish, no histopathologic lesions were perceived. Upon examination of the H&E stained gill sections of the fish that died following challenge with one of the highly virulent isolates, the discerned gill lesions ranged from mild to severe. In a quarter of these fish, mild to moderate

tissue damage consisting of desquamation of gill epithelial cells was observed coinciding with the presence of filamentous bacteria; no inflammatory cells nor fusion of the lamellae were noted. In a second quarter of the fish, lesions were moderate to severe comprising multifocal fusion of lamellae with lamellar loss mostly at the tips and in the middle of the filaments. Numerous filamentous bacteria clustered around the disintegrated lamellae and exfoliated epithelial cells. In addition, mild haemorrhage and the occasional presence of inflammatory cells with the morphology of macrophages were recorded. No fusion of the filaments was discerned. The gill sections of half of the fish depicted more severe lesions with diffuse merger of the filaments and extensive lamellar loss. The lamellae were replaced by necrotic debris and desquamated and inflammatory cells. On top of the filaments, huge clusters of long and slender bacteria embedded in an eosinophilic matrix and necrotic debris were perceived (Figure 2). In the few animals that died following inoculation with the low virulent isolate CDI-A, a minority of the lamellae showed mild fusion and only sporadically were filamentous bacteria noted. No other histopathologic lesions were present. In the fish that survived the CDI-A isolate challenge, no abnormalities were observed.

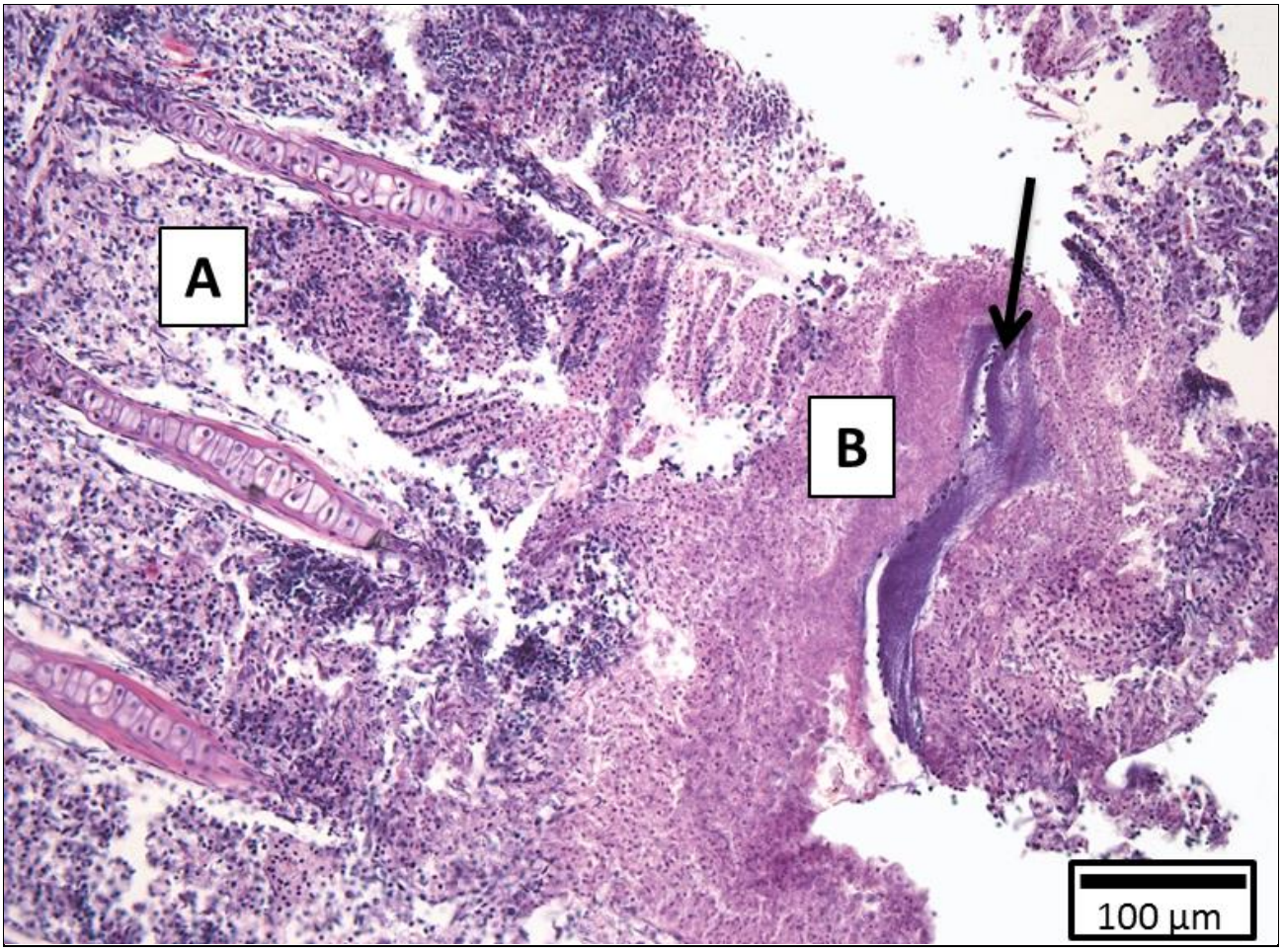

Figure 2. H&E-staining of the left gill of a carp fry 12 h after challenge with a highly virulent *F. columnare* isolate (0901781). Diffuse merging of the filaments (A) is visible with loss of all lamellae. Extensive micro-colonies of *F. columnare* cells (arrow) embedded in an eosinophilic matrix and necrotic debris (B) are present. Bar = 100µm.

Electron microscopic examination

The SEM and TEM pictures of the gills of the control fish did not reveal any abnormalities.

The severity of gill lesions as observed with SEM of the carp inoculated with one of the highly virulent isolates corresponded to the histopathologic traits as described above. Carp challenged with one of the highly virulent isolates revealed diffuse fusion of filaments and lamellae with extensive clumps attaching to the full length of filaments obscuring the epithelial finger printing pattern (Figure 3a).

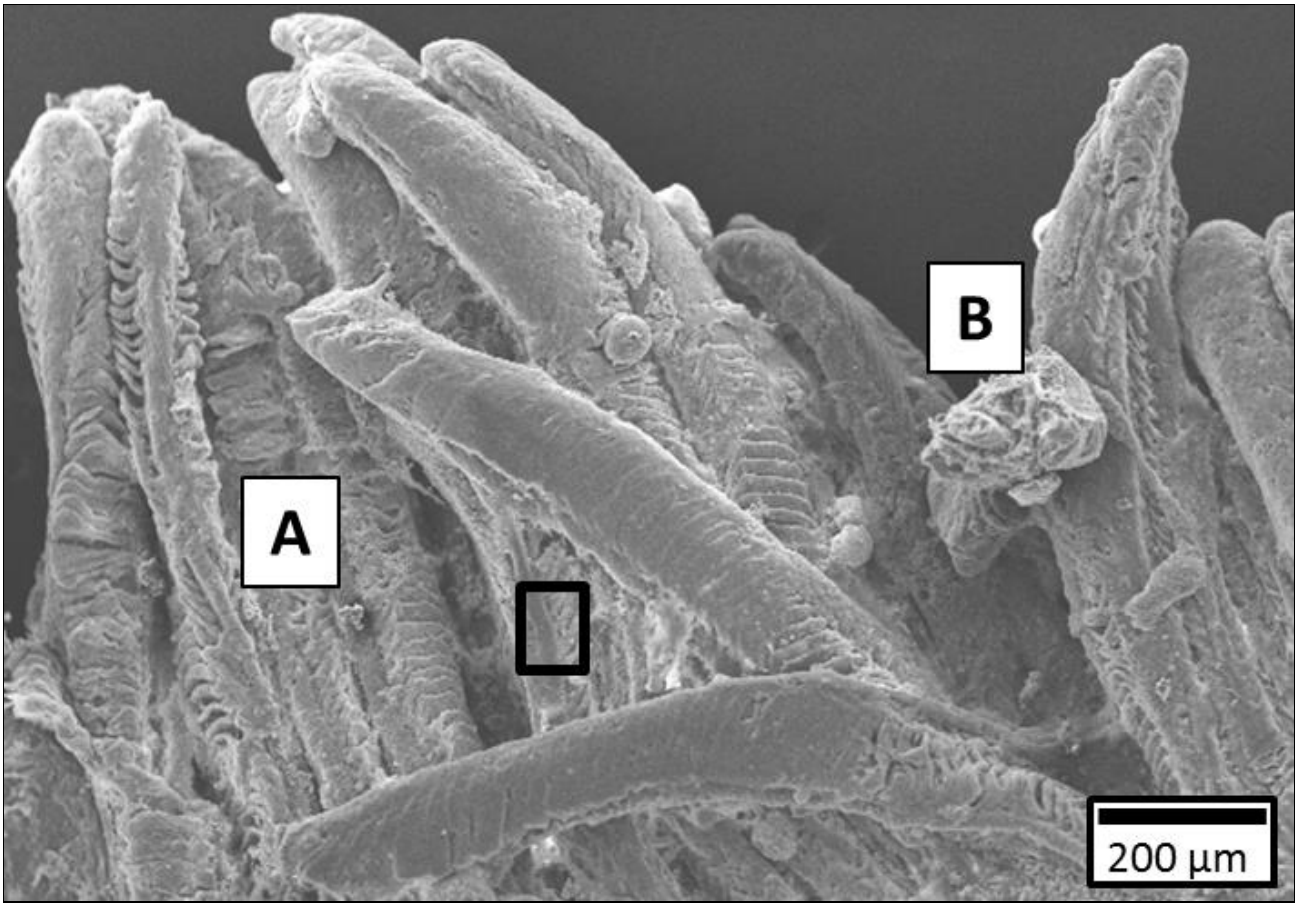

Figure 3a. SEM-picture of the gill tissue of a carp fry 12 h post-challenge with a highly virulent isolate (0901781). Diffuse merging of the filaments (A) is visible and alignment of filaments is lost. Mucus clots (B) are seen in between gill filaments. Inset : see figure 3b. Bar = 200µm.

On a higher magnification, these lumps were composed of microcolonies of long, slender, rod-shaped bacterial cells approximately 0.3-0.5 µm wide and 3-10 µm long. The bacteria were aggregated rather than spread individually throughout the epithelial surface. In between the dense twirl of bacterial cells, red blood cells and cellular debris were visible (Figure 3b). In the fish inoculated with the low virulent isolate, no abnormalities were noted.

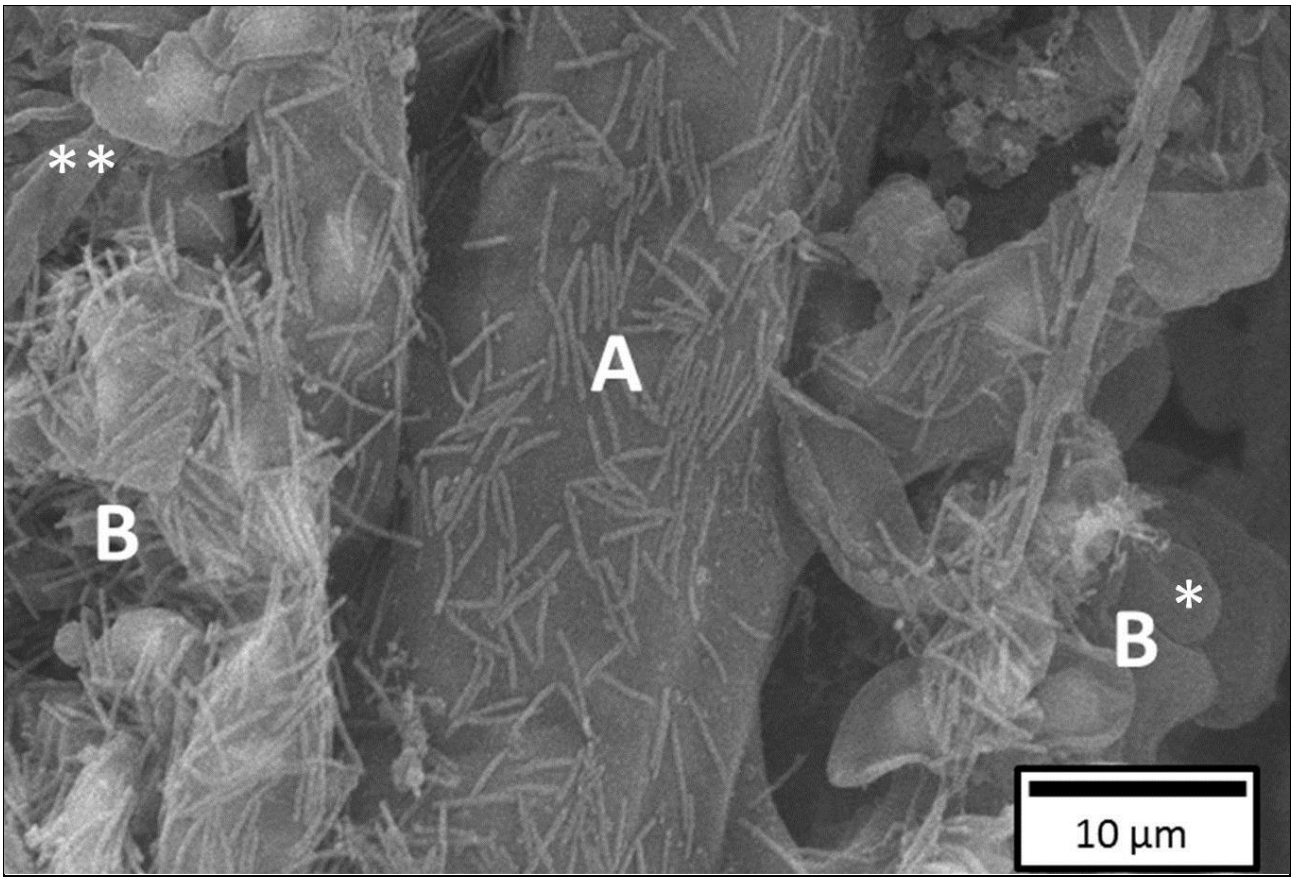

Figure 3b. Detail of figure 3a with slender bacterial cells (A) lining up along the gill filament. In between the lamellae, large clusters of bacterial cells are gathered and enwrapped in mucus clots, shed erythrocytes (*) and epithelial cells (**) (B). Bar = 10µm.

TEM-pictures of the gill tissue of the carp following challenge with one of the highly virulent isolates all revealed severe damage with oedema and cell necrosis prevailing and long and slender bacterial cells lining up along the lamellar epithelium and in between lamellae, wrapped in cellular debris (Figure 4). In the gills of fish surviving a challenge with the low virulent isolate and of fish euthanized at the end of the experiment, no abnormalities were discerned, apart from some slight oedema in only a minor part of the gills.

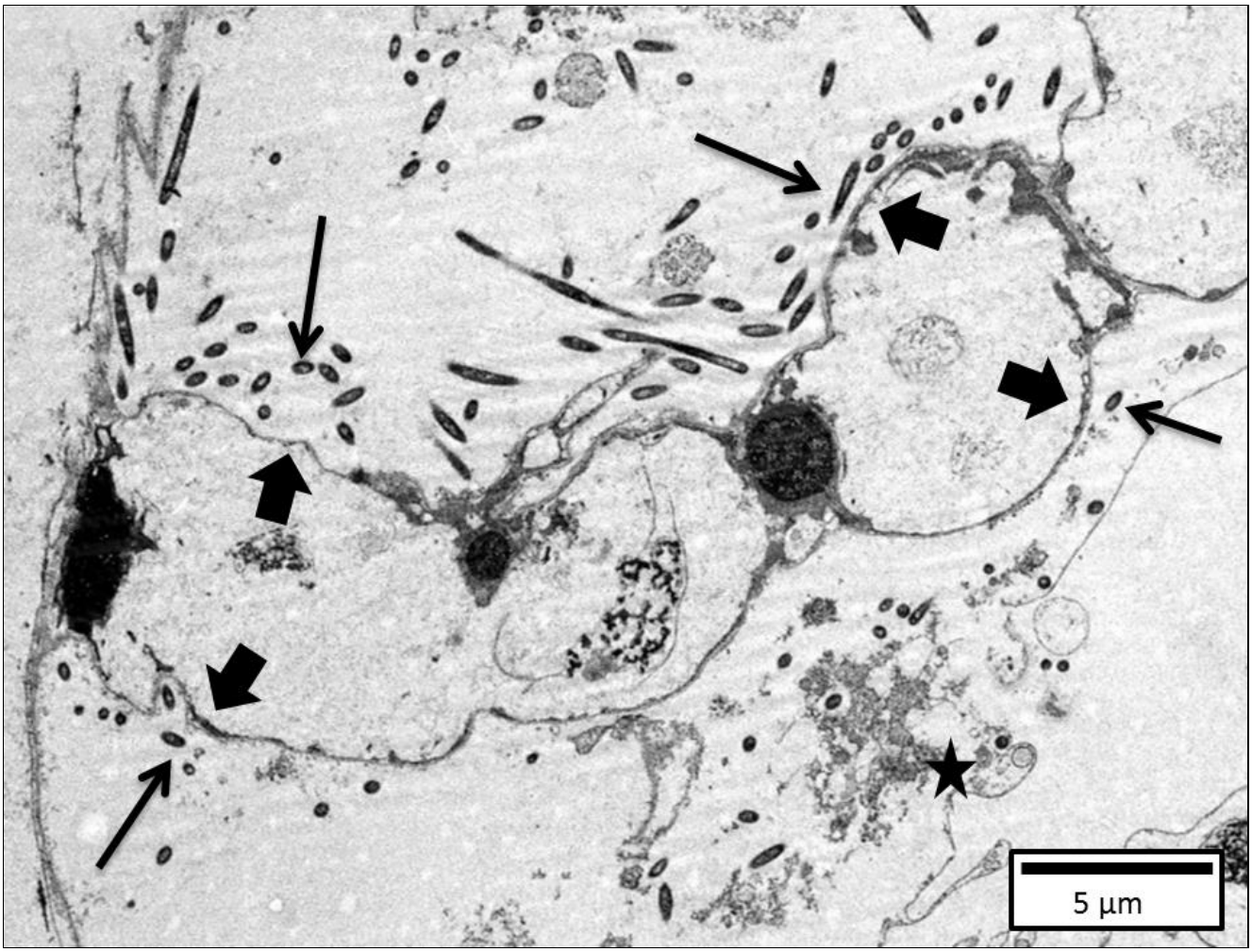

Figure 4. TEM-picture of a necrotic gill lamella 12 h post-challenge with a highly virulent isolate (0901781) in a carp fry. The pillar cell plasma membrane (thick arrows) is exposed and lies in close association with the bacterial cells (thin arrows). Notice the necrotic debris (asterisk). Bar = 5µm.

4.4.3 Necropsy findings in trout

Macroscopic examination

The control fish did not display any abnormal features.

In the rainbow trout that died following challenge with a highly virulent isolate, gill lesions mostly occurred on one side and were only visible in the gill filaments of the first two gill arches. The lesions appeared as discoloured foci of 5-10 mm scattered throughout the gill tissue (Figure 5).

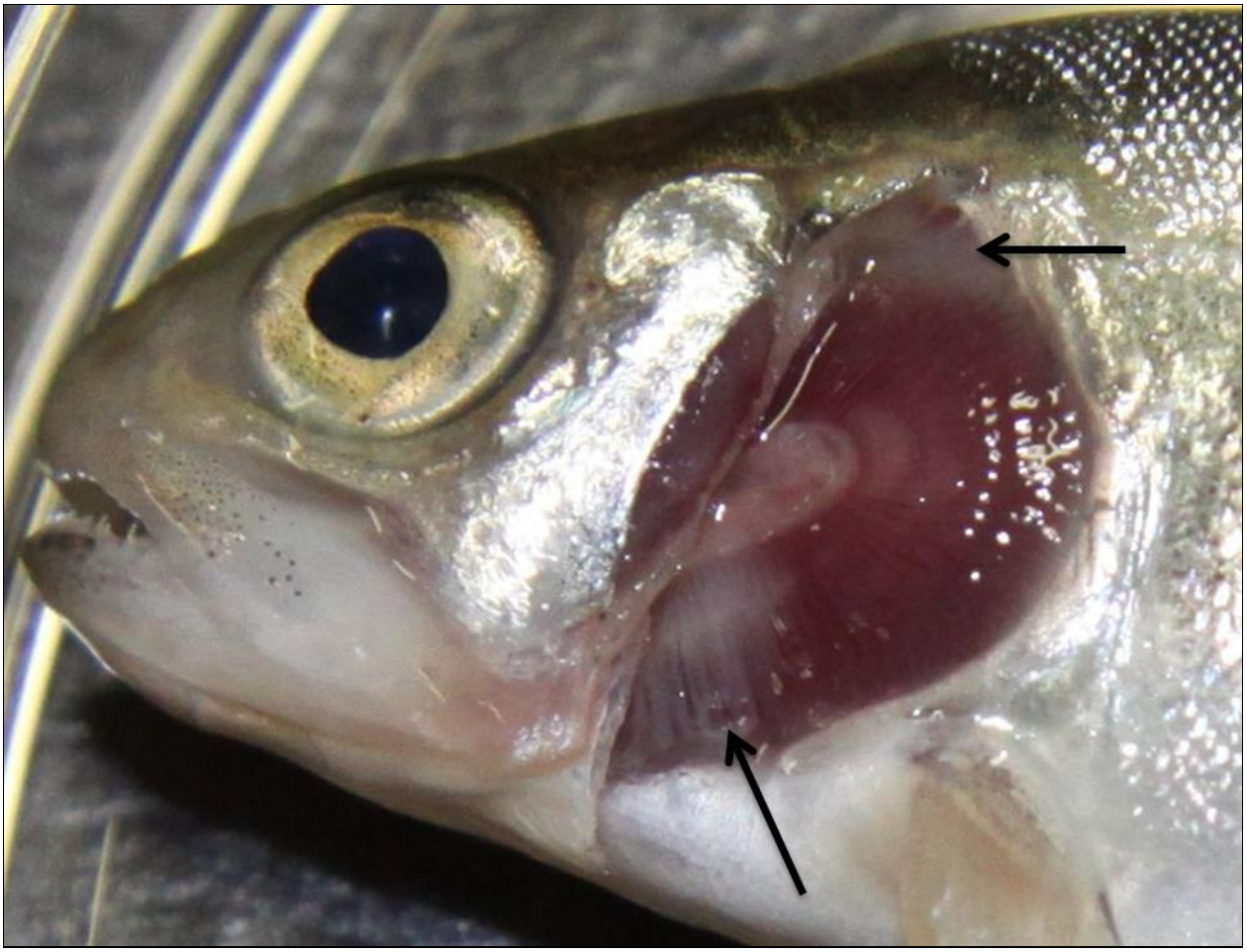

Figure 5. Gill lesions (arrows) appearing as two well-delineated discoloured areas (arrows) after removal of the left operculum in a rainbow trout fry 24 h post-challenge with a highly virulent *F. columnare* isolate (B259).

Half of the fish that died following challenge with a highly virulent isolate displayed pale discolourations of the skin typically starting around the dorsal fin and spreading laterally resembling a saddle. The lesions spread further ventrally and caudally towards the pelvic and anal fin. Skin lesions were also noted surrounding the pectoral fin. Progressively, the delicate tissue in between the fin rays disappeared, exposing the latter, especially at the level of the dorsal fin (Figure 6). The fish died within 12 h following the appearance of the skin lesions.

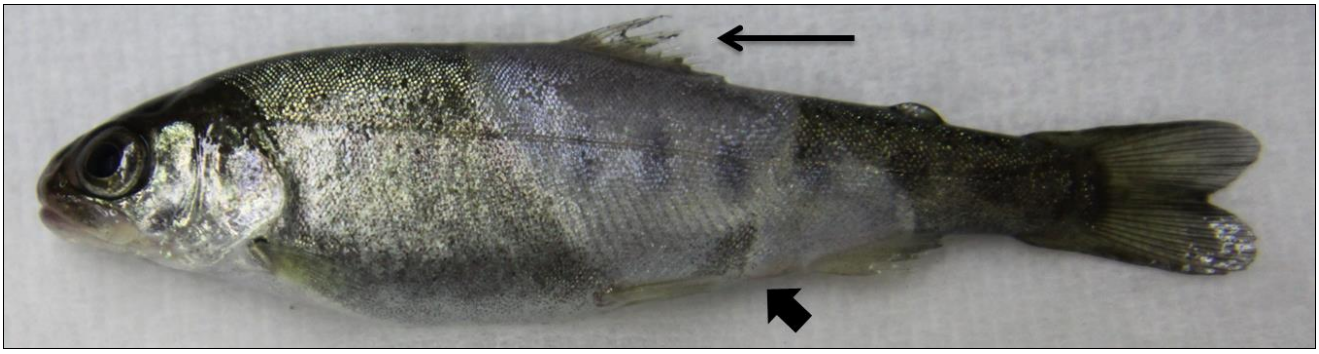

Figure 6. Rainbow trout fry showing typical skin lesions 24 h following challenge with a highly virulent *F. columnare* isolate (B259). Skin discolouration starting dorsally and spreading laterally towards the pelvic fin and caudally towards the anal fin, resembling a saddle (thick arrow) is evident. Discoloured areas of the skin surrounding the pectoral fin are also noted. The dorsal fin rays are exposed due to disappearance of the delicate fin tissue (thin arrow).

The rainbow trout that died after inoculation with a moderately or low virulent isolate or that were sacrificed at the end of the experiment, did not reveal any macroscopic lesions.

Light microscopic findings

In trout, the gills of the control fish did not display any abnormalities.

The gill lesions in the fish challenged with the highly virulent isolates were distributed focally, involving up to half of the gill filaments. In these areas, the lamellar epithelium was necrotic or had disappeared completely and was replaced by a microcolony of filamentous bacteria encased in an eosinophilic matrix (Figure 7 left and right). Leukocytes with the morphology of macrophages and eosinophilic cells, and red blood cells were also noted. In adjacent lamellae, mild to moderate oedema was present. One fourth of the animals that died following inoculation with a highly virulent isolate showed less severe histopathological lesions. The gill epithelium of fish that had died following challenge with the moderately and low virulent isolates were mildly dissociated from the capillary bed. Fusion of the lamellae and the presence of inflammatory cells were also noted. The gill tissue of the fish that survived the challenge showed no abnormalities apart from mild oedema.

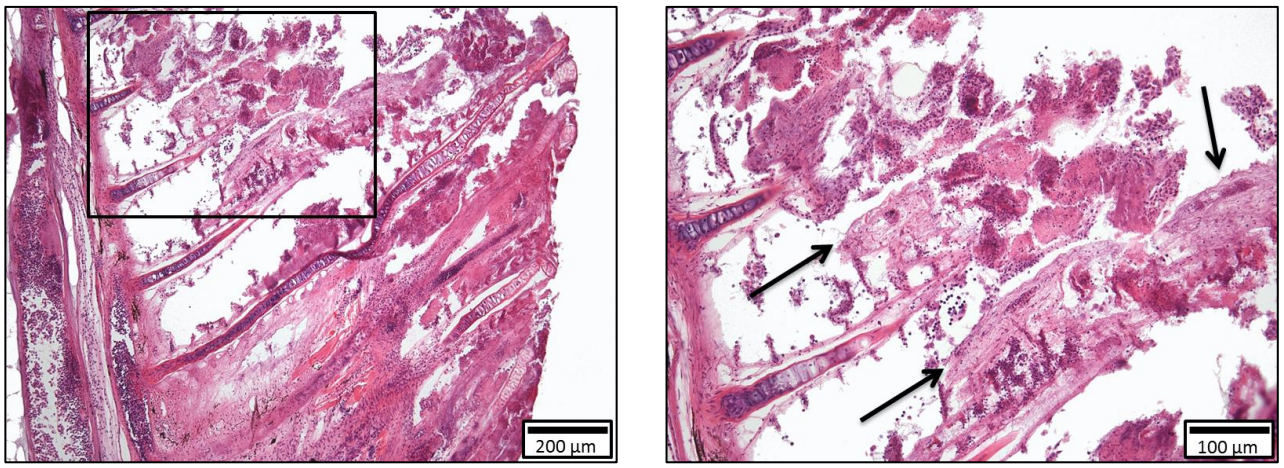

Figure 7. Left. H&E-staining of the first left gill arch of a rainbow trout fry 27.5 h post-challenge with a highly virulent *F. columnare* isolate (JIP P11/91). The upper four gill filaments reveal lamellar necrosis and fusion of the lamellae at the middle parts and at the tips. The four gill filaments situated in the lower figure half are fused, their gill lamellae are fully necrotic and replaced by *F. columnare* bacterial cells. Square = inset. Bar = 200µm. **Right.** Detail of left figure. The necrotic lamellae are nearly completely replaced by vast microcolonies of long, slender bacteria (arrows). Bar = 100µm.

Histopathology of the skin lesions revealed epidermal ulceration and necrosis with loss of scales accompanied by haemorrhage, massive infiltration of long, slender bacteria embedded in an eosinophilic material and the presence of inflammatory cells. Bacterial cells even invaded the deeper skin layers resulting in interstitial infiltration of the fat and muscle tissue with myositis.

Electron microscopic examination

The SEM and TEM pictures of the gills of the control fish did not reveal any abnormalities.

For the trout inoculated with one of the highly virulent isolates, the affected tissue sites as observed using SEM showed multifocally distributed merger of filaments and lamellae. Massive bacterial clusters embedded in between mucus, red blood cells and cellular debris were present. The bacterial cells measured 0.3-0.5 µm in width and 3-10 µm in length. The epithelial fingerprinting pattern of the gill filament was still discernible in sites adjacent to the *F. columnare* microcolony formation (Figure 8). SEM sections of the gills of the trout challenged with the moderately and low virulent isolates and the animals sacrificed at the end of the experiment did not reveal any abnormalities.

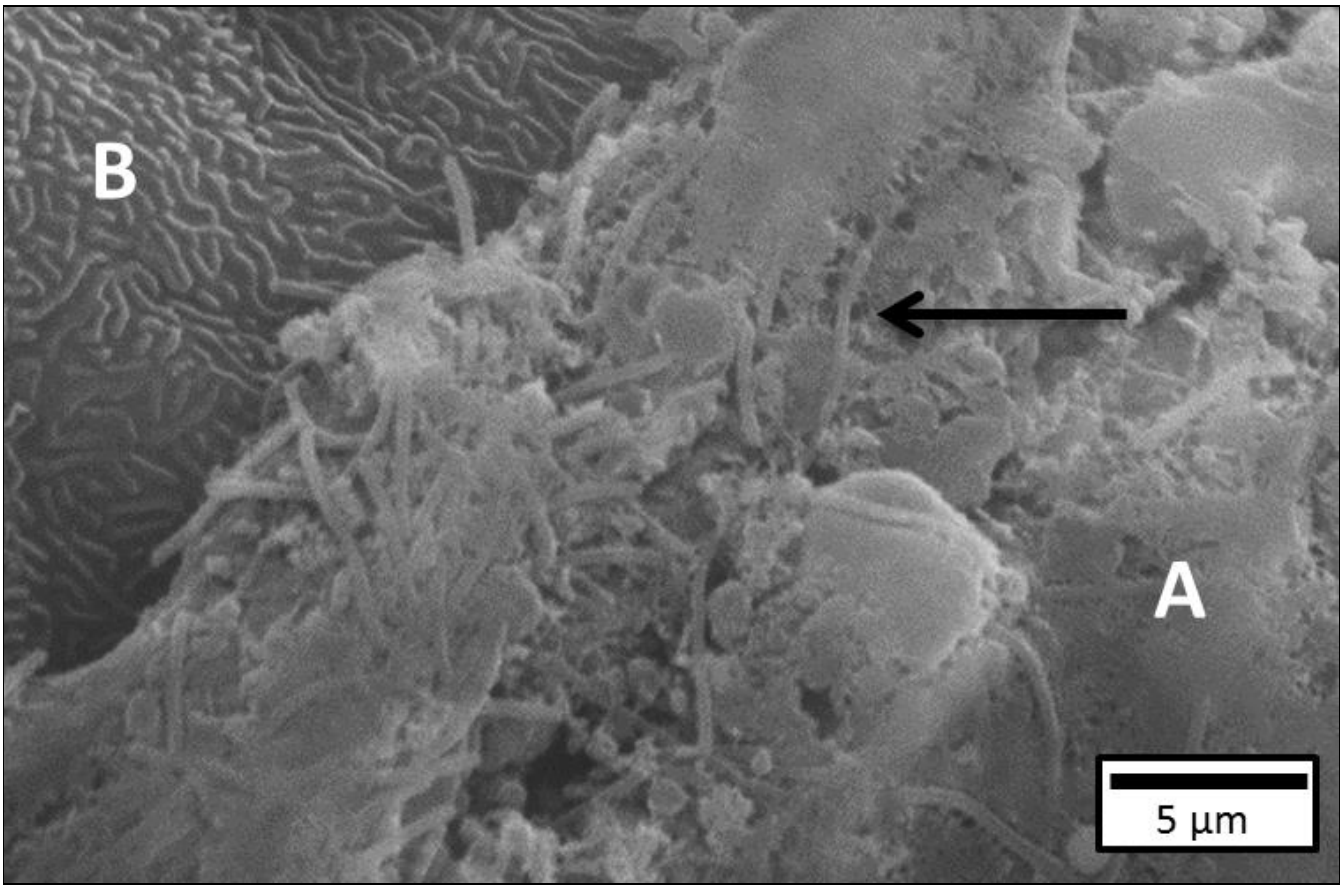

Figure 8. SEM-image of the gill tissue of a rainbow trout fry 27.5 h following challenge with a highly virulent *F. columnare* isolate (JIP P11/91). In the right part of the picture, clusters of long and slender bacterial cells (arrows) enwrapped in a matrix of mucus, red blood cells and cell debris are seen (A). In the left upper part, adjacent to the bacterial cluster, the normal fingerprinting pattern of the gill filament (B) is still recognizable. Bar = 5µm.

TEM of the gills of rainbow trout challenged with one of the highly virulent isolates revealed the lamellar epithelium to be surrounded by bacteria, cellular debris and inflammatory cells. Loss of the normal lamellar structure with lifting of the epithelium and consequent lamellar necrosis were noted, especially in the middle and at the tips of the filaments. In one of the gills of a fish that died following challenge with a moderately virulent isolate, the presence of a few long, slender bacterial cells adjacent to the gill epithelium could be visualized and oedema and lamellar necrosis were visible. In all other samples of fish inoculated with the moderately or low virulent isolates, no bacterial cells were discerned. Lifting of the epithelium did occur in the latter samples.

4.5 DISCUSSION

Hitherto, the virulence mechanisms of *F. columnare* are still far from fully elucidated. Especially the way this pathogenic agent interacts with and causes lesions to the gill tissue remains to be explored, with even a detailed description of the elicited gill lesions currently lacking. This scarcity of data is largely rooted in the absence of a reproducible infection model inducing gill lesions as observed in the field. To our knowledge, this study is the first to pinpoint an experimental infection model in carp and rainbow trout through immersion by means of which such severe gill lesions typical for columnaris disease are elicited. In addition, in this study the gill lesions are described in detail on a macroscopic, a light microscopic and an ultrastructural level.

In the challenge protocols for columnaris disease that were published so far, cultivation and harvesting methods, inoculation temperatures, incubation periods and whether or not tissues need to be scarified, are only some of the existing variances, even those implementing the same fish species. These variations have recently been reviewed by Declercq et al. (2013b) and point towards the delicate balance and complex interplay between bacterial cells, fish and environment in the successful reproduction of this bacterial disease. Indeed, for the latter, there are a number of obstacles to overcome. Firstly, there is the tendency of some *F. columnare* isolates to clump in broth culture and adhere strongly to the agar surface (Bernardet and Bowman, 2006; Darwish et al., 2008; Kunttu et al., 2011), rendering it difficult to make a homogeneous suspension and thus adequately determine bacterial titres in challenge inoculum and tissues. This phenomenon indeed poses a true challenge for developing standardized inoculation procedures. This non-homogeneous growth was also exhibited by various isolates used in the current study, as amongst others reflected by the lack of correlation between OD and bacterial titration values determined by plate count. Clumpy cultures make it difficult to take a reliable subsample for determining bacterial titres. This clump-like bacterial growth could additionally cause an underestimation of the bacterial numbers as obtained by plate culturing, when more than one bacterial cell gives rise to one colony. Using quantitative polymerase chain reaction (qPCR) to determine bacterial titres of the inoculation broth could in this respect pose an aid to more reliably determine bacterial inoculation titres. This technique has been described for *F. columnare* (Panangala et al. 2007) and in merely a few hours, researchers could consult the results and decide whether or not to include bacterial isolates in the challenge. A disadvantage compared to bacterial titration is that dead bacteria are also counted, a problem which could be circumvented by combining an ethidium bromide monoazide (EMA) treatment and real-time (RT)-PCR, allowing differentiation of viable from dead bacteria, as recently described for quantitative detection of *Helicobacter suis* in pork (De Cooman et al., 2013). With regard to determining the number of *F. columnare* bacteria in tissues and comparing these values in between

isolates, besides using plate counting, it is advisable to substantiate these findings using e.g. qPCR or microscopy imaging techniques as was done in the current study. Secondly, in order to be able to develop a reliable challenge model, specific *F. columnare* free fish are needed. Various research groups have described that fish may dwell in a clinically healthy carrier status housing an isolate remaining from a previous infection with columnaris disease (Fujihara and Nakatani, 1971; Suomalainen et al., 2005a). Should carrier fish be included, then the interaction patterns of the test isolates may be ruffled and hamper a reliable outcome of the experiment. Therefore, in this study, before purchasing the fish, several individuals from carp farms and retailers of varying origin were sampled for the presence of *F. columnare* using both cultivation and the *F. columnare* specific PCR. All fish were negative by culture but positive in PCR (data not shown). Indeed, based on results of Suomalainen et al. (2005a), culture-based methods proved to be insufficiently reliable for diagnosing flavobacterial diseases from fish tissues. For this reason, this study used PCR to label the fish as specific *F. columnare* free. The carp that were eventually included in this study were purchased as two-day old larvae from a farm with no history of columnaris disease. The latter also was the case for the rainbow trout farm from which the trout fry were retrieved. All animals were housed under strict quarantine conditions. Adopting both cultivation and PCR techniques to demonstrate the *F. columnare* free status of the included animals has to our knowledge not been done before, giving this study a marked surplus value.

A remarkable feature was that the highly virulent trout isolates JIP P11/91 and B259 induced no or only 20% mortality, respectively, in one out of the three trials in which they were adopted. This provides the rationale for the fact that the trial with both isolates was carried out three times in the course of time. Noteworthy is that in the trials where no or only low mortality was induced, the cultivated broth was much less viscous compared to the other trials in which these two isolates displayed highly viscous cultures and induced 100% mortality. It has been described by Kunttu et al. (2009, 2011) that one isolate may indeed produce different colony types with varying virulence. The authors stated that colony morphology changes might be caused by phase variation, and that different colony types isolated from infected fish might indicate different roles of the colony morphologies in the infection process of columnaris disease (Kunttu et al., 2011). Unfortunately, no correlations or speculations on the viscous character of the associated broth cultures were given, lending this topic merit for further research with the factors determining the degree of viscosity of the culture and the possible interplay between viscosity, colony type and virulence to be elucidated.

In channel catfish, genomovar II is considered to be more virulent than genomovar I (Olivares-Fuster et al., 2007a, Shoemaker et al., 2008). Challenge of rainbow trout with genomovar I and II isolates of *F. columnare* demonstrated in one study to induce significantly higher cumulative percent

mortalities (LaFrentz et al., 2012) while in another study genomovar I demonstrated a high degree of pathogenicity against rainbow trout fry at 15°C (Evenhuis et al., 2014). In this study, all adopted isolates belonged to genomovar I. Hence, a correlation between virulence and genomovar could not be made.

Both macro- and microscopic examination of the affected gills revealed a different distribution pattern of the lesions as induced by the highly virulent *F. columnare* isolates in carp compared to trout. Indeed, the gills of the affected carp showed a diffuse distribution of the lesions, affecting the filaments of all gill arches bilaterally. At least half of the gill tissue was destroyed, with filamentous bacterial cells spread diffusely, clustering on top of the filaments and forming vast microcolonies. In trout, the distribution pattern of the gill lesions was more focal, mostly located unilaterally and only present in the gill filaments of the two first gill arches.

Data resulting from the bacteriologic and histopathologic examination of the gill tissue of fish with the low and moderately virulent isolates, do not seem to correlate. Indeed, the *F. columnare* gill titres following challenge with the low virulent carp (CDI-A) and trout (Coho 92) isolate and the moderately virulent trout (LVDJ) isolates were high whereas upon histopathologic examination of the sampled gill tissue, hardly any filamentous bacteria were discernible. The possible explanation for this phenomenon may be two-fold. Firstly, one should consider the fact that only a limited part of the gill tissue is examined by means of histological examination. If adhesion is rather localised or if the gills are only focally colonised, the bacteria may not be detected by histology but can be found by means of bacteriology, as was noted previously by Decostere et al. (1999). Secondly, the few bacterial cells observed within the gill tissue inoculated with the low virulent isolates were not in close contact with the host epithelium. These bacteria most likely are part of the aqueous biofilm covering the gill tissue and can hence be noted upon bacteriological examination when plating out the tissue samples. However, the processing of the gill tissue for histological examination involves several staining and washing steps with the resulting tissue sections visualizing only firmly attached bacteria. The presence of the latter seems to be the case only for the highly virulent isolate and to a much lesser extent for the moderately and low virulent isolates, explaining the sparsely present bacterial cells upon histological inspection.

Indeed, upon examination of the H&E stained gill sections of the carp and trout challenged with a highly virulent isolate, massive infiltrates of *F. columnare* cells were seen attached to the gill tissue along the gill filaments and lamellae and entangled in between necrotic tissue. This feature typically resembles biofilm formation, a phenomenon which has recently been studied *in vitro* for *F. columnare* (Cai et al., 2013). To our knowledge, this is the first time massive clusters

of microcolonies resembling biofilm formation are being visualized in the gill tissue of both carp and rainbow trout following experimental challenge. These *in vivo* findings are consistent with the demonstration of genes in *F. columnare* encoding for biofilm formation by the research group of Tekedar et al. (2012). The filamentous bacterial cells were seen encased in an eosinophilic matrix. It is tempting to speculate on the origin of this observed matrix, viz. whether it is bacterium- or host-related. This embedment may afford protection for the bacterial cells against outer factors, such as fish mucus containing antimicrobial substances, and even impede the penetration of antimicrobial agents that are administered via the water. Hence, this matrix-formation may constitute an important step in the pathogenesis of columnaris disease and needs to be studied in greater detail.

In conclusion, the current study describes an immersion inoculation model in carp and rainbow trout that can be used to elicit gill lesions typical for columnaris disease as observed in the field. In this way, an in-depth study of the interaction of this pathogen with its target tissue is facilitated. In addition, the gill lesions described in detail urge to study the progression from intact to partially or completely destroyed gill tissue as a means to clarify the pathogenesis of columnaris disease and contribute to the development of efficient methods to combat the disease without having to resort to antimicrobial agents.

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CHAPTER 5: INTERACTIONS OF HIGHLY AND LOW VIRULENT *FLAVOBACTERIUM COLUMNARE* ISOLATES WITH GILL TISSUE IN CARP AND RAINBOW TROUT

This chapter is based on:

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CHAPTER 5: INTERACTIONS OF HIGHLY AND LOW VIRULENT *FLAVOBACTERIUM COLUMNARE* STRAINS WITH GILL TISSUE IN CARP AND RAINBOW TROUT

5.1 ABSTRACT

The interactions of *Flavobacterium columnare* isolates of different virulence with the gills of carp (*Cyprinus carpio* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum) were investigated. Both fish species were exposed to a differing highly (HV) or a low virulent (LV) isolate and sacrificed at seven predetermined times post-challenge. Histopathological and ultrastructural examination of carp and rainbow trout inoculated with the HV-isolate disclosed bacterial invasion and concomitant destruction of the gill tissue, gradually spreading from the filament tips towards the base, with outer membrane vesicles surrounding most bacterial cells. In carp, 5-10% of the fish inoculated with the LV-isolate became moribund and their gill tissue displayed the same features as described for the HV-isolate, albeit to a lesser degree. The bacterial numbers retrieved from the gill tissue were significantly higher for HV- compared to LV-challenged carp and rainbow trout. TUNEL-stained and caspase-3-immunostained gill sections demonstrated significantly higher apoptotic cell counts in carp and rainbow trout challenged with the HV-isolate compared to control animals. Periodic acid-*Schiff*/alcian blue staining demonstrated a significantly higher total gill goblet cell count for HV- and LV-isolate challenged compared to control carp. Moreover, bacterial clusters were embedded in a neutral matrix while being encased by acid mucins, resembling biofilm formation. Eosinophilic granular cell counts were significantly higher in the HV-isolate compared to LV-isolate inoculated and control carp. The present data indicate a high colonization capacity, and the destructive and apoptotic-promoting features of the HV-isolate, and point towards important dynamic host mucin–*F. columnare* interactions warranting further research.

5.2 INTRODUCTION

Columnaris disease, caused by the Gram-negative bacterium *Flavobacterium columnare*, is notorious in freshwater aquaculture, amongst others of carp (*Cyprinus carpio* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum), in which it induces severe economic losses due to gill, skin and fin lesions often resulting in high mortality (Bernardet and Bowman, 2006; Decostere et al., 1998; Figueiredo et al., 2005; LaFrentz et al., 2012; Morley et al., 2010; Řehulka and Minařík; Shoemaker et al., 2011; Soto et al., 2008; Suomalainen et al., 2009; Tripatho et al., 2005). Recently, the bacterium-host interactions of columnaris disease were reviewed, whereby the various prevailing knowledge gaps were highlighted (Declercq et al., 2013b). The mechanisms adopted by the pathogen to establish itself and to maintain a grip on the skin and the gill tissue, and consequently to elicit disease and mortality, are far from fully elucidated. Especially the interplay of *F. columnare* with the gill tissue still puzzles the research community. Hitherto, only a few studies explored the interaction between *F. columnare* and the gill tissue (Decostere et al., 1999a; Peatman et al., 2013; Sun et al., 2012) focussing on host mucosal responses. Sun et al. (2012) studied the transcriptomic profiling of host responses in the gill tissue to columnaris disease following experimental challenge in catfish and found a rhamnose-binding lectin with putative roles in bacterial attachment and aggregation, and several immune suppressive pathways being stimulated after infection with *F. columnare*. Accordingly, Peatman et al. (2013) found resistant catfish to have a higher expression of immune stimulating genes in the gills following challenge with *F. columnare* as compared to susceptible fish which showed high expression levels of a rhamnose-binding lectin and several mucosal immune suppression factors, possibly predisposing them to *F. columnare* infection.

In a recent study, variation in virulence between different *F. columnare* isolates retrieved from carp and rainbow trout was shown and the highly virulent strains induced severe gill lesions in experimentally infected carp and rainbow trout (Declercq et al., 2015). The carp showed a diffuse lesion pattern, affecting the gill filaments of all gill arches bilaterally and the animals died within 12 h after inoculation. In rainbow trout, the distribution pattern of the gill lesions was more focal and only present in the gill filaments of the first gill arches. Mortality started 15 to 18 h after inoculation, also reaching 100% within 72 h.

To obtain better insights in the interaction of *F. columnare* isolates of differential virulence with the gills of carp and rainbow trout, the sequence of events taking place at the level of the gill tissue following challenge with a highly and a low virulent isolate was mapped. Gill health status, pathogen localisation and spread, degree of apoptosis, changes in chloride cell number, quantitative and qualitative mucus changes and bacterial cell counts were investigated at seven predetermined

sampling points post-challenge. By merging the retrieved data, we sought to further elucidate the *F. columnare*-gill interplay.

5.3 MATERIALS AND METHODS

5.3.1 Fish

Two-day old carp fry were obtained from a Belgian hatchery and grown to a mean length of six centimetres before inclusion in the experiment. Rainbow trout with an average length of five centimetres were purchased from a Belgian hatchery (Villers-le-Gambon, Belgium) and acclimatized for one month in our facilities. The fish were maintained in one cubic metre stocking tanks filled with 800 L of recirculating and aerated tapwater. The water temperature was 22 ± 1°C for the carp and 19 ± 1°C for the rainbow trout. Starting from two weeks before the experimental challenge, the water temperature of the stocking tanks was gradually increased by 1°C every two days until a temperature of 25 ± 1°C and 22 ± 1°C was reached for the carp and rainbow trout, respectively. This water temperature was then kept constant until the onset of the challenge. Free and ionized ammonia and nitrite concentrations were determined daily and were below detectable levels at all times. The pH of the water was 8. A photoperiod of 12 h light / 12 h darkness was provided and the fish were fed a commercial diet (rainbow trout: Trouw Nutrition, carp: Fin Perfect Feed, Sonubaits) to satiation twice daily. Fish were deprived from food 24 h prior to the experimental challenge. Twenty-five carp and twenty-five rainbow trout were sacrificed with an overdose of benzocaine (ethylaminobenzoate; Sigma, Belgium; 10g/100mL acetone). Parasites were not observed during microscopic examination of wet mount preparations, made from scrapings of the gill and skin tissue. Gill and skin were also screened for the presence of *F. columnare* by means of Polymerase Chain Reaction (PCR) and bacteriological examination using cultivation onto modified Shieh agar (Shieh, 1980; Song et al., 1988) containing 1µg/mL tobramycin (Decostere et al., 1997). For the PCR, DNA from the tissue samples was extracted using a DNeasy blood and tissue kit (Qiagen, Venlo, the Netherlands), according to the guidelines of the manufacturer. PCR mixtures, primer sequences and cycle conditions were as described before (Declercq et al., 2013a; Panangala et al., 2007). *F. columnare* or its DNA were not detected in these samples.

5.3.2 Bacterial propagation

For each fish species, a highly virulent (HV) and a low virulent (LV) isolate with a known virulence profile, as described by Declercq et al. (2015) were used. Isolates that were able to elicit 80% mortality or more within 72 h were assigned as highly virulent, whereas isolates causing 20%

mortality or less were designated low virulent (Declercq et al., 2015). Carp were experimentally inoculated with isolates 0901393 (HV) and CDI-A (LV), obtained from diseased carp. Rainbow trout were challenged by isolates P11/91 (HV) and JIP 44/87 (LV), sampled from diseased trout (Declercq et al., 2013a). All four isolates belonged to genomovar I, as determined at the Aquatic Microbiology Laboratory of Auburn University (USA) using 16S-Restriction Fragment Length Polymorphism according to the protocol described by Olivares-Fuster et al. (2007). For more information concerning origin of the isolates, the reader is referred to Declercq et al. (2013a).

The isolates were grown for 36 h at 28°C on modified Shieh agar plates (Shieh, 1980; Song et al., 1988). For each isolate, five colonies per plate were sampled and transferred to 15 mL Falcon tubes filled with 4 mL of modified Shieh broth, which were placed overnight on a shaker at 28°C at 100 rpm. The content of two Falcon tubes of these initial broth cultures was added to an additional 392 mL of modified Shieh broth in 500 mL glass bottles and again incubated for 24 h at 28°C on a shaker at 100 rpm. The content of these bottles was used in the immersion challenge studies and the bacterial titres were determined by making tenfold dilution series in triplicate on modified Shieh agar plates.

5.3.3 Experimental challenge

A group of 27 randomly chosen carp or rainbow trout was removed from the stocking tanks and placed in a 10 L inoculation tank filled with 4.6 L of aerated water at 27 ± 1°C (carp) or 23 ± 1°C (rainbow trout). Per fish species, three predetermined groups were included in duplicate; fish to be inoculated with either the HV- or LV-isolate and a control group. Then, 400 mL of cultivated modified Shieh broth containing the *F. columnare* isolates was added to the proper water tank. The bacterial titres of the immersion baths ranged between 1.6 and 6.4 x 10^7 CFU/ml for the carp and between 3.2 x 10^7 and 1.6 x 10^8 CFU/ml for the trout isolates. A control group was included, constituting fish immersed in a tank with water supplemented with 400 mL modified Shieh broth not containing *F. columnare*. After a 90 min inoculation period, each group of 27 fish was transferred to a 60 L tank with trickling filter filled with 48 L of recirculated aerated tapwater of 25 ± 1°C for the carp or 22 ± 1°C for the rainbow trout. The fish were clinically monitored every 30 minutes and three fish per tank were sacrificed with an overdose of benzocaine (10g/100ml acetone) at nine predetermined time-points i.e., 1, 2.5, 4, 6, 8, 9.5, 15.5, 24 and 36 h post inoculation (PI). As soon as the humane endpoints (isolation in a corner, swimming at the water surface, loss of balance) were reached, the fish were sacrificed with an overdose of benzocaine. In case dead fish were encountered, these were immediately removed from the aquaria and sampled only for bacteriological analysis, and not for histological nor ultrastructural examination. Of all sacrificed fish, the filaments of the gill arches were inspected and the first two left gill arches were removed

and sampled for histological, (immuno)histochemical, scanning (SEM) and transmission (TEM) electron microscopic examination. In all animals, the counterpart right gill arches served for bacteriological examination for *F. columnare* by means of bacterial titration by making tenfold dilution series of the gill tissue in triplicate on modified Shieh agar plates; additionally in the control animals PCR was performed as described before (Declercq et al., 2015). All experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University under the project number EC2012/60.

5.3.4 Histological and (immuno)histochemical examination

Histological sections were used to enable a step-by-step tracking of microscopically discernible gill lesions in the course of time. Particular attention was paid to the localisation and arrangement of long slender bacterial cells with the typical *F. columnare* morphology, possible shifts in amount and type of mucins, and the type and spread of gill lesions (top, middle or base of the filaments and lamellae).

The gill tissue sections were fixed for 24 h in 4% phosphate-buffered formaldehyde, dehydrated in graded alcohol-xylene series and embedded in paraffin wax using the STP 420 Microm Tissue Processor and the embedding station EC 350-1 and 2 (Microm, Prosan, Merelbeke, Belgium), respectively. All tissues were sectioned (8µm) (Microm microtome HM 360, Prosan) and stained with haematoxylin and eosin (H&E). A combined periodic acid *Schiff*/alcian blue (PAS/AB) stain at pH 2.5 was additionally applied, allowing mucous cells/mucin to stain blue (AB-positive, acid mucins), purple (PAS/AB-positive, neutral combined with acid mucins) or magenta (PAS-positive, neutral mucins).

In addition, (immuno)histochemistry was adopted to visualize apoptotic cells and ATP-ase activity of chloride cells. Therefore, 5µm thin paraffin embedded tissue sections were mounted on 3-aminopropyl-triethoxysilane-coated slides (APES, Sigma, St. Louis, MO, USA), dried for 1 h at 60°C on a hot plate and further dried overnight at 37°C. To discern apoptosis, the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end-labelling (TUNEL) methodology was used for discerning DNA fragmentation. The TACS^{TM} TdT in situ apoptosis detection kit (R&D Systems Europe Ltd, Abingdon, UK) was adopted following the protocol as described by Van Cruchten et al. (2003). In addition, caspase-3 activity was determined using a polyclonal rabbit IgG human/mouse active caspase-3 antibody (1/400, R&D Systems Europe Ltd, Abingdon, UK) and the Anti-Rabbit HRP-AEC Cell & Tissue Staining Kit (R&D Systems Europe Ltd, Abingdon, UK). The protocol employed was modified from Van Cruchten et al. (2003)

with the difference that 50 µl of a labelled polymer of the Dako EnVision + System/HRP, Rabbit (DAB+) kit was used according to the instructions of the manufacturer. To detect the ATPase activity of the chloride cells, a monoclonal mouse antibody Na, K- ATPase (1/200, University of Iowa, Department of Biological Sciences) and the Dako EnVision + System/HRP, Mouse (DAB+) (DakoCytomation, Glostrüp, Denmark) staining kit (Ref.K4007) were applied according to the instructions of the manufacturer.

The (immuno)reactive cells on the (immuno)histochemically stained sections and the PAS-positive, PAS/AB-positive, AB-positive goblet cells and eosinophilic granular cells (EGC) on PAS/AB stained sections were quantified on three randomly selected gill filaments. Counting was done at the tip, middle and base of these filaments. For all goblet, EGC- and chloride cell counts, results are expressed as the number of cells per 100 µm gill filament. For the TUNEL- and caspase-3-techniques, the results are presented as the number of positive cells per 1000 µm lamellar contour. Apoptosis and chloride cell activity were only determined at the first four sampling time points.

5.3.5 Electron microscopy

For SEM, the gill samples were preserved in a HEPES-glutaraldehyde solution. Tissue samples were postfixed in 1% buffered osmium tetroxide for 2 h and dehydrated in an increasing alcohol series followed by increasing ethanol–acetone series up to 100% acetone. The samples were then dried to the critical point with a Balzers CPD 030 critical point drier (Sercolab bvba, Merksem, Belgium) and further mounted on metal bases and sputtered with platinum using the JEOL JFC 1300 Auto Fine Coater (Jeol Ltd, Zaventem, Belgium). The samples were examined with a JEOL JSM 5600 LV scanning electron microscope (Jeol Ltd). For TEM processing, a protocol as described by De Spiegelaere et al. (2008) was used. For examination of the TEM-samples, a JEM-1400 plus Jeol electron microscope (Jeol Ltd) operating at 80 kV was used. Micrographs were taken digitally.

5.3.6 Statistical analysis

The effect of three independent variables (degree of virulence of bacterial strain, time point after inoculation and localisation on the gill filament) on nine dependent variables (number of recovered bacteria, presence of chloride cells, EGC (only in carp), PAS-positive, PAS/AB-positive, AB-positive, total goblet, TUNEL- and caspase-3 positive cell counts), was assessed. Since a clearly distinct result was noted in some carps inoculated with the LV-isolate in terms of mortality and macroscopically discernible gill lesions, this group was further subdivided into a group of fish

displaying no macroscopic abnormalities (further denoted as the carp inoculated with the LV-isolate) and a group of fish that died with grossly visible gill lesions comparable to the macroscopic lesions as seen in fish exposed to the HV-isolate (further denoted as the LV-isolate affected fish).

As the bacterial titration counts for carp and rainbow trout were not normally distributed, the data were log transformed for further statistical analysis.

Depending on the distribution of the dependant variables, two different statistical assays were performed. Firstly, the effect of the independent variables on the outcome of bacterial titrations, PAS/AB-positive goblet cells, total goblet cells, chloride cells, TUNEL and caspase-3 positive cells in carp, and chloride cells, TUNEL and caspase-3 positive cells in rainbow trout were assessed using a multivariate linear mixed model. When a significant effect on one of the independent variables was observed in the multivariate model, post hoc comparisons were performed using Scheffe or least significant difference (LSD) tests.

Secondly, the data of PAS-positive goblet cells, AB-positive goblet cells and EGC in carp, and PAS-positive goblet cells, PAS/AB-positive goblet cells, AB-positive goblet cells and total gill goblet cells in rainbow trout, were transformed into a binary dataset with values "absence of cells (=0)" and "presence of cells (=1)" and analyzed by means of a multivariate logistic regression model.

Statistical results were considered to be significant when p-values were below 0.05. All analyses were performed using SPSS version 21.0.

5.4 RESULTS

At the last two SPs i.e. 24 h and 36 h PI, no fish in the groups challenged with the HV-isolates were remaining. Therefore, these SPs were omitted. As the disease progressed markedly faster in trout challenged with the HV-isolate from SP 5 onwards, the remaining trout of all groups were sacrificed 3.5 h earlier compared to the carp hence advancing the last SP to 12 h instead of 15.5 h PI.

The control animals of both carp and rainbow trout remained clinically healthy throughout the experiment and no mortality occurred. No lesions nor *F. columnare* bacterial cells were observed upon macroscopic, light microscopic and ultrastructural examination of the gill tissue. Skin lesions were not observed in any of the animals during the trials.

5.4.1 Carp

5.4.1.1 Chronological changes in type and extensiveness of gill lesions

Following challenge with the HV-isolate

Sampling-points (SPs) 1 and 2 (1 and 2.5 h post inoculation (PI), respectively)

No macroscopic lesions were noted and none of the 12 fish sampled or any of the fish present in the tank displayed any clinical signs of discomfort or disease. No dead fish were encountered.

Histological imaging revealed bacteria clustered focally in an eosinophilic matrix encompassing the tips of one to three (first SP) and two to six (second SP) out of the six discernible filaments. The lamellae that were in the vicinity of these bacterial clusters were oedematous at the first SP. The lamellae visualized on the sections of the second SP revealed fusion and necrosis. In these necrotic areas, the bacteria were closely associated with the denuded lamellar epithelium with only pillar cells still showing an intact structure. In addition, the bacterial cells had further migrated to the middle of the filaments (Figure 1). Filamental architecture was safeguarded in all fish examined.

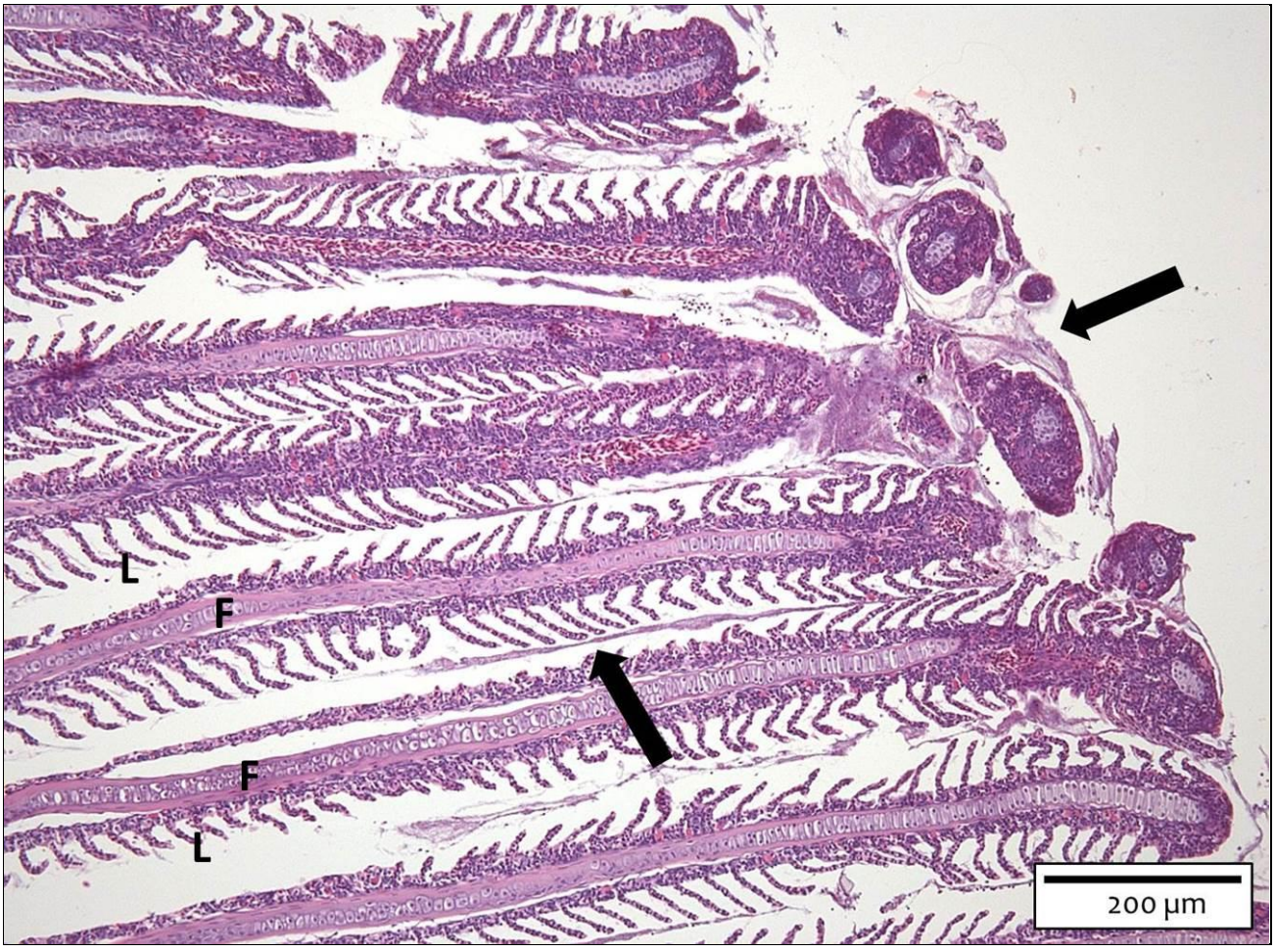

Figure 1. Gill section of a carp challenged with the HV-isolate at SP 2. The bacterial cells (arrows) are clustered focally in an eosinophilic matrix encompassing the tips of the gill filaments (F) and extending to the middle of the gill filaments. L = gill lamella (H&E). Bar = 200 µm.

PAS-staining showed a PAS-positive matrix immediately surrounding the bacteria that in turn was enclosed by AB-positive mucins (Figure 2). Both layers exhibited the same thickness of 3-30µm, with the thickness of the surrounding layer increasing with the size of the bacterial cluster.

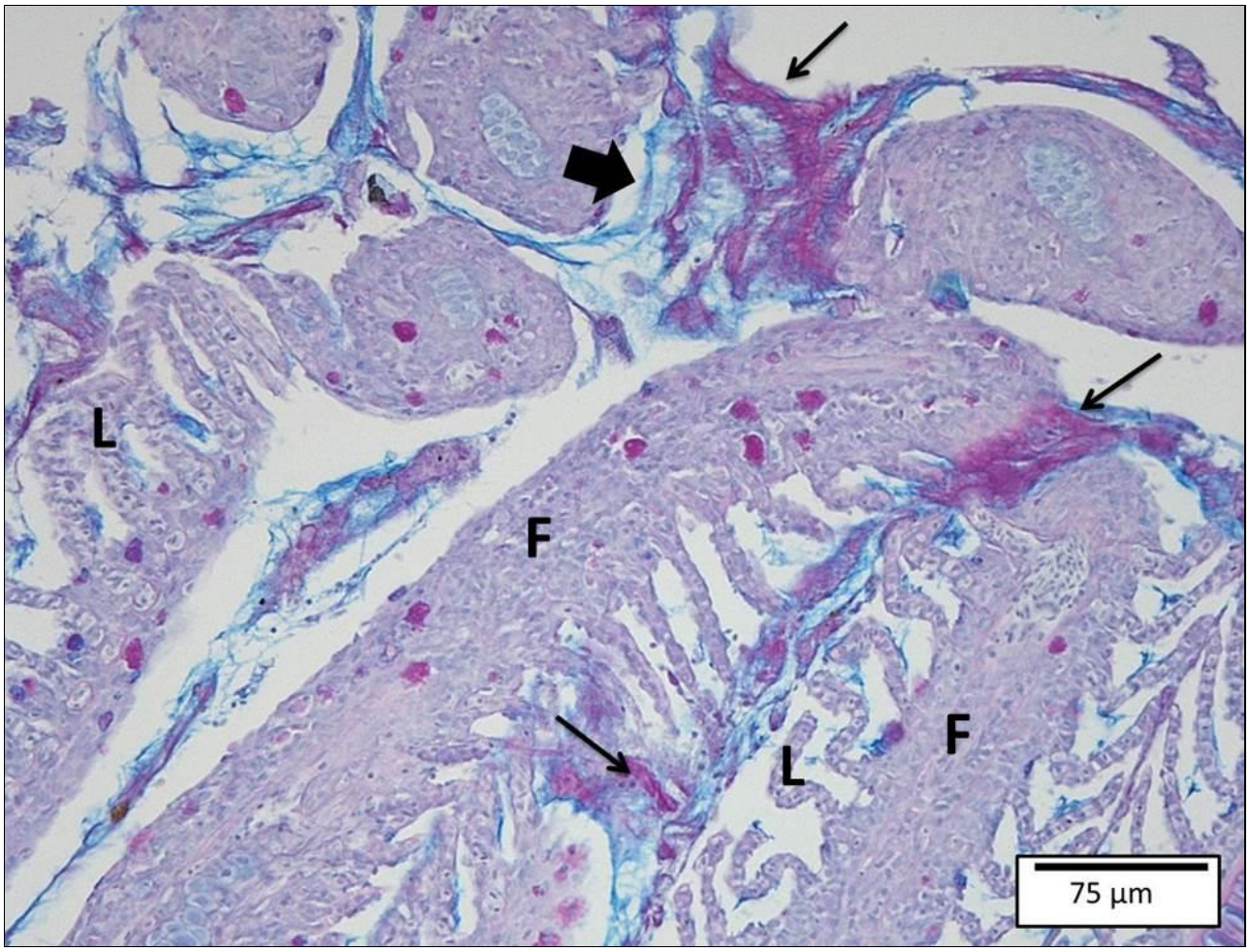

Figure 2. Gill section of a carp challenged with the HV-isolate at SP 2. The bacterial cells are surrounded by a PAS+ matrix (thin arrows, purple) enveloped by AB+ mucins (thick arrow, blue). F = gill filament; L = gill lamella (PAS/AB). Bar = 75 µm.

SEM and TEM revealed aggregates of long, slender bacterial cells with at the second SP large mats of these bacterial cells encompassing the filament tips.

SP 3 and 4 (4 h and 6 h PI, respectively)

At 6 h PI, the first moribund fish appeared. These two fish were hanging at the surface or in a corner of the tank, displayed loss of balance and were gasping for air. These two (out of the six) sampled fish displayed macroscopic lesions. The latter consisted of foci of whitish discolouration of the gill filaments of the first gill arch on both sides. One dead fish was encountered.

Upon inspection of the histological sections from the six fish sacrificed at 4 h PI, assembled bacterial cells were noted in close proximity to the lamellar epithelium of the filament tips with focal loss of filamental architecture. In addition, bacterial cells had pursued their way to the filament base. The gill tissue of the fish sampled at 6 h PI displayed multiple bacterial micro-colonies smothering half to all filament tips coinciding with lamellar fusion and filament destruction.

SEM confirmed the presence of huge clusters of densely packed bacterial cells wrapped in cellular debris and covering the gill tissue. TEM-examination revealed bacterial cells directed parallel to and in intimate contact with the gill epithelia. In conjunction with bacterial presence, severe gill damage with oedema and cell necrosis was noted. The outer membrane of the bacteria was remarkably knurled and regularly surrounded by outer membrane vesicles (OMV) (Figure 3).

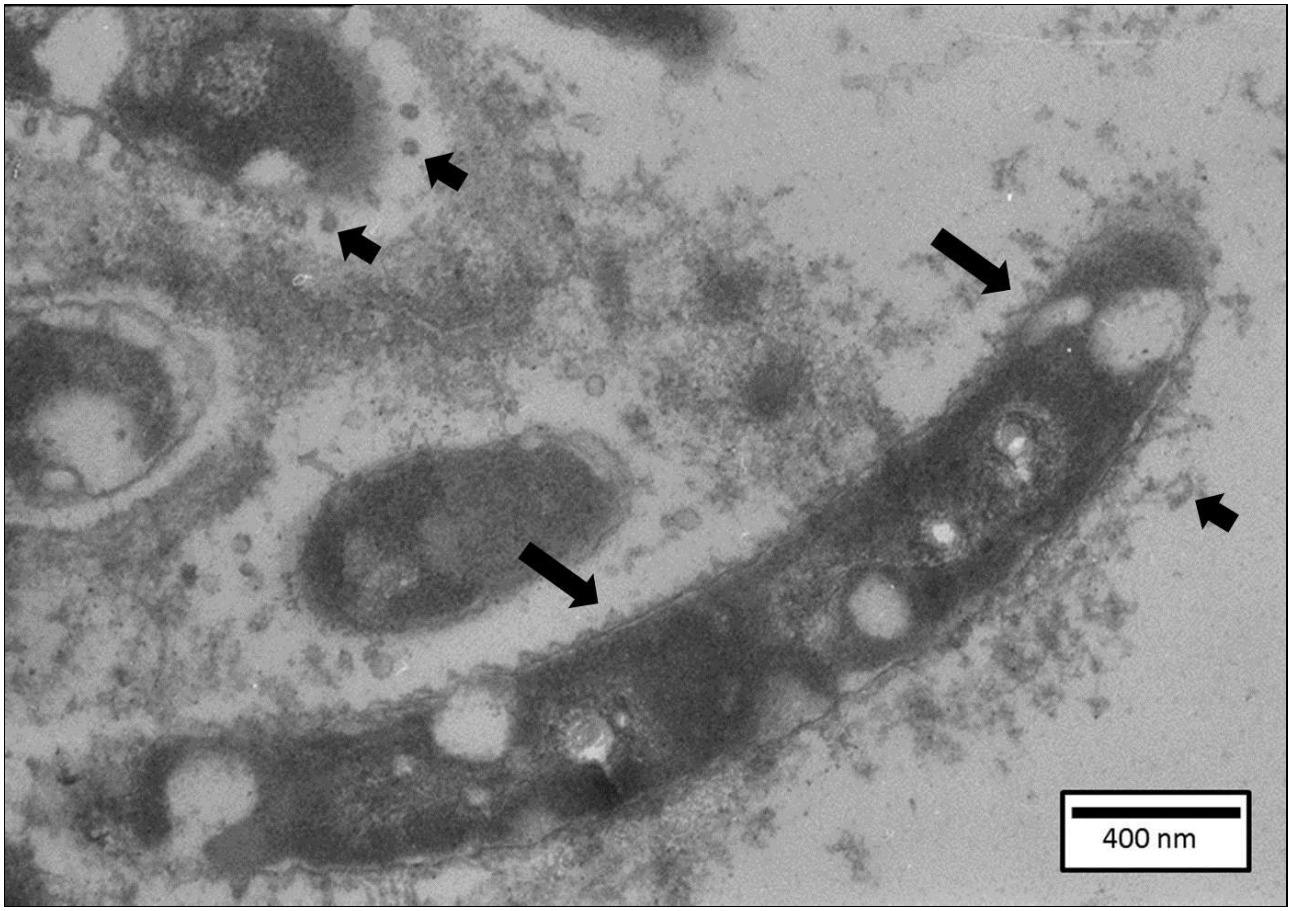

Figure 3. Carp gill after inoculation with the HV-isolate at SP 3. Bacterial cells present a knurled outer membrane (long arrows), and are regularly surrounded by outer membrane vesicles (short arrows) (TEM). Bar = 400 nm.

SP 5, 6 and 7 (8, 9.5 and 15 h PI, respectively)

The vast majority of fish displayed overt signs of disease as exhibited by loss of equilibrium, isolation and respiratory distress, with an increase in severity at later SPs. All these fish showed macroscopic lesions, as manifested by multifocal discolouration of the gill filaments of the first gill arches bilaterally. Thirteen dead fish were encountered. Two out of 18 sampled fish remained clinically healthy with no apparent lesions. At the last sampling point, only four fish (two out of each aquarium) were remaining and sampled for (immuno)histochemical, ultrastructural and bacteriologic analysis.

The histological image changed into severe pathological lesions in all fish sampled, except for the four clinically healthy animals. Large clumps of bacterial microcolonies, embedded in an eosinophilic matrix, covered all filament tips. Multifocally spread bacterial cells were seen in close contact with the lamellar epithelium over the full length of the gill filaments. Focal lamellar fusion and tissue necrosis were associated with the bacterial cells with significantly more fusion of the lamellae at the filament tips compared to the control animals. Areas of complete architectural loss in close proximity of bacterial clumps were additionally noted. At SP 7, overall oedema and total lamellar and filamental fusion were apparent (Figure 4).

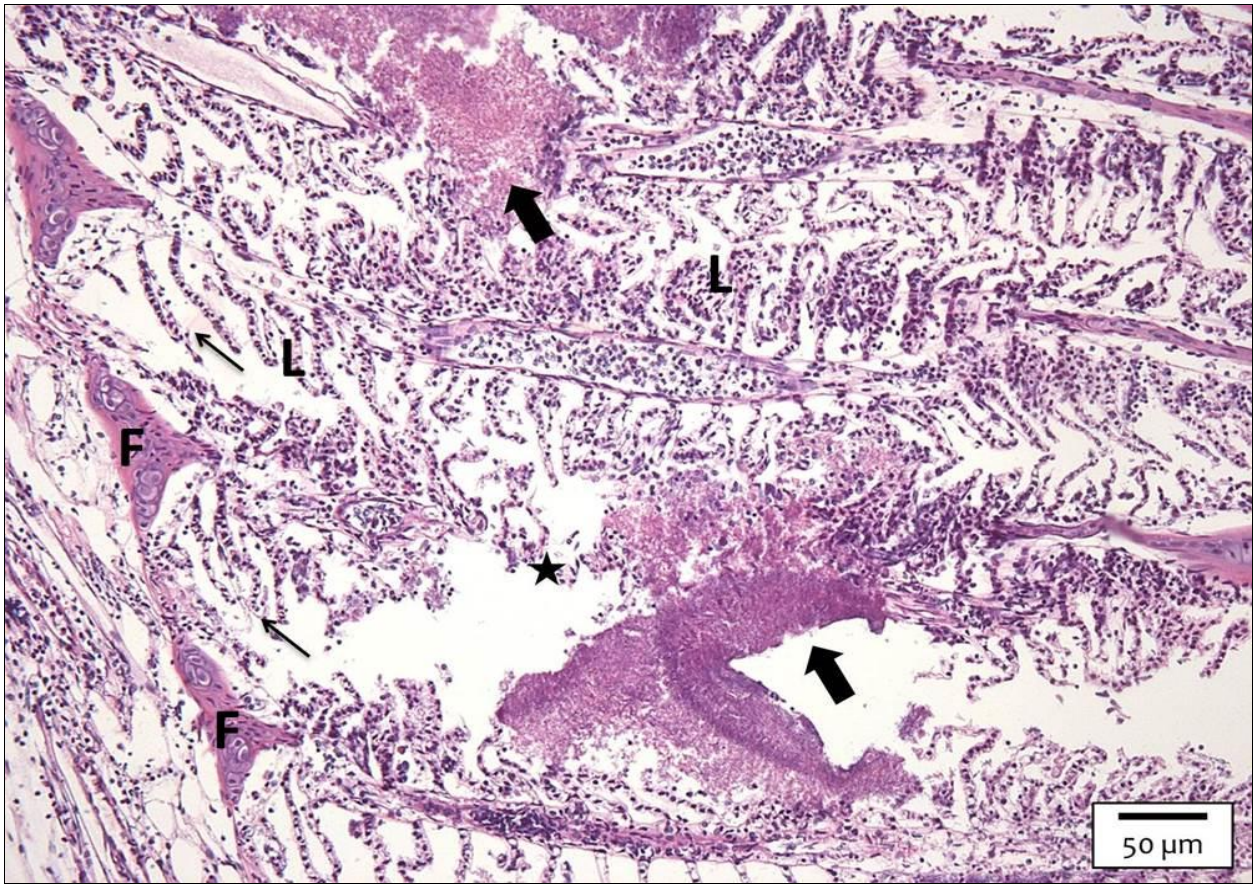

Figure 4. Gill section of a carp challenged with the HV-isolate at SP 7. Large clumps of bacterial micro-colonies (thick arrows) embedded in an eosinophilic matrix are discerned. Areas of complete architectural loss (asterisk) are noted in close proximity of bacterial clumps. Overall oedema (thin arrows) and total lamellar (L) and filament (F) fusion are apparent (H&E). Bar = 50 µm.

TEM revealed all bacterial outer membranes consistently surrounded by OMV. SEM confirmed the histological findings and was demonstrated by large clumps of bacterial micro-colonies, embedded in a matrix, covering all filament tips.

Following challenge with the LV isolate

SP 1 and 2 (1.5 and 2.5 h PI, respectively)

No clinical signs of discomfort nor macroscopic lesions were seen. No mortality was discerned.

Histological examination revealed the presence of clusters of bacterial cells in close contact with one to three (SP1) and two to three (SP2) out of six filament tips in all 12 fish sampled. These bacterial aggregates were also found in the middle of the filaments. The gill filament and lamellae did not exhibit any abnormalities, except for focal lamellar oedema in the filament tips in the presence of bacterial cells.

PAS-staining showed a PAS-positive matrix immediately surrounding the bacteria that in turn was enclosed by AB-positive mucins with the larger the bacterial cluster, the thicker the surrounding layers.

TEM examination revealed bacterial cells that appeared to be separated from the epithelium by a translucent layer (Figure 5). In one fish sampled at 2.5 h PI, close contact between the bacterial cell and the gill epithelium was noted, with a parallel orientation of the bacterium towards the lamellar epithelial cells and apparent bacterial cell division. The bacterium showed a cell wall with an apparently less knurled outer membrane compared to the bacterial cells of the HV-isolate. OMV were observed only in a minority of the bacterial cells. Bacteria could not be visualized using SEM examination. Indeed, only huge mucus clots and packed cells were evident mostly at the gill filaments tips, covering the gill tissue. In sites not covered by mucus, the normal fish gill fingerprinting pattern was visible.

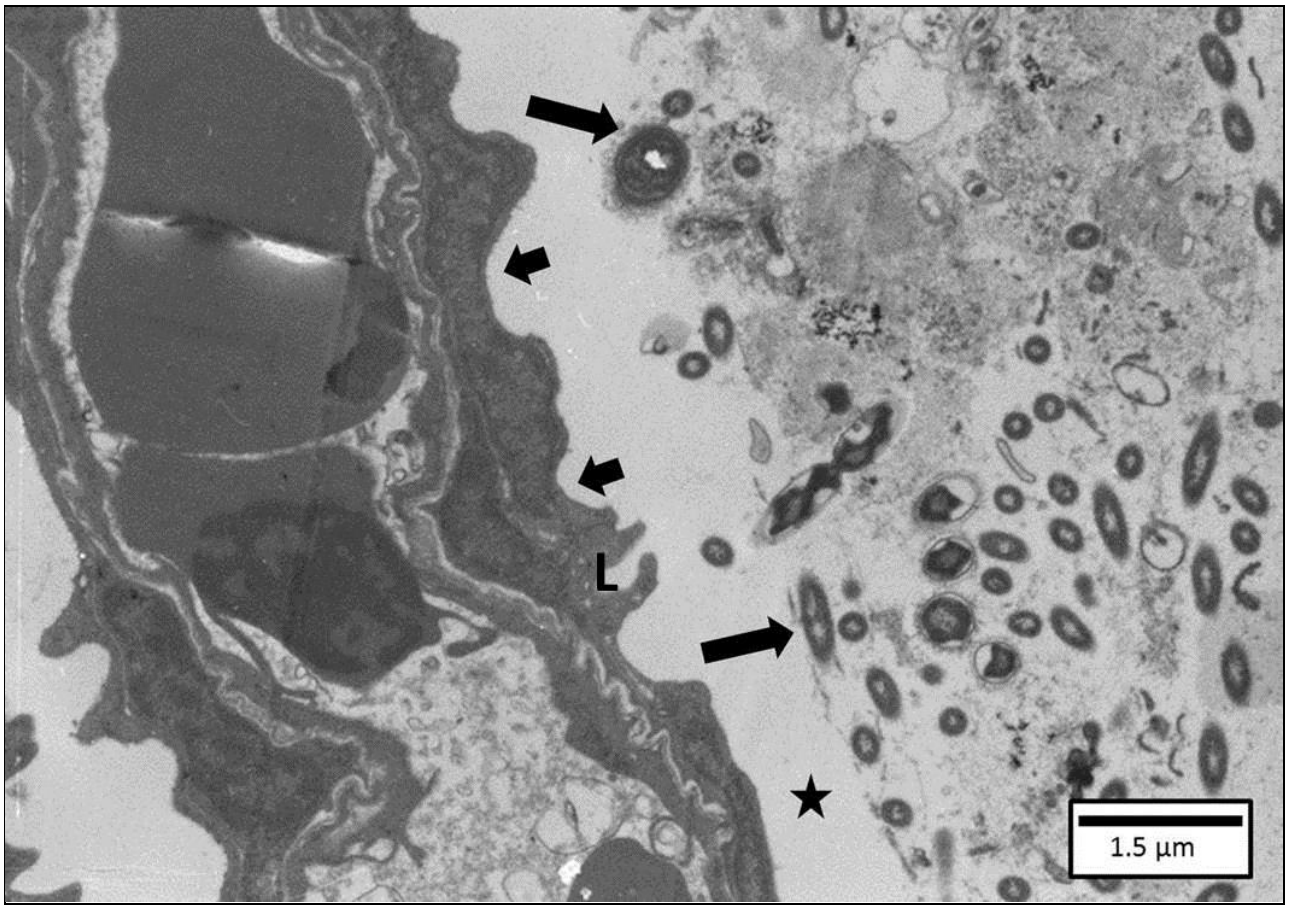

Figure 5. Gill of a carp inoculated with the LV-isolate at SP 2. A cluster of bacterial cells (long arrows) is separated from the lamellar epithelium (L) (short arrows) by a translucent layer (asterisk) (TEM). Bar = 1.5 µm.

SP 3 and 4 (4 and 6 h PI, respectively)

Two fish out of twelve exhibited severe signs of discomfort and were swimming at the surface, gasping for air. However, no macroscopic lesions were encountered in any of the fish sampled. No other clinical abnormalities nor dead fish were encountered.

Histological examination revealed the presence of bacterial cells in nine out of the twelve fish sampled. Bacteria forming small micro-colonies surrounded by an eosinophilic matrix and necrotic cells were observed in close contact with the gill epithelium at the tips and middle of the filaments. The two clinically affected fish showed multifocal histological filament destruction as seen in the fish inoculated with the HV-isolate. No abnormalities were discerned in the outnumbering clinically healthy fish that were sacrificed.

SEM and TEM endorsed the histological results with bacterial cells being noted enclosed in mucus clots and in close contact with the gill epithelium, respectively. As in the HV-isolate, the outer membrane of the bacterial cell wall was surrounded by OMV, but only a minority of the bacterial cells revealed this phenomenon. Again, a less knurdled outer membrane was noted.

SP 5, 6 and 7 (8, 9.5 and 15 h PI, respectively)

Three fish were moribund at SP 6 and 7 and revealed macroscopic lesions consisting of multifocal whitish discolourations of the gill filaments of the first gill arches. One dead fish was encountered.

The gills of the clinically healthy fish did not reveal any macroscopic or microscopic abnormalities and no bacterial cells were noted. The histological findings of the gills of the three moribund fish were comparable to those as described in the fish inoculated with the HV-isolate in terms of the elicited lesions and spread of the bacterial cells throughout the gill tissue. Indeed, huge clusters of bacteria embedded in an eosinophilic matrix with necrotic debris were sited at the filament tips, with offshoots of these in close contact with the gill epithelium over the entire length of the gill filaments. In these sites, severe oedema, lamellar fusion and lamellar gill necrosis were present. Significantly more fusion of the lamellae was observed in the gills, especially at the filament tips, of fish inoculated with LV-isolate as compared to the control animals. At no time, the cartilaginous tissue was lysed nor were the gill filaments fused.

SEM and TEM of the gills of the moribund fish showed bacterial cells wrapped in a matrix of cells and mucus and contact with epithelial cells, respectively. No abnormalities in the clinically healthy fish were encountered.

5.4.1.2 Chronological changes in apoptotic, eosinophilic granular, goblet and chloride cells

With regard to the first four SPs, the mean difference between the TUNEL-positive cell count per 1000 µm gill filament contour in the gills of carp challenged with the HV-isolate compared to the control animals was 1.65 ± 0.64 ($p < 0.05$). No other significant differences were noted for the TUNEL-positive cell counts. The mean difference between the caspase-3 immunoreactive cell counts per 1000 µm gill filament contour in the gills of carp inoculated with the HV-isolate and the control animals was 2.3 ± 0.5 ($p < 0.01$); a comparable difference was noted between gill tissue of carp challenged with the LV-isolate and the control animals ($p < 0.01$). Most caspase-3-immunoreactive cells were noted at the second SP, followed by a decreasing trend. The vast majority of cells staining positive with either TUNEL or caspase-3 were epithelial cells, with only occasionally a positive goblet cell discerned. At no time, significant differences between the various groups were noted for the number of chloride cells per 100 µm gill filament length. Lysis of these cells was found, especially when oedema was present. The gills of fish exposed to the HV-or LV-isolate displayed lysis of chloride cells, although to a higher degree in the HV-isolate inoculated fish which showed more oedema.

For the goblet cell counts, the mean difference between the total gill goblet cell count per 100 µm gill filament length of fish inoculated with the HV-isolate and the control animals was 0.83 ± 0.27 ($p < 0.05$), while the mean difference between fish inoculated with the LV-isolate and the control animals was 1.06 ± 0.31 per 100 µm gill filament length ($p < 0.01$), with no other significantly different results occurring in total goblet cell counts. For the PAS/AB-positive cell counts, no significant differences occurred between any of the groups. Statistical data revealed 2.86 and 4.76 times significantly higher odds ($p < 0.01$) for PAS-positive and AB-positive cell counts per 100 µm gill filament length, respectively, when comparing the cell counts in the gill tissue after inoculation with the HV-isolate and the control animals. Higher counts were noted in the vicinity of bacterial cells, mostly at the tips of the gill filaments. Moreover, 3.85 times higher odds ($p < 0.01$) for the AB-positive cells per 100 µm gill filament length were encountered in the gills after challenge with the LV-isolate compared to the control animals and even 5.22 times higher odds ($p < 0.01$) were found for the AB-positive cells in the gills of fish inoculated with the LV-isolate with similar macroscopic lesions as seen after challenge with the HV-isolate compared to the control fish. A 4.44 times higher odd per 100 µm gill filament length ($p < 0.01$) was calculated for the EGC in the gill tissue of fish challenged with the HV-isolate compared to the control fish. The number of EGC per 100 µm gill filament length of control fish, of the fish exposed to the LV-isolate and of the LV-isolate affected fish odds ratio that were 0.23, 0.32 and 0.24 times lower, respectively, compared to the fish challenged with the HV-isolate ($p < 0.05$). No other significant differences were found in the EGC count. In gill sections of fish exposed to the HV-isolate, upon inspection of sites of tissue damage, degranulation of EGC was noted along with their migration onto the gill lamellae. A dynamic overview of PAS-positive goblet cells, AB-positive goblet cells and EGC in carp is presented in Figure 6.

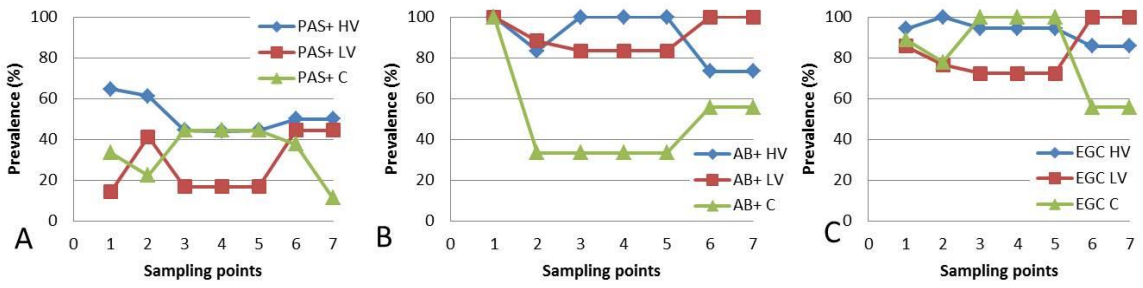

Figure 6. Prevalence (in %) of PAS-positive (A), AB-positive (B) and EG (C) cells calculated, per sampling point, for the fish belonging to a certain virulence group sampled. Significantly higher odds were found for PAS-positive and AB-positive cells counted in the gills after inoculation with the HV-isolate (blue) compared to the control animals, and for the AB-positive cells in the gill tissue of the LV-isolate (red) challenged fish compared to the control animals (green). Significantly higher odds were encountered for the EGC counted in the gills of fish challenged with the HV-isolate compared to the control fish, and for the HV-isolate compared to the LV-isolate inoculated fish.

5.4.1.3 Temporal changes in bacterial cell counts

The bacterial titres retrieved from the gill tissue at the first SP were the highest during the course of the experiment (7.8 x 10^8 CFU/g gill tissue and 5.6 x 10^7 CFU/g gill tissue for the fish inoculated with the HV- and LV-isolate, respectively). A tenfold decrease was noted towards the third SP, after which titres increased again approaching the values of the first SP. Subsequently, the bacterial titres either stagnated or decreased gradually. An overview of the average bacterial titres retrieved from the gill tissue after exposure with the HV- and LV-isolates can be found in Figure 7. Overall, the mean bacterial logarithmic titres retrieved from gill tissue in the HV- and LV-challenged fish were ^{10}log 7.92 ± 0.15 CFU/g and ^{10}log 6.70 ± 0.18 CFU/g, respectively. The LV-isolate challenged fish showing the same macroscopic lesions as seen in the HV-isolate inoculated fish had a mean bacterial logarithmic titre of ^{10}log 7.15 ± 0.39 CFU/g. The mean difference for the bacterial cell counts between the HV- and the LV-isolate challenged fish and the LV-isolate inoculated fish displaying the same macroscopic lesions as seen in the HV-isolate infected fish was ^{10}log 1.15 ± 0.21 ($p < 0.01$) and ^{10}log 1.10 ± 0.38 ($p < 0.05$) CFU/g, respectively. No *F. columnare* bacterial cells were detected in the gill tissue of the control animals.

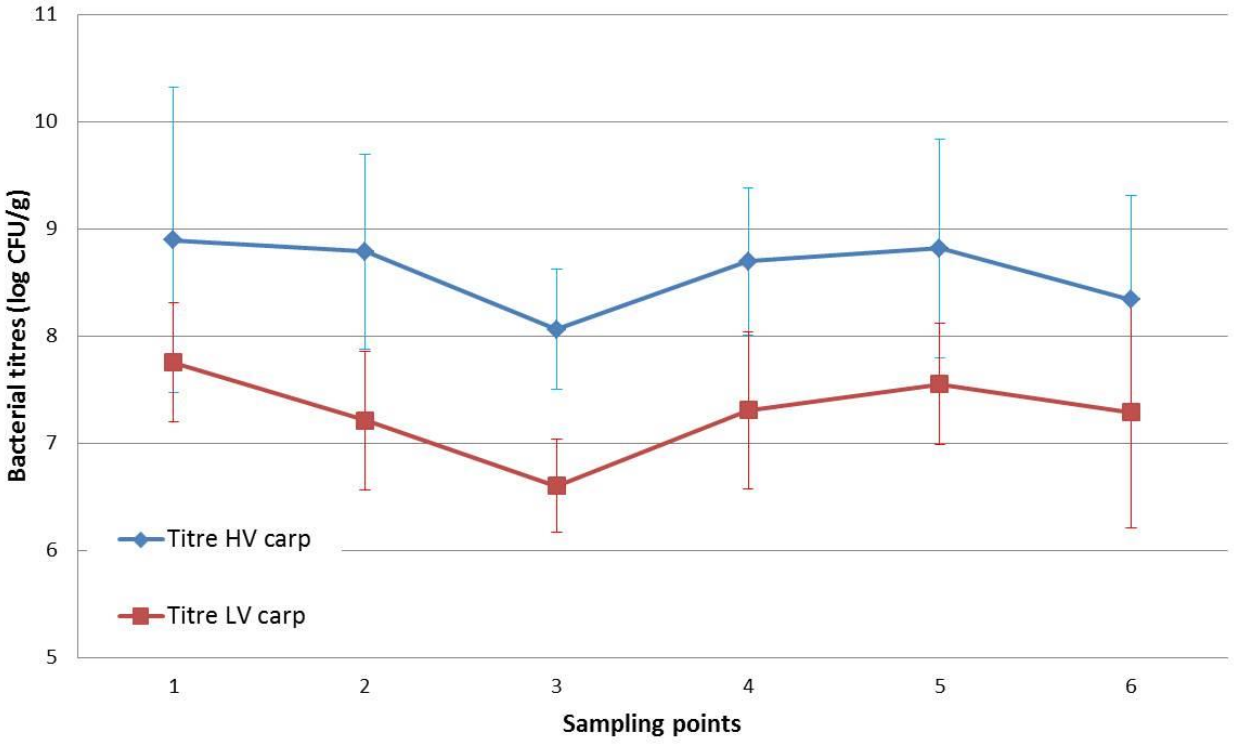

Figure 7. Gill bacterial titres (log CFU/g) after challenge with the HV- and LV-isolate in carp. The bacterial titres of fish inoculated with the HV-isolate (blue) remain high during the course of the experiments and show a significant higher difference compared to the fish infected with the LV-isolate. The bacterial titres retrieved from the latter carp follow a same trend compared to the HV-isolate and remain high until the end of the experiment. The standard deviations are indicated by the error flags.

5.4.2 Rainbow trout

5.4.2.1 Chronological changes in type and extensiveness of gill lesions

Following challenge with the HV isolate

SP 1 and 2 (1 and 2.5 h PI, respectively)

No clinical signs of discomfort nor macroscopic lesions were noted in any of the 12 sampled fish or any of the other fish present in the tanks. Three dead fish were encountered revealing no macroscopic abnormalities.

Histologically, the first lesions were noted as mild oedema and hyperplasia in six out of twelve sampled fish. The other half of the fish showed focal to generalized severe oedema with detachment of the epithelium from the underlying intact pillar cells. Bacterial cells were spotted focally, mostly encompassing the filament tips and middle of the gill filaments. Their presence was associated with localized lamellar necrosis, while the neighbouring lamellae remained intact, apart from slight oedema of the epithelium on the colonized side.

TEM confirmed the latter finding (Figure 8). Furthermore, TEM revealed the chromatin of the nucleus to be marginized and clumped while the other cell organelles remained intact, as seen in apoptotic cells. SEM did not reveal abnormalities.

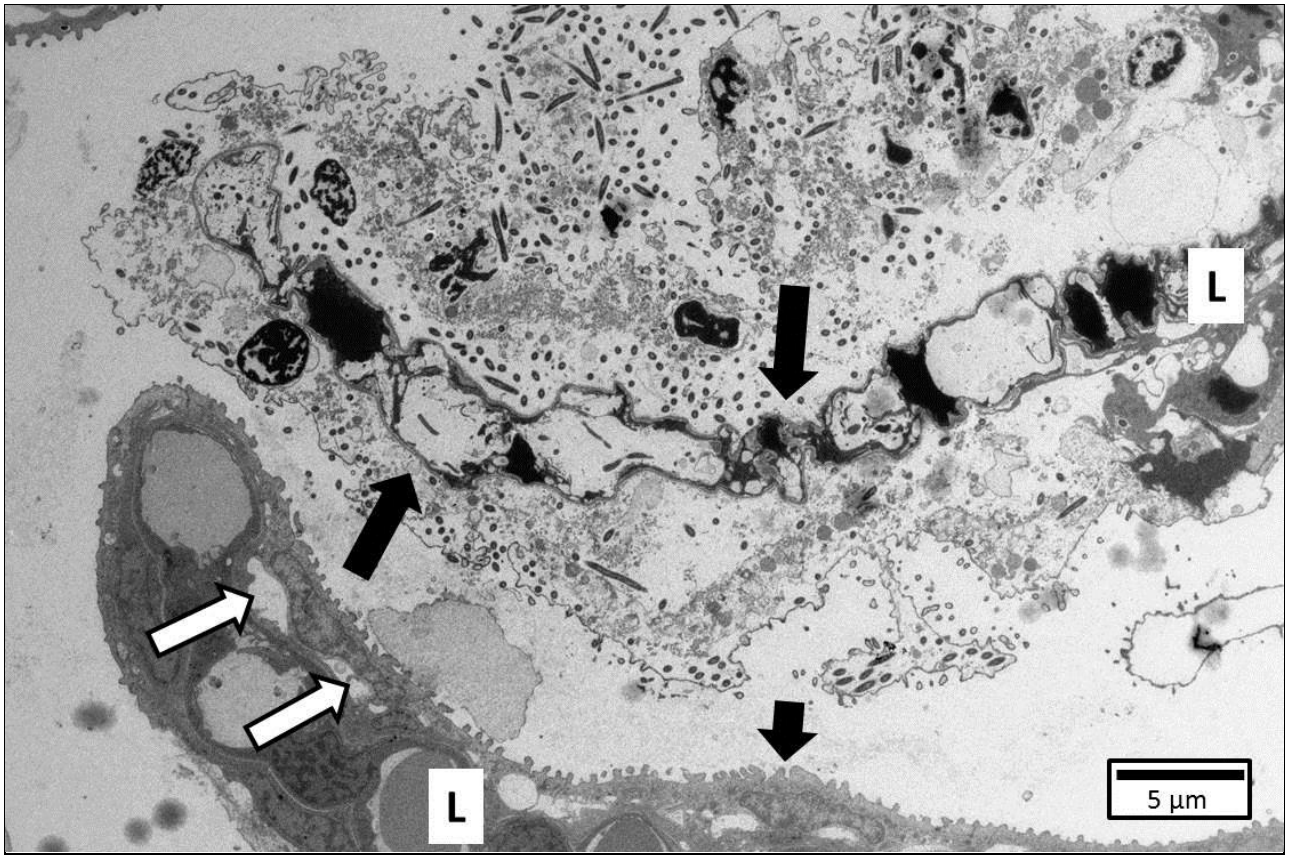

Figure 8. Gill tissue of a rainbow trout following exposure to HV-isolate at SP 1. Localized necrosis (long arrows) is observed in a gill lamella (L) while the neighbouring lamella remains intact (short arrow), apart from slight oedema (white arrows) of the epithelium on the sides where bacteria are present (TEM). Bar = 5µm.

SP 3 and 4 (4 and 6 h PI, respectively)

Clinical signs of discomfort and macroscopic lesions were absent in all fish. One dead fish occurred, showing no macroscopic lesions.

Histologically, bacterial cells were noted in six fish out of twelve fish sampled. Small clumps of bacterial cells were found very focally over the entire length of the filaments and in close contact with the epithelium. Their presence coincided with severe oedema, lamellar necrosis and sloughing of epithelial cells.

PAS-staining revealed the bacterial clusters to be wrapped in a PAS-positive matrix while being encased in AB-positive mucus.

The histological findings were confirmed by SEM- and TEM-pictures revealing bacterial clumps present at the gill filament tips in close contact with the epithelium.

SP 5, 6 and 7 (8, 9.5 and 12 h PI, respectively)

From SP 5 onwards, most remaining rainbow trout exhibited severe discomfort including an altered swimming pattern and gasping for air, and macroscopic focal whitish discolouration of the gill tissue. Eight dead fish were discerned. Only three fish out of the remaining 18 did not exhibit clinical nor macroscopic abnormalities the last three SPs.

Apart from the latter three fish mentioned, histological analysis showed small bacterial clumps in intimate contact with the gill epithelium over the entire length of the filaments, and affecting almost all lamellae. Consistent with the bacterial presence, severe, generalized oedema and lamellar necrosis occurred with shedding of the epithelial cells causing the underlying pillar cells to be denuded. The cartilaginous core of the filaments remained intact.

Likewise, SEM revealed bacterial clusters covering the gill epithelium as blankets and wrapped in between necrotic cells and cellular debris. TEM revealed a knurled outer membrane in the majority of bacterial cells with the consistent occurrence of OMV.

Following challenge with the LV isolate

Neither clinical signs nor macroscopic lesions were seen in rainbow trout challenged with the LV-isolate during the entire experiment. No dead fish were encountered.

At the first three SPs, 14 out of the 18 sampled fish showed mild gill oedema, either focal or generalized, associated with hypertrophia of the epithelial cells situated at the basis of the lamellae. At the last four SPs, two out of 24 fish showed mild oedema with no further abnormalities. Bacterial cells were never discerned in any sample. SEM and TEM showed normal gill tissue with no bacteria present.

5.4.2.2 Chronological changes in apoptotic, eosinophilic granular, goblet and chloride cells

With regard to the first four SPs, the mean difference between the TUNEL cell count per 1000 µm gill filament contour in the gills of fish challenged with the HV-isolate compared to the control animals was 2.67 ± 1.08 ($p < 0.05$). No other significant differences were noted for the TUNEL-positive cell counts. Most TUNEL-positive cells per 1000 µm gill filament contour occurred at the filament tips, showing a mean difference of 3.30 ± 0.98 ($p < 0.01$) TUNEL-positive cells compared to the middle parts of the gill filaments. The mean difference between the caspase-3 immunoreactive cell counts per 1000 µm gill filament contour of the gills of rainbow trout challenged with the HV-isolate and the LV isolate inoculated fish was 2.02 ± 0.54 ($p < 0.01$) while between the HV-isolate inoculated fish and the control animals, this difference was 1.91 ± 0.68 ($p < 0.05$) per 1000 µm gill filament contour. No other significant differences occurred. Cells staining positive with either TUNEL or caspase-3 were predominantly epithelial cells, including occasional goblet cells. No significant differences occurred for the number of chloride cells per 100 µm filament length in between the various groups. As described for the carp, fish exposed to the HV- or LV-isolate displayed lysis of chloride cells but, as more oedema was noted following challenge with the HV-isolate, markedly more lysis of chloride cells was perceived in the fish exposed to the latter isolate.

As for the goblet cell count, no significant differences were noted in between any of the goblet cell counts in between the various groups. An increase in AB-positive cell count was noted in the HV-group from the second to the third SP though.

EGC were not observed in the gill sections of rainbow trout.

5.4.2.3 Temporal changes in bacterial cell counts

Bacterial titres from the gill tissue inoculated with the HV- and LV-isolate were ^{10}log 8.03 ± 0.27 and ^{10}log 2.76 ± 0.42 CFU/g gill tissue, respectively. The mean difference for the bacterial cell counts in between the HV-isolate challenged fish and the LV-isolate inoculated fish was ^{10}log 5.26 ± 0.39 ($p < 0.01$) CFU/g. An overview of the bacterial titres retrieved from the gill tissue at the various SPs after challenge with the HV- and LV-isolate can be found in Figure 9. No *F. columnare* bacteria were retrieved from the control animals.

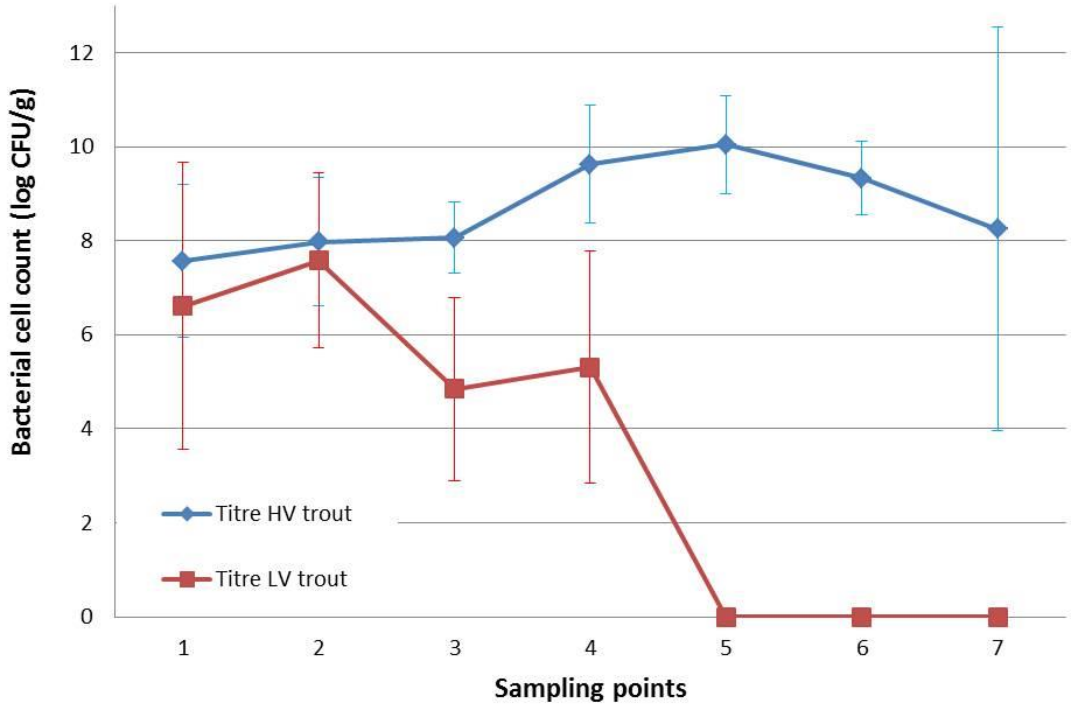

Figure 9. Gill bacterial titres (log CFU/g) after challenge with the HV- and LV-isolate in rainbow trout. The bacterial titres of fish inoculated with the HV-isolate (blue) remain high during the course of the experiments. The bacterial titres retrieved from fish inoculated with the LV-isolate (red) show progressively a substantially decreasing trend. The standard deviations are indicated by the error flags.

5.5 DISCUSSION

The purpose of this study was to track the evolution and discern conspicuous features of the gill lesions in experimentally induced columnaris disease. This is the first description of the sequence of events taking place at the level of the gill tissue before the fish succumb to columnaris disease.

In carp, as soon as 1 h post challenge, bacterial cells of the HV-isolate were found in close contact with the epithelium of the filament tips. Only 1 h later, attachment was seen and the bacteria had pursued their way towards the middle of the filaments. The filament tips eventually were disintegrated and bacterial infiltrates were found in between necrotic tissue. Bacteria further invaded the base of the filaments to ultimately colonize and break down the complete gill filament. Chondrolysis was a dominating feature in the last stages of disease coincided by infiltration of massive clusters of bacterial cells. When necrosis became generalized and severe oedema was evident, the lesions became visible macroscopically as bilaterally whitish discolourations of the gill tissue.

In 75% of the carp inoculated with the LV-isolate, bacterial cells were also able to attach to the epithelium of the filament tips from the first SP onwards and subsequently moved downwards to the middle of the filaments at the third and fourth SP. In contrast, at the fifth and following SPs, the gill sections of only 25% of the fish displayed bacterial cells. Nevertheless, the bacterial titres retrieved from the gill tissue remained high for all carp inoculated with the LV-isolate up until the last SP which seems inconsistent with the histological findings. This may signify that the bacterial cells were no longer as firmly attached to the gill tissue as they were during the first four SPs. Indeed, as described before (Declercq et al., 2015; Decostere et al., 1999), the bacteria might be part of the aqueous biofilm covering the gill tissue and hence be noted by bacteriological examination when plating out the gill sample. However, during processing for histological examination, they might have been washed away as this technique involves several washing steps hence visualizing only firmly attached bacteria. The fifth SP seems to be the turning point for the majority of fish to head for surmounting the LV-isolate *F. columnare* infection with colonization halted at the filament middle section, and bacterial cells allegedly being less firmly attached to the gills. In contrast, the gill tissue of the few carp succumbing to the disease at that time-point (and later) exhibited bacterial colonization over the entire filament length with necrosis of the gill lamellae as a result. Lysis of the cartilaginous tissue was never encountered following exposure to the LV-isolate, which stands in shrill contrast to what was observed in the gill sections of fish challenged with the HV-isolate. The findings for the LV-isolate challenge both in carp and rainbow trout are intriguing

as the bacterial cells seem to be able to colonize the gill tissue, albeit to a significantly lesser extent than those of the HV-isolate. However, it appears that they cannot maintain a firm grip on this vital organ. The latter needs to be slightly toned down for the LV-isolate in carp where the gills of a few fish were colonized by *F. columnare* over the entire filament length. Why these few fish ultimately succumbed to the challenge with the LV-isolate and the majority of fish survived, warrants further research, as this might provide most relevant information concerning the susceptibility to this increasingly important disease. The hypothesis could be raised whether or not the initial fusion of lamellae is directed by bacterial input. However, we cannot confirm nor decline whether the host plays a role in this phenomenon and in case it does, whether it is beneficial to the host, or does it have a deleterious effect? A fusion of the lamellae could cause a sequestration of bacterial cells as such protecting the remainder of the gill tissue; however, fusion also induces a decrease in gill surface possibly causing respiratory distress. This phenomenon of lamellar fusion and how it is elicited, certainly warrants further research.

In natural outbreaks of edible and ornamental fish, the same clinical and pathologic features have been perceived. Fish were described to firstly lose appetite, swim at the water surface with rapid opercular movements to finally lose balance and succumb to the disease. Acute to chronic massive mortality with (Řehulka and Minařík, 2007; Amin et al., 1988; Decostere et al., 2002) or without (Amin et al., 1988) macroscopic visible skin or gill lesions have been described. Histological examination of the affected gill tissue could reveal oedema, distortion of lamellae and clusters of bacterial cells situated at the filament tips (Decostere et al., 2002) and bases (Amin et al., 1988). In some cases, a total loss of gill architecture was observed (Decostere et al., 2002).

Although skin lesions have been described in natural outbreaks of columnaris disease in salmonids (Řehulka and Minařík, 2007), no skin lesions were observed in this study. Most probably, the disease pattern evolved too fast for skin lesions to be discernible, as the latter usually appear in more chronic cases of columnaris disease (Bernardet and Bowman, 2006).

In the present studies, we aimed to mimic the natural situation as close as possible. Therefore, carp were inoculated with *F. columnare* strains isolated from carp, and rainbow trout with isolates from trout. Our results therefore do not allow concluding whether the temporal differences in infection observed between rainbow trout and carp are fish species related or rather *F. columnare* strain dependent.

Virulence is determined by more than the process of colonization. Upon TEM examination of the carp and rainbow trout gill sections of fish inoculated with the HV-isolates, OMV were discerned surrounding the majority of bacterial cells. These OMV were markedly less frequent in

the vicinity of the bacterial cells upon inspection of gill sections of fish exposed to the LV carp isolate. The production and composition of OMV seem to be influenced by ambient factors that microbes sense inside the host during normal cell growth (Kuehn and Kesty, 2005) and not during cell lysis (Whitchurch et al., 2002). OMV have been described to play a role in protein secretion, immune activation and suppression, stress response, attachment, internalization, tissue lysis and virulence (Berleman and Auer, 2013; MacDonald and Kuehn, 2012; McMahon et al., 2012). These bacterial "bombs" have been detected in *F. columnare* after *in vitro* research (Arias et al., 2012, Laanto et al., 2014), but, as far as we know, they have never before been described *in vivo*.

The findings in the present study strongly point towards biofilm development as has been demonstrated for *F. columnare* in *in vitro* experiments (Cai et al., 2013). These *in vivo* findings comply with the demonstration of genes in *F. columnare* encoding biofilm formation by the research group of Tekedar et al. (2012). The recurring features stressing biofilm formation potentially being an important stage in the pathogenicity of *F. columnare*, warrant further investigation.

Another important parameter to investigate in the theme of virulence, is the myriad of ways adopted by the pathogen to escape the host's immune defense. A remarkable and hitherto unexplained lack of inflammatory response is typical of *F. columnare* infections, especially in the early stages, allowing progression of the infection. Apoptosis constitutes a cell death programme with a notably non-inflammatory outcome, which may provide an explanation for the impaired inflammatory response and the acuteness with which columnaris disease can strike. In the present study, TUNEL and caspase-3 staining showed that significantly higher apoptotic cell counts in the gill tissue of fish challenged with the HV- or LV-isolate compared to the control animals and in some cases even between fish gills challenged with either one of the bacterial isolates were perceived. Apoptosis mostly affected epithelial cells and only occasionally goblet cells. Sun et al. (2012) demonstrated an upregulation of apoptosis pathways in the early stages after a challenge with *F. columnare,* which corresponds to our findings. The initiation of an increase in apoptotic cells following exposure to *F. columnare* – as now also demonstrated morphologically – remains enigmatic (Sun et al., 2012). Although different techniques for apoptosis detection are available, none of these are entirely specific or all-inclusive. Therefore, it was chosen to combine different techniques in our apoptosis research. The TUNEL assay was used, because it detects DNA fragmentation. However, DNA fragmentation has also been described to occur in oncosis (cell death with swelling) (Saikumar et al., 1999). Therefore, the immunohistochemical detection of caspase-3 was also applied. It is generally accepted that the latter technique is entirely specific for apoptosis, although apoptosis can be independent of caspase-3 (Hamatake et al., 2000).

The presence of EGC or mast cells in teleosts may be demonstrated by using H&E and Giemsa stainings (Holland and Rowley, 1998; Reite and Evensen, 2006). These EGC were described to stain negative using PAS (Holland and Rowley, 1998). However, precise and fully documented data on the staining characteristics of EGC remain scarce, while information on the impact of the organ and/or fish species and the effect of histological processing is almost fully lacking. In the current experiment, the granules of the EGC clearly stained PAS-positive in the gill tissue of carp. These cells were negative on alcian blue and toluidine blue stained sections (data not shown), with the well-delineated granules and staining features clearly differing from what was observed for the goblet cells. EGC have been associated with host defence actions towards bacteria (Reite and Evensen, 2006) and parasites (Reite, 2005; Reite and Evensen, 2006), although their exact role and the events they elicit are far from fully known. A significantly higher amount of these cells was noted in the carp gill tissue following exposure to the HV- as compared to the LV-isolate and control groups. EGC may be mobilized towards regions attacked by insults with acute tissue damage causing mast cell/eosinophilic granular cell degranulation and release of mediators of inflammation (Reite and Evensen, 2006). Degranulation of EGC was also noted in the carp in sites where tissue damage was visible. Whether this was favourable or disadvantageous for the host at this stage, is unclear. As this is the first time that a marked mobilization of EGC at the level of the gill tissue following *F. columnare* challenge is described, this phenomenon justifies further research into the function and significance of these cells in the pathogenesis of columnaris disease.

In this study, both fish species showed an increase in the total amount of producing goblet cells following challenge with either one of the *F. columnare* isolates compared to the control group, but only the carp showed significantly more producing goblet cells. A similar increase in goblet cells was seen after exposure to various parasites and bacteria (Roberts and Powell, 2005; van der Marel et al., 2010).

Interestingly, gill mucus cell histochemistry demonstrated an increase of neutral mucin production in carp and a significant increase of PAS-positive cell numbers following challenge with the HV-isolate as compared to the control animals. A similar mucus shift has been described in Atlantic salmon after challenge with the causative agent of amoebic gill disease (Roberts and Powell, 2005). After exposure to the HV- or LV-isolate in carp, a significant increase in AB-positive productive goblet cells was also observed compared to the control animals. An increased number of goblet cells containing acid glycoconjugates has been noted in skin mucus of carp after exposure to water containing a high load of non-pathogenic bacteria (van der Marel et al., 2010). Although not significant, an upward trend in acid mucus production was also noted in rainbow trout following challenge with the HV-isolate. Rainbow trout in general have been described to produce less acid

mucins compared to other fish species such as Atlantic salmon (Reite, 2005), which might explain why the observed differences were not statistically significant.

The question arises how the mucus changes observed in this study are elicited and how these may impact both the pathogen and the host. A distinct possibility is that the bacterium itself can alter the composition of the mucins. This has been described for *Helicobacter pylori* infections in the acid environment of the stomach where *H. pylori* uses urea hydrolysis to elevate the pH of its environment. The elevation of pH to neutral transforms the viscoelastic mucin gel to a viscous liquid (Celli et al., 2009), enabling the helical cell-shaped bacterium to swim faster in the viscous solution (Spagnolie et al., 2013). It is unknown whether *F. columnare* can steer the pH of mucus as, to our knowledge, this is unknown for any gill-disease eliciting organism. A consistent finding throughout this study is the fact that *F. columnare* bacteria were immediately surrounded by a PAS-positive matrix whilst being encased by AB-positive mucins. As an acid environment has been described to be adverse for *F. columnare* bacteria (Fijan, 1968), it is most tempting to speculate that the more neutral mucins in the immediate vicinity of the bacterial cells presumably are self-produced whilst the acid AB-positive mucus would be secreted by the host as a self-defence mechanism. Indeed, acid AB-positive mucins at pH 2.5 contain negative charges which reduce bacterial binding and are missing in PAS-positive mucins. In view of the importance of mucus in the fish's arsenal to combat disease, again, this finding should be further investigated.

In conclusion, the present study has revealed that, following immersion challenge, adhesion and aggregation of HV- *F. columnare* isolates adhere first at the tips of the gill filaments before the bacterial cells pursue their way to the middle and base of the filament, and eventually colonize the complete filament. Rainbow trout showed more focal tissue destruction compared to complete gill filament disintegration being most conspicuous in carp. Moreover, lysis of the cartilaginous tissue was perceived in carp. The production of OMV merits further attention, as these structures have been described to play a role in virulence and tissue lysis and have, as far as we know, never before been described in *F. columnare in vivo*. Furthermore, biofilm formation was discerned with bacterial cells wrapped in a PAS-positive matrix while being encased by AB-positive mucus. The observed shifts in goblet cells both quantitatively and qualitatively point towards the complex yet intriguing interplay of *F. columnare* with the gill mucus again warranting further research. The perceived increase in apoptotic cells and the higher EGC counts conclude the list of conspicuous features that provide pointers for future research on the pathogenesis of columnaris disease.

5.6 ACKNOWLEDGEMENTS

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GENERAL DISCUSSION

GENERAL DISCUSSION

According to the FAO (Food Agriculture Organization of the United Nations), presently 53% of the 600 wild fish species with economic value are fully exploited, 28% overexploited and 3% depleted (FAO, 2010, 2014). A growing percent of the world aquatic production derives from aquaculture, and its importance will grow dramatically as a result of overfishing of the world's waters and an increasing demand for seafood (Esteban, 2012; FAO, 2014). World aquaculture is heavily dominated by the Asia-Pacific region, which accounts for 89% of production in terms of quantity and 77% in terms of value. This dominance is mainly due to China's enormous production, which accounts for 67% of global production in terms of quantity and 49% of global value. China produces 77% of all carps (cyprinids) (FAO, 2014), representing the largest group of cultured freshwater fish (Singh, 1997; FAO, 2014) while Norway and Chile are the world's two leading producers of cultured salmons (salmonids), accounting for 33 and 31%, respectively, of world production (FAO, 2014). Farming of ornamental fish also became popular, originating largely from South-East Asia (Michel et al., 2002; Smith et al., 2012; Stickney, 2000). Over 1 billion ornamental fish comprising more than 4000 freshwater and 1400 marine species are traded internationally each year (Whittington and Chong, 2007). Considered a luxury item, the retail value of the ornamental fishes worldwide was estimated between US$ 4,000 and US$ 7,200 million in 2000 (Chapman, 2000) and kept on growing ever since. With the increasing interest in expensive outdoor garden ponds, more specialty producers are raising fish, of which some koi carp are sold for hundreds of dollars each and champion koi carp are even paid over US$ 10,000 (Watson et al., 2004). However, as a negative impact of the growth of the food and ornamental fish sector in the past years, increased intensification has led to more disease outbreaks with concomitant high economic losses. Ornamental fish in trade for example are mostly tropical in origin, and require the same warm and aerated environments that favour bacterial growth (Smith et al., 2012). One of the serious bacterial diseases affecting ornamental fish is columnaris disease (Declercq et al., 2015). The latter disease also causes huge financial losses in important food fishes such as rainbow trout (Pulkkinen et al., 2010) and in cyprinids, e.g. carp (Singh, 1997). The rationale for including rainbow trout and carp in this research is to be situated in the economic relevance of the fish species and their sensitivity to columnaris disease, which is increasingly frequent with only scarce data available on its pathogenesis.

1. Antimicrobial therapy

Although the research in the present thesis did not aim to investigate treatment options against columnaris disease, several antimicrobial administration routes and treatment options are discussed here to shortly introduce the reader into the differences in antimicrobial treatment between aquatic and terrestrial animals.

To control infectious diseases, similar strategies are employed in the aquaculture sector compared to other areas of animal production, e.g. vaccination and the use of antimicrobial agents (Heuer et al., 2009). However, the administration of antimicrobials to animals in aquatic environments differs significantly from terrestrial animals (Park et al., 2012). Antimicrobials in food fish aquaculture may be administered through the water or via the feed (Heuer et al., 2009; Park et al., 2012). Other methods for administering drugs such as injecting the fish or treating them topically, are labour intensive, cause stress in the fish, and mostly require anaesthesia, leaving these methods to be used less frequently (Park et al., 2012). Although simple to administer, water medication gives undesirable exposure of the environment to the drugs (Cabello et al., 2013; Heuer et al., 2009; Love et al., 2011; Park et al., 2012; Shah et al., 2012). The most important limitation of medicated feed is that fish to be treated must be feeding actively, which is cumbersome as diseased fish easily cease to eat (Park et al., 2012; Wall and Widgoose, 2005). Uneaten food may also sink to the bottom and accumulate along with medicated faeces containing unmetabolised drugs, such as described for oxytetracycline (Cabello et al., 2013; Park et al., 2012). In addition, the antimicrobial agent needs to be absorbed by the intestine. Herein, differences may occur in between fish species. Upon administering a single oral dose of 75mg/kg fish of oxytetracycline in trout, the apparent oral bioavailability was 5.6% (Björklund and Bylund, 1991). In carp on the other hand, oral administration of oxytetracycline at 60mg/kg body weight revealed that the bioavailability would only be 0.6% (Grondel et al., 1987). In another study of Hansen and Horsberg (2000), flumequine was administered orally to cod and wrasse at a dose of 10 mg/kg body weight. Pharmacokinetic modelling of the data showed that flumequine had quite different pharmacokinetic properties in cod and wrasse and the oral bioavailabilities were calculated to be 65% (cod) and 41% (wrasse) (Hansen and Horsberg, 2000). Of the ingested medicated feed, approximately 80% of antimicrobials used enter the environment with their activity intact where they may select for resistant bacteria (Armstrong et al., 2005; Cabello et al., 2013).

The safety of human food can also directly be affected by the presence of residual antimicrobial agents in farmed fish products (Cabello et al., 2013; Fortt et al., 2007; Love et al., 2011; Park et al., 2012; Tittlemier et al., 2007). Hence, the withdrawal period, which is the time between the last antimicrobial treatment of the fish and the point at which the fish is processed into a

food product, has to be taken into consideration. The withdrawal period is dependent on the antimicrobial agent used, the fish species in which the drugs are applied (Park et al., 2012) and the ambient water temperature (Park et al., 2012; Wall and Widgoose, 2005). The withdrawal period is expressed in degree days (Wall and Widgoose, 2005), which are the number of (treatment) days multiplied by the water temperature. The usual treatment time is 7 to 10 days and the legal withdrawal period for fish is 500 degree days (Wall and Widgoose, 2005).

In ornamental fish, attention to water quality and husbandry are considered primary in the management of bacterial diseases. The same antimicrobial administration pathways may be applied as in food fish aquaculture. Moreover, antimicrobials are commonly administered by intramuscular injection (Wall and Widgoose, 2005).

The introduction of vaccines has substantially reduced the need for antimicrobial agents. In Norwegian fish farming industry for example, the combination of an improved management and effective vaccine strategies, mostly against furunculosis (caused by *Aeromonas salmonicida*), reduced the antimicrobial use with 99% from 1987 through 2007, despite a substantial increase in Atlantic salmon and rainbow trout production during that period (Heuer et al., 2009). For columnaris disease, two vaccines are commercially available. An attenuated immersion vaccine is registered for the use in channel catfish (Shoemaker et al., 2011) and largemouth bass (Bebak et al., 2009) and a bacterin for use in salmonids (AFS-FCS, 2011). Unfortunately, these vaccines are only registered in the United States of America (USA), making other countries to rely upon the administration of antimicrobial or chemical agents for (curative) treatment of columnaris disease (Declercq et al., 2013).

Antimicrobial agents mostly used in both the aquaculture food and ornamental fish sector are quinolones, oxytetracycline (Cabello et al., 2013; Parker et al., 2012; Shah et al., 2012; Weir et al., 2012) and florfenicol (Cabello et al., 2013). Quinolones are mostly directed towards Gram-negative bacteria, making them suitable for the treatment of most of the bacterial infections in fish (Park et al., 2012). However, second generation quinolones (fluoroquinolones such as enrofloxacin) are among the World Organisation for Animal Health (OIE) “critically important” antimicrobials (CIA) for veterinary health (OIE, 2007), and should be limited or avoided whenever possible (Tuševljak et al., 2013). The efficacy of florfenicol towards several bacterial diseases, including columnaris disease, has been reported in channel catfish, in striped bass and sunshine bass (Darwish et al., 2012; Gaunt et al., 2010). However, florfenicol is listed as “veterinary critically important”, meaning it is

an important antimicrobial, essential against specific infections and there is a lack of sufficient therapeutic alternatives (OIE, 2007).

To be able to choose the most effective drugs for therapy, it is advisable to sample for bacterial culture and perform sensitivity testing on the retrieved isolate (Wall and Widgoose, 2005). However, in case of columnaris disease, two items need to be considered. Firstly, as columnaris disease can strike hard and fast (Bootsma and Clerx, 1976; Davis, 1922; Decostere and Haesebrouck; 1999; Evenhuis et al., 2014; Foscarini, 1989; Hawke and Thune, 1992; Marks and Lewis, 1980; Morrison et al., 1981; Pacha and Ordal, 1967) and since susceptibility results may not be available for several days, treatment may need to be initiated immediately to limit the severity and spread of the disease (Wall and Widgoose, 2005). Secondly, susceptibility testing against identified pathogens should be performed in a standard manner (Park et al., 2012), which can be a real challenge for fastidious organisms such as *F. columnare*. The Clinical and Laboratory Standards Institute (CLSI) proposes the use of the broth microdilution technique for fastidious growing organisms like *F. columnare* (CLSI 2006).

For interpretation of results of minimum inhibitory concentration (MIC) determinations of antimicrobials, either epidemiological cut-off values or clinical breakpoints can be used (Schwarz et al., 2010). Clinical breakpoints aim to predict how a patient will respond to a treatment and they classify bacteria as susceptible, intermediate or resistant (Schwarz et al., 2010). These breakpoints are mainly based on pharmacological and clinical criteria. They try to correlate the *in vitro*-susceptibility of a bacterium towards a certain antimicrobial agent (the MIC-value) with the chance to successfully treat an animal with the normal, recommended dose of this antimicrobial agent (Turnidge and Patterson, 2007; Schwarz et al., 2010). Epidemiological cut-off values on the other hand, do not take into account the results of clinical efficacy studies or the pharmacological parameters. They are mainly based on the microbiological criterion, sometimes in combination with the genetic criterion, and allow to distinguish wild type populations of bacteria from those with acquired resistance (non-wild type). They do not necessarily predict how a patient will respond to antimicrobial therapy (Dung et al., 2008; Schwarz et al., 2010). Contrary to mammals, for fish, clinical breakpoints have not yet been established for *F. columnare*. Hence, in Chapter 3, MIC-values of 97 *F. columnare* isolates collected worldwide from food and ornamental fish species were determined for 12 antimicrobial agents and interpreted using the microbiological criterion.

As there is a need for standardized testing, the broth microdilution method as prescribed by CLSI (2006) was applied, designating two reference isolates, *E. coli* ATCC25922 and *A. salmonicida* ATCC 33658 and employing standardized 96-well plates. Furthermore,

the choice of the growth medium is of extreme importance and should fulfil three requirements (Smith, 1998): (1) the composition of the medium should give sufficient growth for the isolates to be tested, (2) the medium must not contain material that would interfere with the test itself or react with any antimicrobial agent tested, and (3) the media used in performing such tests should be specifically formulated. Different media have been described to determine the antimicrobial susceptibility of *F. columnare*: Ordal's medium (Koski et al., 1993; Soltani et al., 1995), 1:7 dilution of Mueller-Hinton (MH) supplemented with 5% fetal calf serum (Hawke and Thune, 1992), Shieh medium (Zhao et al., 1997; Decostere et al., 1998) and 1:5 dilution of MH (Farmer, 2004). MH medium is a widely accepted standard medium for determining antimicrobial susceptibility of pathogens from terrestrial and aquatic animals (Dalsgaard, 2001; CLSI, 2002; Miller et al., 2005; CLSI, 2006). Darwish et al. (2008) stated that diluted MH at a concentration of 1:5 rather than 1:7 yielded statistically better growth of *F. columnare* ATCC 23463, and the 1:5 dilution of MH broth was found to be the lowest dilution that would not compromise the growth of *F. columnare* (Farmer, 2004). As we did not find a significant difference in growth nor MIC-values of seven *F. columnare* isolates in between 1:5 or 1:7 diluted cation-adjusted MH broth (CAMHB) (data not shown), we opted to adopt the 1:7 dilution according to the guidelines of CLSI (2006). In a recent article by Gieseker et al. (2012) it is stated that the associated quality control ranges applying a 1:5 diluted CAMHB have been approved by the CLSI, and will be included in the next edition of the CLSI M49-A Guideline (Gieseker et al., 2012). However, at the moment the MIC-trials of Chapter 3 were performed, the latter information was not yet available.

Acquired resistance towards the first (flumequin and oxolinic acid) generation quinolones was found in all 9 *F. columnare* isolates from Vietnamese catfish and three isolates moreover displayed acquired resistance towards the second (enrofloxacin) generation quinolones. Vietnam is the third largest aquaculture producing country in the world (FAO, 2012) due to intensive *Pangasius* production in flow through systems, which brought along outbreaks of bacterial diseases and consequently, an increasing antimicrobial use of amongst others enrofloxacin (Andrieu et al., 2015). A recent study indicates that effluents from *Pangasius* farms using enrofloxacin are an important way of antimicrobial pollution into the aquatic environment. The findings in the present study might be indicative of a less prudent application of quinolones and/or a less stringent control on their use.

Indeed, additional statistical analysis of all data collected from the *F. columnare* isolates and comparing region of origin (Europe, USA or Asia) and fish type (ornamental versus food or wild fish) for the acquired resistance found towards the quinolones, oxytetracycline and nitrofuran, indicates that bacterial isolates of food or wild fish collected from Asia displayed significantly more

($p < 0.01$) acquired resistance compared to the ones collected from Europe and USA. However, further research needs to be carried out to verify the former supposition as other elements may have contributed to the encountered resistance. In addition, a full set of data on the origin of the isolates is lacking. For example, we do not know whether the available samples from Vietnamese catfish are fully independent from each other (viz. exact location of farm, common origin of fish from which the isolates were retrieved, …), which might have influenced the results.

Statistical analysis further confirmed significantly more ($p < 0.01$) acquired resistance for all the tested antimicrobials for the isolates procured from ornamental fish species compared to food or wild fishes. This might point towards less prudent use of antimicrobial agents in the ornamental fish sector.

Bacterial antimicrobial resistance can arise from mutations in the target gene or incorporations of foreign genetic material in the chromosome, or, more importantly, from mobile genetic elements containing multiple resistance determinants transmissible to other bacteria (Alekshun and Levy, 2007; Cabello et al., 2013; Heuer et al., 2009; Park et al., 2012; Shah et al., 2012). The possible horizontal spread of these resistance genes from drug-resistant fish bacteria to human pathogens, is assigned as the indirect way of resistance transfer. This transfer of resistance genes between bacteria, could indeed impose a risk for human health (Alekshun and Levy, 2007; Cabello et al., 2013; Dobiasova et al., 2014; Kesarcodi-Watson et al., 2008; Love et al., 2012; Park et al., 2012). Direct resistance transfer arises when resistant zoonotic bacteria, such as *Edwardsiella tarda*, or some *Mycobacterium* and *Vibrio* species infect humans by contact or through the food chain (Cabello et al., 2013; Heuer et al., 2009).

In the present research, acquired resistance of *F. columnare* was found amongst others against the quinolones and oxytetracycline. Drlica et al. (2009) hypothesized that quinolone resistance arises stepwise through selective amplification of mutants when drug concentrations are above the MIC-values of the susceptible population and below the MIC-values of the least susceptible mutant subpopulation. Resistance towards quinolones due to mutations is frequently found in the quinolone-resistance-determining-region of the DNA gyrase subunit A (*gyr*A) (Alekshun and Levy, 2007; Shah et al., 2012).

Preliminary full genomic sequencing results (not shown) of three *F. columnare* isolates proved so far a single point mutation in *gyr*A of the LV-koi carp isolate (CDI-A) resistant towards both first and second generation quinolones. Mutation in *gyr*A has been described to generate resistance to the first generation quinolones and reduced susceptibility to other quinolones (Marien et al., 2007). This coincides with the resistance found towards oxolinic acid and flumequin and the reduced

susceptibility or resistance towards enrofloxacin for CDI-A. Further research is on-going to explore whether more point mutations are present in other gyrase genes.

The antimicrobial activity of tetracyclines is elicited by interference with the 30S subunit of bacteria. Resistance towards tetracyclines is caused by pumps in the inner cell membrane of Gram-negative bacteria that reduce the levels of tetracyclines within the cytoplasm (Chopra and Roberts, 2001; Wang et al., 2014). The best-studied pumps of the Gram-negative bacteria are the ones encoded by the *tet*A, B and C determinants, which have been found on transferable plasmids and in the chromosomes of bacteria. By means of these mobility mechanisms, tetracycline resistance genes have been transferred from species to species involving a large number of genera (Chopra and Roberts, 2001).

Preliminary full genomic sequencing results (not shown) of three *F. columnare* isolates indicated the presence of a *tet*A gene in the HV-koi isolate 04017018. Class A *tet* determinants can confer high-level tetracycline resistance (Wang et al., 2014). Whether or not the *tet*A gene is the only cause of resistance and whether or not it lies on a plasmid and could hence be easy transferable to other bacteria, is still to be investigated.

Taking full account of the various disadvantages and considerations associated with the use of antimicrobials, modern aquaculture industry should direct all efforts towards alternative preventive practices that may help maintain good animal health as well as a healthy environment, resulting in better production and higher profits (Esteban, 2012). The emergence of "green" labels and heightened environmental awareness make the development of sustainable aquaculture necessary (Esteban, 2012), while one should strive for a decrease in the use of antimicrobial agents. A better understanding of the pathogenesis of important diseases such as columnaris disease, is therefore crucial to be able to combat the disease without having to resort to antimicrobial treatment or at least to reserve this for exceptional cases when alternative treatments have failed.

2. Pathogenesis study

As described in the introduction of this thesis (Chapter 1), columnaris disease causes mostly gill and skin lesions. When this research was initiated, hardly any data were available on the *in vivo* interaction between the gill tissue of carp and trout on the one hand, and *F. columnare* on the other hand. The need for research in this field of aquatic veterinary medicine, more specifically with regard to gill lesions caused by columnaris disease in carp and trout, formed the rationale for

investigating the gill pathology in these fish species. Firstly, this was done by developing an infection model inducing gill lesions and simulating the natural way of infection as accurately as possible (Chapter 4). Secondly, we aimed at studying in detail the elicited gill lesions and the localisation, organization and number of the *F. columnare* bacterial cells in the course of time (Chapter 5). In what follows, various important steps in the pathogenesis of columnaris disease are discussed and ideas for further research put forward.

2.1 Adherence to the host tissue

To enter the host, specific bacterial adhesion is of crucial importance, in which both pathogen and host are major influential actors. In addition, the environment plays an important role. Previous studies have shown that in *F. columnare*, adhesion to host tissues was correlated with virulence (Bader et al., 2005; Decostere et al., 1999b) while attachment to polystyrene (Kunttu et al., 2011) and gill cells (Zaldivar, 1985) was not. Hence, contradictory results are found *in vitro* compared to *in vivo* research, stressing the importance of the latter trials to understand interactions occurring between host and pathogen. In the aquatic setting of the trials performed in this thesis, water temperature was a crucial environmental parameter. This soon became clear in preliminary infection trials in trout, in which the animals did not display any signs of discomfort nor disease after immersion challenge with *F. columnare* performed at 21°C (results not shown). The water temperature of the inoculation tank had to be increased to 23°C to enable the successful generation of a challenge model in which the premised aim of creating gill lesions was achieved. A challenge temperature of 23°C in trout might seem high, but during summer the water temperature in rearing facilities of rainbow trout may rise rapidly to approximately 20°C and higher (Suomalainen et al., 2005). Moreover, a general summer water warming trend, amongst others in Finland, has been described (Altermatt et al., 2008). As columnaris disease outbreaks occur mainly during the summer, when the warm water favours its occurrence as described in Chapter 1, we chose to challenge the animals at these representative summer water temperatures. The carp were challenged at 25°C, a temperature falling in the comfort zone of carp and in the optimal temperature growth range of *F. columnare* (24-28°C). High temperature would also increase the adhesion ability of HV-*F. columnare* colony types *in vitro* (Kunttu et al., 2011). Temperature solely does however not seem to offer the key for HV-isolates to induce disease.

2.2 Multiplication and biofilm formation of the bacteria

Both the bacterial titres retrieved from gill tissue and the bacterial number displayed microscopically after inoculation with the HV-isolate in carp and trout, remained high in the course of time. Following challenge with the LV-isolate in carp, bacterial titres also stayed high in those fish displaying lesions in which bacterial cells could be visualised microscopically, but still in significantly lower amounts as compared to the HV-isolate. In contrast, in trout, the retrieved bacterial titres from the LV-isolate inoculated fish decreased to numbers under the detection limit of bacteriological plate counts and microscopical analysis. Hence, although adhesion is needed to initiate an infection, multiplication capacity seems to be an important virulence factor as well, bringing us to the next step of the colonization process.

A remarkable feature in the challenge trials of Chapter 4 was that the HV-trout isolates JIP P11/91 and B259 induced no or only 20% mortality, respectively, in one out of the three trials as described in Chapter 4. In the trials rendering no to low mortality, the cultivated broth was much less viscous compared to the broth adopted in the trials inducing 100% mortality. A single isolate can generate different colony types resulting in varying virulence (Kunttu et al., 2009, 2011) and it may be hypothesized that colonies with lower virulence were selected from the agar plates to be inoculated in the broth used for immersion challenge. The viscosity data of the inoculation broths in the *in vivo* trials of Chapter 4 and 5 combined with later *in vitro* trials proved that adopting rhizoid colonies – linked to high virulence and hence high mortality – for inoculation consistently resulted in a highly viscous broth (results not shown). Inoculation of the rough colonies produced by the LV-isolates effectuated a less viscous broth after 24h incubation at 28°C (results not shown). This is the first time that a possible correlation between the viscous character of the associated broth cultures and the resulting virulence in subsequent immersion challenge trials is raised. The reason why the viscosity may differ between isolates of different virulence has not yet been resolved. Virulence and adherence of bacterial colonies with varying colony morphologies could not be correlated *in vitro* and no molecular differences could be found between different colony types of a single isolate (Kunttu et al., 2009). Rhizoid colony morphology is however linked with the gliding capacity of *F. columnare*. The suggestion was made that both gliding motility and the excretion of the enzyme chondroitin AC lyase are needed for virulence to be induced (Kunttu et al., 2011), but further research is needed to confirm this finding. Chondroitin AC lyase is capable of breaking down acid mucopolysaccharides and of binding together the cells of animal skin, bone, muscle and cartilage tissue (Griffin, 1987) as if to create some protective environment, possibly aiding in the biofilm formation. Although this enzyme has been described to be produced solely by pathogenic

isolates (Griffin, 1987), more recent research shows that LV-bacteria with a smooth colony morphology also produce chondroitin AC lyase, as do the virulent bacteria characterized by a rhizoid colony morphology (Kunttu et al., 2011). In our own research, we did not investigate a possible correlation between virulence and chondroitin AC lyase production, but it would certainly be interesting to measure the activity of this enzyme in future challenge trials. Another explanation might be found in the secretion of bacterial exopolysaccharides. In the human Gram-positive pathogen *Actinomyces oris*, viscosity of the culture broth is associated with virulence and biofilm formation, and the viscosity is determined by the production of exopolysaccharides. This phenotype has been proposed to play an important role for *A. oris* to express virulence through the formation of abscesses. The bacterium can moreover evade destruction and elimination by host phagocytic cells due to protection in the extracellular matrix formed (Yamane et al., 2012). The factors determining the degree of viscosity of the *F. columnare* culture broth need clarification. Moreover, a standardized method should be designed to measure viscosity in an objective way to possibly predict the outcome of a challenge trial based on the viscosity of the adopted *F. columnare* isolates.

Bacterial motility is an important factor for rapid colonization of a surface (Álvarez et al., 2006). The gliding motility in *F. columnnare* is well-known (Bernardet and Bowman, 2006; Decostere 1997, 1998). The rate of motility has been linked to biofilm formation and the production of virulence factors in different pathogenic Gram-negative bacteria (Choy et al., 2004; Gardel and Mekalanos., 1996; Ha et al., 2003; Lee et al., 2004; Pratt and Kolter, 1998). Firstly, the bacterial cells are highly motile and seen gliding over the surface prior to attachment. After this step, the bacterial cells loose the gliding ability and become immotile in a biofilm (Cai et al., 2013). This makes it tempting to appoint biofilm formation from as soon as the gliding motility in *F. columnare* cells has completely disappeared. The developmental switch to the biofilm state is commonly regulated by quorum sensing (QS), a mechanism of gene regulation in which bacteria coordinate the expression of certain genes in response to the concentration of small signal molecules. The *Vibrio fischeri* LuxR/LuxI system was the first bacterial cell–cell communication to be characterized. In Gram-negative bacteria, two important proteins are involved in the regulation of QS, as described by Lazdunski et al. (2004). An I-protein is the enzyme that synthesizes the signalling molecule, usually an *N*-Acyl homoserine lactone (AHL) (Lazdunski et al., 2004). Additionally, a signalling molecule known as autoinducer-2 (AI-2) may be involved (Camilli and Bassler, 2006). The signal molecules are detected by an R-protein, a transcriptional regulator. AHLs accumulate extracellularly and once a certain threshold concentration has been reached, AHL-molecules bind to a response regulator (LuxR homolog). Subsequently, the LuxR/AHL-complex

activates the transcription of QS-regulated genes (Lazdunski et al., 2004). *In vitro* assays implying the simultaneous use of different biosensors were performed to screen both HV- and LV-isolates of *F. columnare* for AHL-production (results not shown). After negative results were found when investigating a non-AHL signal molecule pathway in an attempt to detect AI-2, QS could not be detected in *F. columnare* using the systems investigating QS-mechanisms known so far (results not shown). However, although *F. columnare* might not be capable of producing AHL or AI-2, the *lux*R-gene is present in the genome of the fully sequenced LV- and HV-isolates (results not shown), suggesting that *F. columnare* cells may be able to detect QS-molecules. This is seen in biosensors or reporters that are able to detect the presence of AHL but do not produce these factors themselves. AHL can be detected by binding a functional LuxR-family protein and this complex can then bind with a cognate target promoter which positively regulates the transcription of a reporter gene (Defoirdt et al., 2013; Steindler and Venturi, 2006) such as in *Chromobacterium violaceum* CV026 (McLean et al., 1997). Another QS-pathway in *F. columnare* might be by the production of indole serving as a signal molecule in many pathogenic bacteria such as *E. coli*, several *Shigella* strains, *Enterococcus faecalis*, and *V. cholera* (Lee and Lee, 2010). Several *Flavobacterium* species such as *F. columnare* (Bernardet and Bowman, 2006; Lee and Lee, 2010) have been shown to produce indole. Whether the latter has a signalling function, is hitherto unkown. Therefore, the way by which biofilm is regulated and formed in *F. columnare*, deserves further research.

Other characteristics, besides losing gliding motility, need to be present as well before biofilm formation can be involved. A biofilm is "a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum, interface or to each other, are embedded in a matrix of extracellular substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription" (Donlan and Costerton, 2002). Biofilms are estimated to contain 85% extrapolymeric substances (EPS) which are composed of water, polysaccharides, DNA and proteins (Donlan and Costerton, 2002). The carbohydrate composition of EPS produced by *F. columnare* isolates would predominantly consist of glucose and glucuronic acid, with traces of galactose, mannose and rhamnose (de Alexandre Sebastião et al., 2013). *In vitro*, both HV- and LV-*F. columnare* isolates have been described to form biofilms under static conditions, albeit at a different extent (Cai et al., 2013). In water flow chambers, HV-isolates could form biofilm completely covering the chamber surface, while the LV-isolate biofilm formation would remain confined to the sides of the chamber (Cai et al., 2013). Simulating the water flow, the latter situation more closely resembles the real life environment in fish, denoting the importance of an *in vivo* model to get a better understanding of the complex interactions in the biofilm formation of the

bacterial cells at the gill tissue site. In the pathogenesis study of Chapter 5, the HV bacteria were capable of maintaining high bacterial titres, and of forming biofilms at the gill tissue surface. This thesis is the first to show biofilm formation in the gill tissue presented *in vivo* after inoculation with the HV-*F. columnare* isolates in carp and trout and moreover in a minority of the LV-isolate challenged carp as well. Histologic examination showed the bacterial cells to be embedded in an eosinophilic matrix as soon as 1.5 h post inoculation. The eosinophilic matrix observed, is most probably the extracellular matrix produced by the bacterial cells. Using PAS/AB-stains, the layer immediately surrounding the bacterial cells coloured pink (PAS-positive or neutral mucins) and was surrounded by a second layer which stained purple (PAS/AB-positive or combined neutral/acid mucins respectively). Bacterial cells were never directly surrounded by blue stained (AB-positive or acid) mucins, which strongly suggests that AB-positive stained mucins encountered in the gill samples were produced only by the host defence system.

Host mucus is considered to be a first line defence against infection through the gill epithelium. Mucus has evolved to have robust mechanisms that can trap and immobilize pathogens before these make contact with the epithelial surfaces (Cone, 2009). Entrapped particles can be removed from the mucosa by the water current (Mayer, 2003). For this reason, mucus in most fish is continuously secreted and replaced, preventing the stable colonization of potential infectious microorganisms (Nagashima et al., 2003). Indeed, in our study of Chapter 5, both fish species displayed a higher total gill goblet cell count after challenge with one of the *F. columnare* isolates, although this was only significantly higher in carp. This corresponds to former studies reporting an increase in mucus production after exposure to parasites and bacteria (Roberts and Powell, 2005, van der Marel et al., 2010) as part of the immune host response. The thickness of the mucus blanket is determined by the balance between the rate of secretion and rate of degradation or shedding. Mucus transport requires well-regulated viscoelasticity, regulated by hydration (Cone, 2009). Most likely, mucosal epithelia can change the ionic environment or pH (Forstner, 1995; Thornton and Sheehan, 2004). Lowering the pH would increase viscosity, while a higher pH creates a more liquid mucus layer (Celli et al., 2009). The challenge with *F. columnare* stimulates the host production of AB-positive acid glycoconjugates which have been linked to negative charges in mucins, reducing bacterial binding. The latter is another reason to suggest that these AB-positive mucins are secreted only by the host and not by the bacteria. The AB-positive mucins were seen encasing the bacterial cells, as a means adopted by the host to defend itself. After challenge with the HV-isolate, PAS-positive cell counts were also increased. This was not the case following exposure with the LV-isolate. Perhaps, the HV-isolate is capable of redirecting the mucin secretion towards

a more favourable environmental state, resulting in better binding capacity due to a change in charges in the mucins. None of this has been investigated so far in *F. columnare*. This theory could be reinforced by additionally investigating the expression of mucin genes as a response to an experimental *F. columnare* challenge with both HV- and LV-isolates.

Bacterial biofilms are frequently less susceptible to antimicrobials (Sundell and Wiklund, 2011), and moreover they have an important role as reservoirs of pathogens, enabling these to persist in aquacultural settings for a long period of time (Wingender and Flemming, 2011). Biofilm of *F. columnare* cells was described to preserve virulence, as confirmed by cutaneous inoculation of channel catfish fingerlings with mature *in vitro* prepared biofilm (Cai et al., 2013). Suggested mechanisms by which bacterial biofilms can sustain disease include detachment of cells or cell aggregates, production of endotoxins, resistance to the host immune system, and provision of a niche for the generation of resistant organisms (through resistance plasmid exchange, as described higher) (Donlan and Costerton, 2002). The study of Chapter 5 showed that bacteria attached firstly at the sides of the gill tissue and at the gill tips and then moved their way to the middle and if capable, to the basis of the gill filaments. This means that the bacteria colonized the gill tissue counter-current to the water-flow over the gill tissue, as shown in Figure 1.

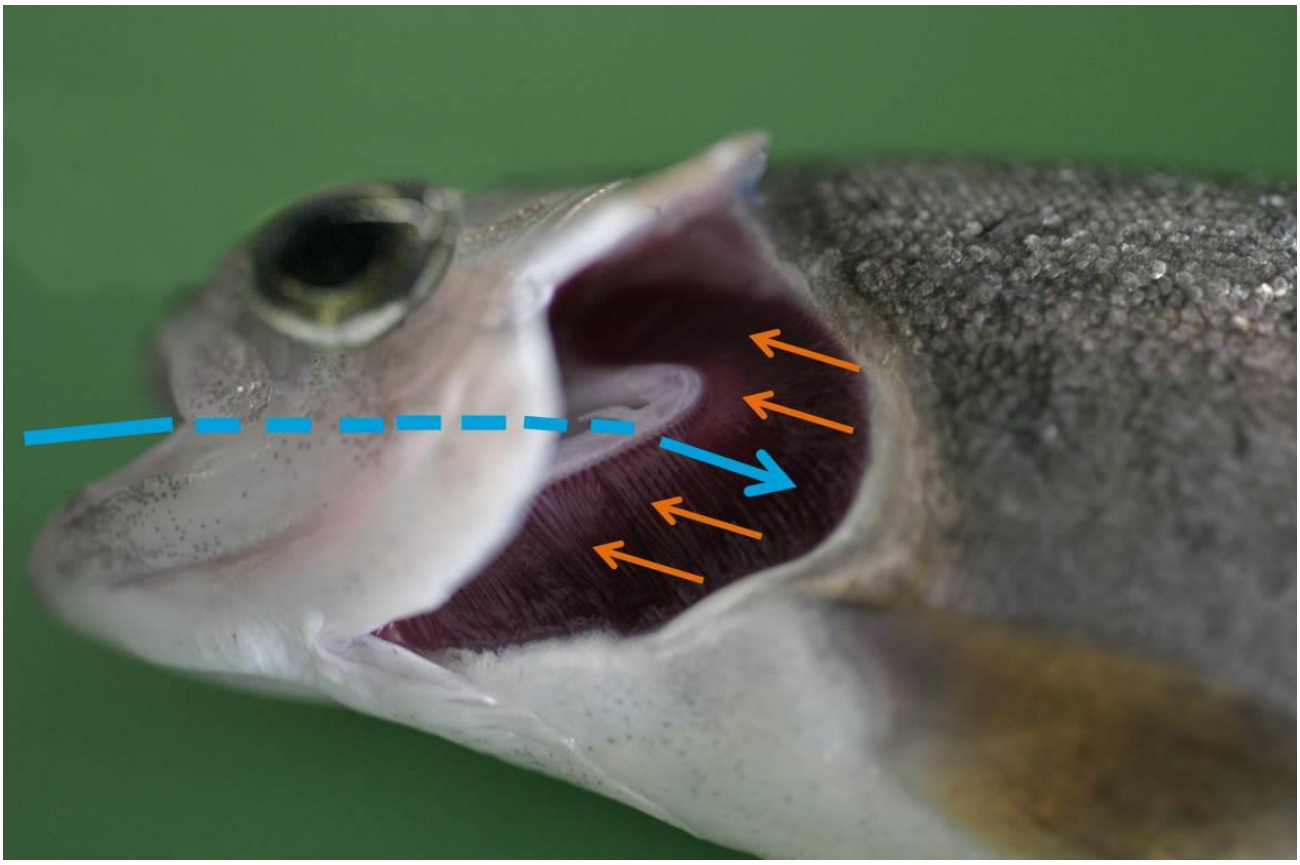

Figure 1. Water flow (blue arrow) and *F. columnare* colonization direction (orange arrows) in a trout.

Engineers speculate that turbulent flow enhances bacterial adhesion and biofilm formation by swaying the planktonic cells on the surface, but whatever the mechanism, biofilms form preferentially at high-shear locations in natural and industrial systems (Donlan and Costerton, 2002). The gill filament tips hence seem to offer an ideal place to start a biofilm and moving against the water stream in this respect becomes logical.

The formation of biofilms is particularly relevant for immunocompromised fish, lacking the ability to combat invading organisms (Donlan and Costerton, 2002). Fish can indeed react differently in behavioural responses to certain stress situations (Castanheira et al., 2013). This might lead to a varying impact of disease agents, such as seen after exposure to the LV-isolate in the carp. The latter bring us to the next required step in the bacterial colonization of the tissue; evading or resisting the immune system of the host.

2.3 Evade or resist host (immune) responses

One of the host immune defense mechanisms is to sacrifice cells infected or damaged by bacteria for the benefit of the other cells. Apoptosis is one of those pathways that can be applied to cause cell death and is critical in the defense of some microbial pathogens (Ashida et al., 2011). Two pathways can trigger apoptosis, viz. the extrinsic pathway and the intrinsic one (Ashida et al., 2011). One of the consequences of triggering the extrinsic pathway is the activation of caspase-3 (Ashida et al., 2011), which was measured in the current thesis to investigate apoptosis in the gill epithelia (Chapter 5). Generally speaking, apoptosis – in contrast to necrosis – does not induce inflammation processes (Van Cruchten and Van den Broeck, 2002). As the cell's innate immune system seeks to destroy invading pathogens by activating inflammatory complexes (Speir et al., 2014), blocking or delaying cell death is an excellent strategy applied by – especially intracellular – pathogens to evade the host detection system (Ashida et al., 2011). Natural outbreaks of columnaris disease indeed display a remarkable and, at the onset of this thesis, a hitherto unexplained lack of inflammatory response, especially in the early stages, allowing progression of the infection. The results from Chapter 5 show that the HV-isolate challenged fish, and in carp even the LV-isolate inoculated fish, display more apoptosis in the gills as an early response after inoculation with *F. columnare*. This supports the hypothesis that *F. columnare* is capable of postponing the immune response to create a time gap to enable itself to attach to and multiply at the level of the gill tissue. As in carp the caspase-3 immunohistochemical staining technique also evidenced higher apoptotic cell counts in the HV- compared to the LV-isolate inoculated fish, the HV-isolate seems to master

this evasion technique better as compared to the LV-isolate. The exact pathways in which *F. columnare* possibly induces apoptosis, are yet to be unraveled.

2.4 Damage the gill tissue

The fish gill is essentially composed of a highly complex vasculature, surrounded by a high surface area epithelium that provides a thin barrier between fish blood and aquatic environment. The entire cardiac output perfuses the branchial vasculature before entering the dorsal aorta and the systemic circulation. The gill tissue is an exceptional gas exchanger and the branchial epithelium is the primary site of transport processes that counter the effects of osmotic and ionic gradients, as well as the principal site of body fluid pH regulation and nitrogenous waste excretion. This makes the gill a multipurpose organ that plays a central role in a series of physiological responses to environmental and internal changes (Evans et al., 2005).

The first lesion noted as described in Chapter 5, is oedema. Although this might seem innocent, oedema is usually associated with important respiratory acid-base disturbances (Goss et al., 1992). To compensate for osmotic imbalances, there is an increased epithelial ion permeability coinciding with a possible influx of water leading to intercellular oedema. This is where the chloride cells come into the histological and physiological picture. The number of these Na+/K+-ATPase immunoreactive cells was however not significantly different between the fish inoculated with the HV- or LV-isolate and the control animals (Chapter 5). Noticeable is, that the more lysis occurs in the chloride cells, the more oedema can be observed in the gill tissue (Chapter 5).

A cell type possibly explaining part of the gill tissue damage is the Eosinophilic Granular Cell (EGC). Although shown to be present before in the gills of trout (Holland & Rowley, 1998), no EGC were perceived in the trout gills in our experiment, neither after using H&E or PAS-staining. This is in contrast to carp in which EGC were noted using both of the latter staining techniques in all fish gills sampled. Indeed, the presence of EGC was also discerned in the gill tissue of the control carp, but in the latter these cells did not migrate nor degranulate, in contrast to the findings after challenge with the HV-isolate. This is the first time that mobilization and degranulation of this cell type is shown in the gill tissue of carp after challenge with *F. columnare*. Mobilization of these EGC only occurs after stimulation by a trigger, which in this case may be presumed to be the bacterial cells. Stress due to the challenge might also indirectly have induced this effect. Indeed, hydrocortisone has been shown to have a pronounced effect on the EGC of the swimbladder of the brown trout, inducing degranulation (Reite, 1997). The degranulation is

followed by margination of neutrophil granulocytes in small blood vessels. This response might be an indication that chemical leucocyte attracting factors are released or produced in connection with EGC degranulation, while production of factors influencing vascular permeability may also be involved in the accumulation of fluid (Reite and Evensen, 2006). The latter might explain the observation of severe oedema induced in the gill tissue. This also evidences that after an initial evading of the immune system via apoptosis, the host does respond with an inflammation reaction (partly) induced by EGC in carp. The inflammatory factors released can then start combatting the bacterial cells, possibly explaining a decrease in the latter as observed microscopically after challenge with the LV-isolate in most carp. The other LV-and HV-bacterial cells have by that time had the chance to form biofilms, offering protection against the inflammation, as described above. The latter is merely a hypothesis, but links several phenomena seen.

The production of outer membrane vesicles (OMV) by HV- *F. columnare* isolates was noted in both fish species (Chapter 5). OMV may contain biologically active proteins important for nutrient acquisition, co-aggregation of bacteria and biofilm development, pathogenesis and bacterial survival (Frias et al., 2010; Kulp and Kuehn, 2010). They are accepted as key virulence factors of which all participate in colonization. Possibly, some factors released by the bacterial OMV may trigger migration and degranulation of the EGC, although this is currently merely speculative and yet to be proven. This is the first time that OMV are evidenced after *in vivo* trials adopting HV- and LV-isolates in *F. columnare*.

The impact of the former colonization steps and all mediators involved, result in severe gill damage.

Indeed, after the adhesive phase, the filament tips and afterwards the middle and bases of the filaments were disintegrated following inoculation with the HV-isolates. In carp, the bacterial colonization evolved into lysis of the cartilaginous tissue ending in the generalized necrosis and severe oedema interspersed with bacterial microcolonies. The lesions caused by the LV-carp isolate have to be spinned because lysis of the cartilagenous tissue does not occur. In trout, the lesions provoked are similar as compared to the ones after challenge with the HV-carp isolate, but these are mostly smaller in size, creating focal lesions. An explanation for the finding that trout lesions stayed more focal compared to the massive and total gill destruction after challenge with the HV-carp isolate, was not found and merits further research. The differences in gill damage severity between the carp and trout emphasize the importance of developing a challenge model for each fish species, as one model cannot easily be extrapolated to another fish species. Due to the development of the challenge models as described in Chapter 4, this is the first time that such severe lesions are

visualized and in detail described using (ultra)microscopic techniques in both carp and trout as described in Chapter 4 and 5. In former trials found in literature, the host tissue had to be scarified to induce lesions or only mortality rates were investigated, neglecting the series of events at the origin of infection, which is the host tissue. Moreover, no macroscopic gill lesions as observed in the field were induced in any model described previously. These formerly published models do not enable to gain insight in the interactions between the pathogen and its host at the very initial steps of the infection, at which stage the adhesion and/or colonization could possibly be prevented, offering important opportunities for testing treatments for columnaris disease. Several hypotheses have been launced as to why fish die as a result of columnaris disease. Necrosis of the lamellar respiratory epithelium would result in respiratory and osmoregulatory distress (Roberts and Rodger, 2012). An impaired gill vascular blood circulation would cause severe changes in heart performance. Owing to the close relationship between gill blood circulation and cardiac performance, the interaction of the two phenomena may result in the death of fish (Foscarini, 1989).

3. Conclusion

In conclusion, *F. columnare* has been an important research subject for over the past 100 years, which can be attributed to its worldwide distribution, its ability to infect all freshwater fish species, and its significant impact on both the food and ornamental fish sector. To treat the disease, antimicrobial agents may be adopted, although this may lead to an increase in antimicrobial resistance of *F. columnare*. The latter may imply health risks associated with the transfer of possible antimicrobial resistance genes to other bacteria or directly impacting humans consuming fish products. This urges the need not only to monitor the antimicrobial resistance in *F. columnare*, as was initiatied in the present thesis, but also to find alternatives for the treatment of columnaris disease. The latter was done in the second part of this research, where the aim was to unravel the formation of lesions developed at the gill tissue site of both carp and trout, not only in terms of evolvement in time and localisation, but also in correlation with the presence of bacterial cells. Chapter 4 succeeded in developing a model consistently resulting in severe gill lesions after challenge with HV-isolates in carp and trout, and even in some carp after challenge with the LV-isolate, implementing the basis of further interaction studies between *F. columnare* and the gill tissue. It was shown that it is important to optimize a challenge model for each fish species, as these can react differently in the displayed lesions, making it impossible to extrapolate all results to other fish species. In chapter 5, the colonization pattern of *F. columnare* isolates of differing virulence in both carp and trout was assessed and several challenging speculations and hypotheses were launched with respect to biofilm formation, apoptosis and inflammatory pathways, bringing the research

another step closer in understanding the way *F. columnare* interplays with its host colonization process of columnaris disease. This study also enforces multiple questions, which certainly are worthwhile to be addressed in future experiments.

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SUMMARY

SUMMARY

With the rapid expansion in both the food fish farming and ornamental fish sector over the past years, an increasing number of microorganisms has been identified as important fish pathogens, including *Flavobacterium columnare* (*F. columnare*), the causative agent of columnaris disease. This bacterium affects both cultured and wild freshwater fish including many susceptible commercially important fish species such as rainbouw trout and carp. *F. columnare* infections may result in skin lesions, fin erosion and gill necrosis, with a high degree of mortality, leading to severe economic losses. Especially in the last decade, various research groups have performed studies aimed at elucidating the pathogenesis of columnaris disease, leading to significant progress in defining the complex interactions between the organism and its host. Despite these efforts, the pathogenesis of columnaris disease hitherto largely remains unclear, compromising the further development of efficient curative and preventive measures to combat this disease. Besides elaborating on the agent and the disease it causes, **Chapter 1** aims to summarize these pathogenesis data emphasizing the areas meriting further investigation. The specific aims of the thesis can be found in **Chapter 2**. These were determining the antimicrobial susceptibility pattern of *F. columnare* by using reliable screening methods on a representative number of *F. columnare* isolates collected worldwide (**Chapter 3**), to develop an infection model generating gill lesions typical for columnaris disease in carp and rainbow trout and to classify *F. columnare* field isolates regarding virulence (**Chapter 4**) and to study the pathogenesis of columnaris disease with the gill tissue in carp and rainbow trout, adopting both highly and low virulent isolates (**Chapter 5**).

Up until now, only a limited number of effective preventive measures against columnaris disease are available, including an attenuated immersion vaccine registered for use in channel catfish and largemouth bass in the US only. Other countries hence rely upon other aids to combat the disease, amongst which antimicrobial agents. The possibility exists though that harmful bacteria can acquire resistance to previously effective antimicrobial agents, thereby giving rise to uncontrolled bacterial disease outbreaks. Hence, antimicrobial susceptibility monitoring is a necessary prerequisite to inform the veterinary practitioners on the therapeutic value of antimicrobial agents enabling them to make an informed choice. Despite the importance of columnaris disease, the antimicrobial susceptibility pattern of *F. columnare* is not well-studied. Therefore, 97 bacterial isolates of *F. columnare* were collected worldwide between 1987 to 2011 from 17 fish species of both the aquaculture and ornamental fish sector, to test their *in vitro* antimicrobial susceptibility towards 12 antimicrobial agents, as described in **Chapter 3**. This study is the first in its kind in view of the high number and mixed origin of *F. columnare* isolates in terms of fish species, year of

isolation and geographical area. The broth microdilution technique was utilized for reliable testing of the fastidious organisms. None of the isolates displayed acquired resistance to florfenicol, gentamicin, ormetoprim-sulfadimethoxin and trimethoprim-sulfametoxazole. Acquired resistance to chloramphenicol, nitrofuran, oxytetracycline, flumequin, oxolinic acid and enrofloxacin is reported for the first time in *F. columnare*. Furthermore, acquired resistance towards erythromycin and ampicillin was detected.

The isolates displaying acquired resistance originated from Vietnamese catfish or ornamental fish species except for two isolates coming from wild channel catfish in which acquired resistance was encountered towards oxytetracycline only. Moreover, fifty percent of the resistant isolates from ornamental fish were assigned as multiple resistant. The results obtained in this study indicate less prudent use of antimicrobials, especially in the ornamental fish industry and therefore urges to limit their use and to focus on preventive measures or alternatives for treatment of columnaris disease.

The modern aquaculture industry hence needs alternative preventive practices or treatments which allow the use of a "green" label, as environmental awareness grows and urges to help maintain a high animal welfare as well as a healthy environment, resulting in better production and higher profits. Crucial in the development of such techniques in combatting columnaris disease, is the understanding of the pathogenesis of this disease, starting at the initial phase of colonization: the induction of lesions, amongst which the important but understudied gill lesions. The need for research in this neglected field of aquatic veterinary medicine, more specifically with regard to gill lesions caused by columnaris disease in carp and trout, formed the basis for investigating the gill pathology in these fish species by firstly developing an *in vivo* infection model inducing gill lesions **(Chapter 4)**.

The gill tissue of carp and rainbow trout fry were exposed to different *F. columnare* isolates by means of immersion challenge. The fish were allowed to swim for 90 min in a suspension of one out of five (carp) or six (trout) available *F. columnare* isolates (approximating 1-7 x 10^8 CFU/ml, contact infection). For carp, four isolates (0401781, 0901393, 10009061-1 and 10012573/2) resulted in 100% mortality within 12 h, assigning these as highly virulent (HV). The other isolate (CDI-A) gave around 5% mortality within 18 h, being assigned to the low virulent (LV) group. From 100% of the moribund carp, *F. columnare* cells could be retrieved from the gill tissue. In trout, two isolates (B259 and JIP P11/91) were able to induce mortality in 100% of the fish, being assigned to the HV-group. One trout isolate (LVDJ (D7461)) was able to kill around 50% of the fish and was hence designated as moderately virulent. The other three isolates (H2, Coho 92 and JIP 44/87) with which

the challenge resulted in a mortality rate of less than 10%, got assigned a LV-label. Reisolation of *F columnare* cells from the gill tissue of moribund trout varied from 67% of the inoculated fish in one LV-isolate to over 90% in the fish challenged with the moderately or HV-isolate.

Both macro- and microscopic examination of the affected gills revealed a different distribution pattern of the lesions as induced by the HV- *F. columnare* isolates in carp compared to trout. The gills of the affected carp showed a diffuse distribution of the lesions, affecting the filaments of all gill arches bilaterally. At least half of the gill tissue was destroyed, with large parts of the filaments replaced with necrotic debris entangled with massive clusters of *F. columnare* bacterial cells, enwrapped in an eosinophilic matrix. In trout, the distribution pattern of the gill lesions was more focal, mostly located unilaterally and only present in the filaments of the two first gill arches. Scanning and transmission electron microscopic observations of the affected gills pictured long, slender bacterial cells attained in an extracellular matrix and in close contact with the destructed gill tissue. This is the first study to reveal gill lesions typical for columnaris disease at a macroscopic, light microscopic and ultrastructural level in both carp and rainbow trout following challenge with *F. columnare*, opening ample research opportunities regarding pathogen-gill interaction.

Subsequently, in **Chapter 5**, the colonization pattern in the course of time of *F. columnare* isolates of different virulence and their interaction with the gill tissue were investigated adopting the infection model as described in **Chapter 4**. For this purpose, carp and trout were exposed to a HV- or LV-isolate and sacrificed at predetermined times post-challenge. Histopathological and ultrastructural examination of carp and trout inoculated with the HV-isolate disclosed the bacterial cells invading the gill tissue spreading from the gill filament tips to the basis. In both carp and trout, significantly higher bacterial cell counts could be retrieved from the gill tissue after an inoculation with the HV- compared to the LV-isolate. Another important parameter investigated in the theme of virulence, was the way the pathogen can escape the host's inflammation response adopting apoptosis. In both carp and trout, TUNEL-staining showed a significant increase in the number of apoptotic cells in gill sections of fish exposed to the HV-isolate compared to control animals. In trout, the caspase-3 staining affirmed these results. In carp, caspase-3 staining could moreover display significantly more apoptotic cells in the gill tissue of fish challenged with the HV-isolate compared to the LV-isolate and between the LV-isolate inoculated fish and the control animals. Apoptosis was mostly seen in epithelial cells. Trends in gill mucus cell histochemistry included an increase of neutral mucin production in carp, with a significant increase in PAS-positive productive cell numbers following challenge with the HV-isolate compared to the control animals.

H&E- and PAS/AB- stained sections depicted basophilic bacteria embedded in an eosinophilic matrix, and bacteria immediately surrounded by a PAS-positive matrix enveloped by AB-positive mucins, respectively. These findings strongly point towards biofilm development. Most bacterial cells of the HV-isolate were seen surrounded with outer membrane vesicles. Another investigated cell type are the eosinophilic granular cells (EGC), or so-called stress cells. While not perceived in trout, EGC were demonstrated in all carp gills investigated using both H&E- and PAS-staining. Mobilization and degranulation of this cell type was however only noted in the gill tissue of carp challenged with the bacterial cells, making this the first study to report this feature.

In the general discussion part, the results of the different studies are summarized and discussed. Additionally, future research perspectives are provided to further elucidate possible virulence factors of *F. columnare*.

SAMENVATTING

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De afgelopen jaren breidde het kweken van voedselvissen en de productie van siervissen uit. Hiermee steeg het voorkomen van enkele belangrijke vispathogenen, waaronder *Flavobacterium columnare* (*F. columnare*), het oorzakelijk agens van columnarisziekte. Deze bacterie infecteert zowel gekweekte als wilde zoetwatervissen, waaronder verscheidene belangrijke commerciële vissoorten zoals forel en karper. Een infectie met *F. columnare* kan huidlesies, vinerosie en kieuwnecrose induceren met een hoge sterfte en zware economische verliezen tot gevolg. Het afgelopen decennium nam het aantal studies over de pathogenese van columnarisziekte toe. Dit leidde tot een beter inzicht in de complexe interacties tussen gastheer en bacterie. Niettemin is de pathogenese van columnarisziekte nog niet volledig achterhaald, wat de ontwikkeling van efficiënte preventie- en behandelingsmethoden bemoeilijkt. **Hoofdstuk 1** geeft een overzicht van de pathogenese van columnarisziekte en de domeinen die verder onderzoek verdienen. In **Hoofdstuk 2** worden de doelstellingen van deze thesis belicht, startend bij het bepalen van de antimicrobiële gevoeligheid van een groot aantal *F. columnare* isolaten aan de hand van een betrouwbare screeningsmethode (**Hoofdstuk 3**). Vervolgens typeert een infectiemodel de kieuwletsels voor columnarisziekte bij karper en forel en beschrijft het de *F. columnare* isolaten volgens hun virulentiepatroon (**Hoofdstuk 4**). Een laatste doelstelling betreft de studie van de kieuw-gastheer interacties bij karper en regenboogforel (**Hoofdstuk 5**), gebruik makend van het infectiemodel ontwikkeld in **Hoofdstuk 4**.

Momenteel zijn de preventieve maatregelen voor de bestrijding van columnarisziekte beperkt, waaronder het gebruik van een geattenueerd immersievaccin. Dit laatste is enkel voor gebruik bij kanaalmeerval en forelbaars in de Verenigde Staten van Amerika geregistreerd. Andere landen zijn voornamelijk op antimicrobiële middelen aangewezen. Nochtans bestaat de kans dat pathogene bacteriën resistent worden. Dit noopt tot opvolging van de antimicrobiële gevoeligheid om zo dierenartsen te kunnen adviseren inzake de therapeutische waarde van antimicrobiële middelen. Niettegenstaande het belang van columnarisziekte is de antimicrobiële gevoeligheid van *F. columnare* onvoldoende gekend. **Hoofdstuk 3** beschrijft daarom de *in vitro* gevoeligheid van 97 *F. columnare* isolaten tegenover 12 antimicrobiële agentia. Tussen 1987 en 2011 werden deze isolaten wereldwijd bij 17 vissoorten uit zowel de siervis- als voedingsindustrie verzameld. De studie is uniek in het aantal gebruikte stammen en de gemengde oorsprong van de *F. columnare* isolaten volgens vissoort, isolatiejaar en geografisch gebied. De microdilutietechniek werd gehanteerd om op een betrouwbare wijze de gevoeligheid van deze moeilijke groeier na te gaan. Geen van de isolaten vertoonde verworven resistentie tegenover florfenicol, gentamicine,

ormethoprim-sulfadimethoxine en trimetoprim-sulfametoxazole. Verworven resistentie van verscheidene *F. columnare* isolaten tegenover chloramfenicol, nitrofuran, oxytetracycline, flumequine, oxolinezuur en enrofloxacine werd hier voor de eerste keer gerapporteerd. Verder werd ook verworven resistentie tegenover erythromycine en ampicilline gedetecteerd.

De isolaten die verworven resistentie vertoonden waren afkomstig van Vietnamese katvis en siervissen, uitgezonderd twee isolaten afkomstig van wilde kanaal katvis bij dewelke verworven resistentie tegenover enkel oxytetracycline werd aangetroffen. Meer dan de helft van de isolaten van siervissen werd multiresistent bevonden. Dit kan wijzen op een ondoordacht gebruik van antimicrobiële middelen bij siervissen. Het benadrukt het belang om hun gebruik te beperken en bij voorkeur andere, preventieve behandelingsmethoden in te zetten in de strijd tegen columnarisziekte.

Door de groeiende bewustwording voor milieuverontreiniging, het belang van dierenwelzijn en een gezond leefmilieu, is de moderne aquacultuurindustrie op zoek naar preventieve maatregelen of behandelingen met een "groen label" om tot een betere productie en grotere winst te komen. Cruciaal in de ontwikkeling van dergelijke bestrijdingsmethoden tegen columnarisziekte is het begrijpen van de pathogenese, startend bij de initiële fase van het kolonizatieproces: de aanloop naar de inductie van lesies, waaronder de weinig bestudeerde kieuwlesies. Gezien de nood aan onderzoek in dit domein van de aquatische diergeneeskunde, meer bepaald met betrekking tot kieuwlesies veroorzaakt door columnarisziekte bij karper en forel, wordt in **Hoofdstuk 4** beschreven hoe een *in vivo* model werd ontwikkeld voor het bestuderen van de kieuwpathologie bij deze vissoorten.

Het kieuwweefsel van juveniele karper en regenboogforel werd via immersie blootgesteld aan verschillende *F. columnare* isolaten. De vissen zwommen gedurende 90 tot 120 minuten in een suspensie van één van de vijf (karper) of zes (forel) beschikbare *F. columnare* isolaten (bacteriële titers 1-7 x 10^8 kolonie vormende eenheden (KVE)/ml). Bij karper veroorzaakten vier isolaten (0401781, 0901393, 10009061-1 en 10012573/2) binnen de 12 u 100% sterfte, waardoor deze als hoogvirulent (HV) werden bestempeld. Het overige karperisolaat, CDI-A, gaf 5% mortaliteit binnen 18 u en werd als laagvirulent (LV) aangeduid. *F. columnare* bacteriën konden bij 100% van zieke vissen uit het kieuwweefsel worden gereïsoleerd. Bij forel werden de twee isolaten (B259 en JIP P11/91) die 100% sterfte gaven tot de HV groep gerekend. Eén isolaat (LVDJ (D7461)) veroorzaakte 50% vissterfte en kreeg een matig-virulent label. De overige drie isolaten (H2, Coho 92 en JIP 44/87) gaven minder dan 10% sterfte in de geïnoculeerde vissen en werden tot de LV-groep gerekend. Reïsolatie van *F. columnare* bacteriën uit het kieuwweefsel bij forel varieerde van 67% bij vissen die met de LV-stam werden geïnoculeerd tot 90% bij de vissen die aan de matig- of HV-stam werden blootgesteld.

Macro- en microscopisch onderzoek van het aangetaste kieuwweefsel na inoculatie met de HV-stam wees op een verschillende distributie van lesies bij karper en forel. Bij de karper waren deze kieuwletsels diffuus verspreid en kwamen ze voor op beide kieuwhelften. Meer dan de helft van het weefsel was aangetast, waarbij grote delen van de filamenten door necrotisch debris waren vervangen en omhuld waren met enorme clusters *F columnare* bacteriën in een eosinofiele matrix. Bij de forel waren de letsels meer focaal en meestal slechts voorkomend in één kieuwhelft waarbij enkel de eerste kieuwbogen waren aangetast. Scanning en transmissie elektronenmicroscopisch onderzoek van de aangetaste karper- en forelkieuwen vertoonden lange, dunne bacteriën die in een extracellulaire matrix waren gewikkeld en in nauw contact met het vernielde kieuwweefsel stonden. Voor het eerst konden de kenmerkende kieuwletsels van columnarisziekte na blootstelling aan *F. columnare* bij karper en forel worden aangetoond en dit zowel op macroscopisch, microscopisch als ultrastructureel niveau. Met dit model kan uitvoerig de pathogeen-kieuwinteractie worden onderzocht.

Vervolgens werden in **Hoofdstuk 5** het kolonisatieproces van *F. columnare* isolaten met verschillende virulentiegraden en de interactie met het kieuwweefsel bestudeerd door gebruik van het in **Hoofdstuk 4** ontwikkelde infectiemodel. Hiervoor werden karper en forel blootgesteld aan een HV- of LV- *F. columnare* isolaat en op vooraf bepaalde tijdstippen geëuthanaseerd. Histopathologisch en ultrastructureel onderzoek van karper en forel geïnoculeerd met het HV-isolaat toonden aan dat de bacteriën zich vanaf de toppen naar de basis van het kieuwfilament verspreidden. In tegenstelling tot na blootstelling aan de LV-isolaten, konden na inoculatie met de HV-isolaten zowel bij de karper als bij de forel hoge aantallen bacteriën in het kieuwweefsel worden aangetoond. Een belangrijke, onderzochte parameter is de mogelijke manier waarop de bacteriën in staat zijn om het immuunsysteem van de gastheer door toepassing van apoptose te omzeilen. In vergelijking met controledieren kon de TUNEL-kleuring zowel bij karper als forel een significante verhoging van het aantal apoptotische cellen aantonen in de kieuwstalen van vissen die met het HV-isolaat waren geïnoculeerd. Deze resultaten werden bij forel met de caspase-3 techniek bevestigd. Deze caspase-3 techniek kon bij karper significant meer apoptotische cellen aantonen na blootstelling aan het LV-isolaat in vergelijking met het HV-isolaat.

Apoptose werd voornamelijk opgemerkt in de epitheliale cellen. In vergelijking met de controledieren werd na inoculatie met de HV-stam bij de karper een verhoogde productie van neutrale kieuwmucines aangetoond met een significante stijging van het aantal PAS-positieve cellen. H&E- en PAS-kleuringen toonden de basofiele bacteriën in een PAS-positieve matrix, omringd door een laag van AB-positieve mucines. Dit zijn sterke indicaties voor biofilmvorming. Bij de meeste bacteriën van de HV-isolaten werd de productie van buitenste membraanvesikels waargenomen.

De eosinofiele granulaire cellen (EGC), of zogenaamde stresscellen, zijn een ander onderzocht celtype. Na gebruik van zowel H&E- als PAS-kleuring konden deze in alle onderzochte kieuwstalen van de karper worden aangetroffen maar niet bij de forel. Mobilisatie en degranulatie van deze cellen werd bij blootstelling aan de HV-isolaten van *F. columnare* enkel in de kieuwstalen van karpers gezien. Dit is de eerste keer dat dit werd beschreven.

In de algemene discussie worden de resultaten van de verschillende studies samengevat en nader besproken. Daarenboven wordt het toekomstig onderzoek belicht dat de pathogenese van columnarisziekte verder kan ontrafelen en virulentiefactoren kan onderkennen.

DANKWOORD

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CURRICULUM VITAE

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Annelies Maria Declercq werd geboren te Tielt op 7 mei 1985. Ze doorliep haar schoolperiode aan het Nieuwen Bosch instituut te Gent waar ze in 2003 afstudeerde in de richting Latijn-Wetenschappen. Ze startte haar studies Diergeneeskunde aan de Universiteit Gent waar het diploma van Master in de Diergeneeskunde in 2010 werd behaald.

Op 1 januari 2011 trad ze als doctoraatsbursaal in dienst aan de vakgroep Morfologie van de Gentse faculteit Diergeneeskunde. Naast het onderzoeken van de pathogenese van columnarisziekte bij vissen, wat geleid heeft tot voorliggende thesis, hielp ze ook mee aan het op punt stellen van bloedonderzoek bij vissen en bij onderzoek naar de invloed van voedingssupplementen bij karpers. Verder bestond haar taak in het verzorgen van de anatomiepractica van vissen aan de bachelorstudenten in de Diergeneeskunde. Ze assisteerde eveneens bij de practica en oefeningen van de cursus Fish Diseases aan de masterstudenten in de Aquacultuur (Faculteit Bio-ingenieurswetenschappen). Voor de basiscursus Laboratory Animal Science hielp ze mee practica voor te bereiden en uit te voeren. Ze was promotor van 3 masterproeven en fungeerde ook als leescommissaris.

Gedurende haar mandaat werkte Annelies nauw samen met de vakgroep Pathologie, Bacteriologie en Bijzondere Huisdieren te Merelbeke en met het Artemia Reference Centre te Gent. Verder kreeg ze de mogelijkheid om in 2013 een buitenlandse stage te volgen aan het laboratorium voor Medische Biochemie en Celbiologie aan de Universiteit van Göteborg, Zweden. Ze is op regelmatige basis reviewer voor internationale tijdschriften.

Ze behaalde het diploma van Master of Laboratory Animal Science in 2010. Het diploma van Master in de Aquacultuur werd verworven in 2014. Ze is auteur van meerdere publicaties in nationale en internationale tijdschriften en nam actief deel aan verschillende nationale en internationale congressen.

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AWARDS

1. Student award European Association of Fish Pathologists 2013, Tampere, Finland, September 2-9th 2013.
2. Student award Vlaams Diergeneeskundig Tijdschrift 2011, Merelbeke, Belgium.