Åbo Akademi University

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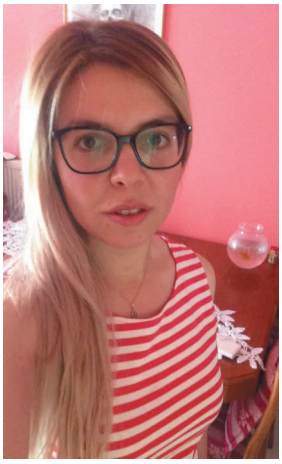
Flavobacterium psychrophilum adhesion and biofilm formation



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στους γονείς μου

to my parents

ABSTRACT

Flavobacterium psychrophilum causes serious infections in farmed rainbow trout (*Oncorhynchus mykiss*) and subsequent substantial economic losses to fish farmers, globally. Over the past years, few studies have conclusively suggested that adhesion to and biofilm formation on surfaces, are of importance for *F. psychrophilum*, probably conferring a selective advantage by increasing its ability to persist in fish-farming environments. It is known that phase variation occurs in *F. psychrophilum* with two different morphological colony phenotypes, smooth and rough, however the role of these phenotypes in adhesion and biofilm formation are not well known yet.

The aim of this thesis was to provide information of the adherence of different *F. psychrophilum* phenotypes to host tissues and screen the temporal persistence of bacterial cells on tissues. Another aim of this thesis was to assess the inhibiting effect of different compounds and bacteriophages on adhesion and the biofilm formation and mature biofilm of both phenotypes. The role of environmental conditions and factors on the biofilm formation of both phenotypes of *F. psychrophilum in vitro* was also evaluated.

Cells of both phenotypes initially adhered to mucosal surfaces of rainbow trout, but only the rough cells were present on tissues for a longer time. Both phenotypes showed a tissue tropism with the fin tissue being the most adhered. Skin mucus promoted the growth of both colony phenotypes but none of the isolates were able to form biofilms in mucus. The results of this thesis showed that a treatment with a combination of carbohydrates, D- and L-amino acids, A-type proanthocyanidins, EDTA and proteinase K reduced the adhesion of mainly smooth cells. Smooth cells produced stronger biofilms compared to rough cells in nutrient-rich media, and development of these biofilms was Ca^{2+} dependent. Most of the examined compounds inhibited the biofilm formation of smooth isolates reaching up to 80% inhibition, while only 2-aminoimidazole, emodin, parthenolide and D-leucine reduced the biomass of mature biofilms at a concentration-dependent manner. Lytic bacteriophages inhibited the biofilm formation of *F. psychrophium*, while they partially reduced the biomass of the mature biofilms.

In conclusion, mucosal surfaces of rainbow trout colonized by *F. psychrophilum*, and the growth of bacterial cells in skin mucus suggest that mucus-binding sites were available for the bacterial adhesins. The inhibition of mainly smooth cell adhesion to surfaces by most of the

compounds was probably due to disruption of the protein-protein interactions that hold the cells together and to cell-surface hydrophobicity. In contrast, the examined rough isolates exhibited resistance to most inhibiting compounds during their adhesion to and biofilm formation on inert surfaces. The ability of *F. psychrophilum* cells to use fish feed for biofilm formation suggests that there are probable enough nutrients in fish-farming environments for the *F. psychrophilum* to form biofilms. The results of this thesis also showed that the biofilm formation and mature biofilms can be modulated by using certain compounds and a combination of bacteriophages indicating the potential to reduce the *F. psychrophilum* biofilms in fish farms, if *F. psychrophilum* produce biofilms on fish-farming surfaces.

ABSTRAKT

Bakterien *Flavobacterium psychrophilum* förorsakar allvarliga infektioner hos odlad regnbågslax med påföljande ekonomiska förluster för fiskodlare runtom i världen. Under de senaste åren har en del undersökningar gett antydningar om att adhesion, eller vidhäftning, och biofilmbildning vid ytor är viktiga egenskaper hos *F. psychrophilum*, vilka kan ge bakterien en selektiv fördel i fiskodlingsmiljöer genom att öka dess förmåga att persistera. Det är känt sedan tidigare att *F. psychrophilum* kan genomgå fasvariation, som yppar sig i två morfologiskt särskiljbara kolonifenotyper, släta och marmorerade, men deras roll i adhesion och biofilmbildning har tillsvidare varit okända.

Målsättningen med denna avhandling var att bidra med kunskap om adhesion av de olika kolonifenotyperna av *F. psychrophilum* till värdvävnad och undersöka persistensen av bakteriecellerna på vävnaden i tid. Ett annat mål var att uppskatta den inhibitoriska effekten av exponering för olika kemiska föreningar samt bakteriofager på adhesion och biofilmbildning hos *F. psychrophilum*, samt undersöka effekterna av exponeringen på mogna biofilmer av både släta och marmorerade kolonifenotyper. Dessutom undersöktes betydelsen av olika miljöfaktorer på biofilmbildningen hos släta och marmorerade kolonifenotyper av F. psychrophilum in vitro.

Resultaten visade att celler av både släta och marmorerade kolonifenotyper kunde fästa sig vid mukosala ytor hos regnbågslax, men endast marmorerade celltyper var vidhäftade under en längre tid. Både släta och marmorerade kolonityper av *F. psychrophilum* uppvisade vävnadstropism och fäste sig bäst vid fenvävnad. Fiskmukus främjade tillväxten av F. psychrophilum-celler av båda kolonifenotyperna, men varken släta eller marmorerade typer kunde bilda biofilm i mukus. Resultaten i denna avhandling visar också att behandling med en kombination av kolhydrater, D- och L-aminosyror, A-typ proantocyanidiner, EDTA och proteinas K minskade adhesionen främst hos släta kolonityper. I näringsrikt odlingsmedium producerade släta celltyper kraftigare biofilmer jämfört med de marmorerade och utvecklingen av dessa biofilmer var Ca^{2+} beroende. De flesta av de undersökta kemiska föreningarna hade en inhibitorisk effekt på biofilmbildningen hos de släta kolonityperna som uppgick till 80%, men endast 2-aminoimidazol, emodin, partenolid och D-leucin hade en reducerande koncentrationsberoende effekt på biomassan hos mogna biofilmer. Lytiska

bakteriofager hämmade biofilmbildningen hos *F. psychrophilum* och minskade delvis biomassan av mogna biofilmer.

Sammanfattningsvis tyder koloniseringen av *F. psychrophilum* på mukosala ytor hos regnbågslax och tillväxten av bakterien i fiskmukus på att bindningsplatser på slemytorna kunde utnyttjas av bakteriella adhesiner. Den inhibitoriska effekten som de flesta av de undersökta kemiska föreningarna hade på adhesionen till ytor hos den släta celltypen, var troligen en följd av störningar i protein-protein interaktioner, som håller cellerna vidhäftade vid varandra, eller en reduktion av cellytans hydrofobicitet hos *F. psychrophilum*. I motsats uppvisade de undersökta marmorerade celltyperna resistens mot inhibitoriska effekter av kemiska föreningar under adhesion till och biofilmbildning på inerta ytor.

Förmågan hos *F. psychrophilum* att utnyttja fiskfoder som näring vid biofilmbildning antyder att näringstillgången i fiskodlingsmiljöer är tillräcklig för bakterien att bilda biofilmer. Biofilmbildningen och mogna biofilmer kan dock moduleras genom behandling med olika kemiska föreningar eller genom exponering för en kombination av olika bakteriofager; ett resultat som kunde utnyttjas till att reducera eventuella biofilmer av *F. psychrophilum* i fiskodlingar.

LIST OF ABBREVIATIONS AND SYMBOLS

A-PACs: A-type proanthocyanidins

BCWD: Bacterial Cold Water Disease

CFU: Colony Forming Unit

CRISPR: Clustered Regularly Interspaced Palindromic Repeats

EDTA: Ethylenediaminetetraacetic Acid

EPS: Extracellular Polysaccharides

ECPs: Extracellular Products

FF: Fish Feed

Ig: Immunoglobulins

LPS: Lipopolysaccharide

LW: Lake Water

MALDI-TOF: Matrix-Assisted Laser Desorption/Ionization-Time of Flight

MIC: Minimum Inhibitory Concentrations

NB: Nutrient Broth

OD: Optical Density

OM: Outer Membrane

PBS: Phosphate-Buffered Saline

PTL: Parthenolide

QS: Quorum Sensing

RT: Rainbow trout

Sub-MICs: Sub-Minimum Inhibitory Concentrations

TYES: Tryptone Yeast Extract Salts

2-AI: 2-aminoimidazole

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which are referred to by their Roman numbers (**Paper I-IV**) in the text. The original articles have been reprinted with the kind permission of the copyright holders; John Wiley & Sons Ltd (**Paper I**) and Oxford University Press (**Paper II**)

I. Papadopoulou A, Dalsgaard I, Lindén A and Wiklund T (2017) *In vivo* adherence of *Flavobacterium psychrophilum* to mucosal external surfaces of rainbow trout (*Oncorhynchus mykiss*) fry. Journal of Fish Diseases 40, 1309–1320.

II. Papadopoulou A, Howell A and Wiklund T (2015) Inhibition of *Flavobacterium psychrophilum* adhesion *in vitro.* FEMS Microbiology Letters, 362, 2015, fnv203.

III. Papadopoulou A and Wiklund T (2018) *In vitro* biofilm formation by *Flavobacterium psychrophilum*. Manuscript.

IV. Papadopoulou A, Dalsgaard I and Wiklund T (2018) Inhibition of *Flavobacterium psychrophilum* biofilm formation *in vitro.* Manuscript.

1 INTRODUCTION

1.1 Rainbow trout

Aquaculture is one of the fastest growing food-producing industries in the world, and one of the most important sources of high quality proteins for human consumption globally. The National Food and Agriculture Organization recognize the contribution of European and the United States salmonid aquaculture, with rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) representing the most prominent species (FAO 2012; Mente and Smaal 2016). Over the last four decades, the government of Finland has been trying to encourage the rainbow trout production due to geographic position of the country with priority to fish health and welfare (European Union 2017). Under farming conditions RT females spawn in winter, when eggs are manually stripped by man. After external fertilization, eggs of trout normally develop in oxygen rich waters (in running well water) and the development of fertilized eggs is dependent on water temperature. Fry with yolk sacs stay in the gravel for some weeks and feed off theirs yolk sacs until emerge as free-swimming fry (Hoitsy et al. 2012). Nowadays, fertilized eggs can be shipped all over the world from hatcheries to other rearing farms research institutes. Rainbow trout tolerates a range of water temperature with optimum growth temperature at 16 °C and a highest feed intake at 19 °C for their energy requirements (Wurtsbaugh and Davis 1977; Jobling 1981). Although, rainbow trout farming is source of high quality proteins for human consumption, only healthy fish can produce safe and quality food for human consumption (Villarroel et al. 2009).

In fish farming environments, the continual and intimate surface contact of potential pathogens to mucosal tissues of fish body is always a challenge for host innate immunity (Magnadóttir 2006). Rainbow trout have a well-functioning mucosal immune defence system in their external organs such as fins, gills, skin and eyes, which aim to block the penetration of pathogens into the fish (Esteban and Cerezuela 2015). The mucosal layer that covers these organs not only has the capacity to trap the invader (mechanical-clearance mechanism) but it also contains various antimicrobial factors such as agglutinins and precipitins (lectins), enzyme inhibitors (lysozyme), pentraxins, complement proteins, macrophages, antibacterial peptides and natural antibodies (humoral substances), for eliminating infectious agents (Ellis 2001; Uribe et al. 2011). Skin is one of the largest mucosal tissues that significantly contributes to immune defence against bacterial pathogens (Salinas et al. 2011), but it is often susceptible to the adherence of most pathogens (Brumell and Finlay 2000). In addition to the

skin epithelium, both gills and fins are considered to be important portals of entry for several bacterial pathogens including *F. psychrophilum* (Henriksen 2013; **Paper I**).

In teleost fish, several different immunoglobulins (Ig) are present in mucosal secretions including IgD, IgT and IgM (Salinas 2015). It has been suggested that IgD has a significant functional role at the gill surfaces (Ramirez-Gomez et al. 2012). IgM levels in gills and skin mucus increase in immunized and infected rainbow trout; while it has been found that higher levels of IgM than IgT in mucosal secretions are present, highlighting the importance of IgM in mucosal immunity (Zhang et al. 2010; Henriksen et al. 2015; Makesh et al. 2015). IgM is produced by B cells at mucosal tissue sites, and is the first antibody to appear in response to initial exposure to an antigen in fish (Parra et al. 2015). It is thought that a successful adhesion and invasion via the mucosal surfaces can be achieved if a pathogen is able to confront the humoral and cellular components of host's innate immunity. Bacterial pathogens bind to host cell surface glycans (mucus) through surface carbohydrate-binding proteins (lectins or other endogenous legends) present on the pathogen's surface, inducing immune response for the recognition of the pathogen (cell-to-cell interaction or homeostatic regulation). *In vivo* challenge experiments can provide a realistic approach to screen the host-pathogen interaction, and thus the adhesion of *F. psychrophilum* to mucosal surfaces was investigated in this thesis (**Paper I**).

1.2 Diseases

Disease is universally recognised as one of the most serious threats to the commercial success of aquaculture. As in other Nordic countries, rainbow trout farms in Finland have met with disease problems caused by bacteria e.g. furunculosis, enteric red-mouth disease, columnaris disease and bacterial cold water disease (BCWD). Stress appears to play a considerably large role in causing disease in fish depending on the severity and type of stress, its duration, and the physiological state of the fish (e.g. nutritional status). Poor water quality is one of the key factors that can negatively affect the fish production in farming systems (Noble and Summerfelt 1996). Bacterial fish diseases lead to economic problems for the fish farmers and their treatment contributes to antibiotic resistance, one of the biggest threats to global health and food security today. Fish vaccination plays an important role in large-scale commercial aquaculture of high-value species including the rainbow trout. At present, there are some commercial vaccines against many of the serious bacterial diseases in fresh water fish farming industries in Europe for example against vibriosis, furunculosis and yersiniosis (Sommerset et

al. 2005), but not against BCWD due to the small size (1 to 2 g) of the infected fish (fry) (Gómez et al. 2014).

1.3 *Flavobacterium psychrophilum*

F. psychrophilum is a member of the family Flavobacteriaceae, phylum Bacteroidetes. *F. psychrophilum*, a gram negative bacterium, which prefers reduced nutrient media such as Tryptone Yeast Extract Salts (TYES) (Holt et al. 1993). Daskalov et al. (1999) suggested a modified medium by adding sugars and skimmed milk for better isolation.

1.3.1 Bacterial Cold Water Disease

F. psychrophilum is the etiological agent of BCWD and the pathogen affects mainly rainbow trout, coho salmon (*Oncorhynchus kisutch*) and ayu (*Plecoglossus altivelis*) leading to serious economic losses for fish farmer's (Verner-Jeffreys and Taylor 2015). Additionally, *F. psychrophilum* has also been isolated from a number of different non-salmonid fish (Barnes and Brown 2011). *F. psychrophilum* occurs in a wide geographical area; Australia, Chile, Peru, Korea, North America, Turkey, Japan and many countries within Europe such as Denmark, Finland, France, Germany, Italy, Spain and UK mainly in fresh-water fish farms (Starliper 2011). In Finnish aquaculture, *F. psychrophilum* still remains a serious pathogen, causing substantial mortalities.

BCWD is a serious septicaemic condition of rainbow trout fry and also larger fish at water temperatures ranging from 4 to 15 °C. At temperatures below 10°C, BCWD outbreaks may be more severe and prevalent, causing mortalities with a rate up to 90% in fry if left untreated, while the mortality rate seems to be much lower in larger trout (Starliper 2011). Fish surviving the infection often show deformities. In the first stages, rainbow trout infected with *F. psychrophilum* show clinical signs as lethargy, cessation of feeding, eroded fin tips, anaemia, and hyperpigmentation of the skin. At later stages, clinical signs such as spiral or erratic swimming behaviour, blackened skin regions and spinal deformation, bilateral exophthalmia, pale gills, swollen abdomen with enlargement of spleen and haemorrhages on the gills, liver and kidney often occur (Starliper and Schill 2011). It has been hypothesized that under natural conditions, *F. psychrophilum* can gain entrance via the oral cavity, gills, nostrils, disruptions in the skin or fin mucus, and microscopic lesions (Madsen and Dalsgaard 1999; Kondo et al. 2002; Nematollahi et al. 2003b; Martinez et al. 2004; Lorenzen et al. 2010).

1.3.2 Characteristics of *F. psychrophilum*

F. psychrophilum is a strictly aerobic, flexible and slender rod with rounded ends forming yellow glossy colonies with diameter of 1–5 mm on agar plates. It has a diameter of 0.2–0.7 μm and a length of 2–7 μm depending on the culture media, although isolated bacteria seem to have smaller cell dimensions in the lesions of naturally infected fish (Holt et al. 1989). The gliding motility of *F. psychrophilum* is slow and weak, and sometimes difficult to observe (Lorenzen and Olesen 1997), but it is suggested that gliding motility of *F. psychrophilum* could be involved in its ability to move and cause erosions in the fins of infected Atlantic salmon (Martinez et al. 2004). However, when a bacterium closely attach to surfaces, the motility is slowed (Watnick and Kolter 2000). Álvarez et al. (2006) identified that the gliding motility and biofilm formation appear to be antagonistic properties, which are both affected by a thiol oxidoreductase-like protein gene.

F. psychrophilum is actively proteolytic with a capacity to degrade albumin, casein, fibrinogen, gelatin, fish muscle, litmus milk, tyrosine, and type IV collagen, but it cannot use simple and complex carbohydrates such as cellulose, chitin and xanthine (Bernarde and Kerouault 1989; Ostland et al. 2000; Nematollahi et al. 2003a). *F. psychrophilum* has also been shown to have weak catalase and oxidase activity and does not produce hydrogen sulfide and indole. There are studies showing elastin hydrolysis, nitrite reduction, tributyrin degradation and chondroitin sulfate lysis by *F. psychrophilium* isolates (Nematollahi et al. 2003a; Starliper and Schill 2011). In general, *F. psychrophilum* isolates are biochemically homogeneous, with the exception of some variation in the presence or absence of caseinases, gelatinases, and elastases (Bertolini et al. 1994, Lorenzen and Olesen 1997, Madetoja et al. 2001; 2002). Molecular diagnostic techniques are routinely applied for identification and/or detection of *F. psychrophilum* including biochemical characteristics, PCR assays and MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization-Time of Flight) (Del Cerro et al. 2002, Soule et al. 2005; Pérez-Sancho et al. 2017). Multilocus sequence typing of *F. psychrophilum*, a technique in molecular biology for the typing of multiple loci, has revealed many sequence types indicating the existence of an epidemic population of *F. psychrophilum* with specific sequence types in Nordic countries (Nilsen et al. 2014; Sundell 2015).

Bacterial lipopolysaccharides (LPS) are the major outer surface membrane components present in both smooth and rough *F. psychophilum* isolates (Högfors-Rönnholm and Wiklund 2010). LPS consists of three domains: (i) the lipid A, a hydrophobic domain that associates with the outer membrane; (ii) an oligosaccharide core, and (iii) a terminal, immunogenic

polysaccharide domain the O antigen, which gives rise to different diversity of O-antigens linked to sugar compositions (carbohydrates) (Raetz et al. 2009). The LPS of *F. psychrophilum* is composed of an O chain composed of a repeating trisaccharide containing the unusual sugar N-acylated bacillosamine (Crump et al. 2001). Omp-A family preotiens have been identified on *F. psychrophilum* surfaces as adhesions-facilitating binding of host cell receptors (Dumetz et al. 2007; Dumetz et al. 2008).

The complete genome of the *F. psychrophilum* has been sequenced by genomics studies in different countries (Duchaud et al. 2007; Wu et al. 2015, Rochat et al. 2017), although *F. psychrophilum* possesses an open pan genome (Castillo et al. 2016). Extracellular metalloproteases (Fpp1 and Fpp2) have been identified as virulence factors of *F. psychrophium* and these are involved in the destruction of host tissue (Secades et al. 2001; 2003). Another virulence factor of *F. psychrophilum* is the *tlpB* (thiol oxidoreductase-like protein gene) locus, which is involved in gliding motility, growth on iron-depleted media, and has an extracellular proteolytic activity (Álvarez et al. 2006).

1.3.3 Phase variation

Phase variation in bacterial pathogens, most visible as a colony variation, is often associated with bacterial virulence (van der Woude and Bäumler 2004). Two different colony phenotypes of *F. psychrophilum* have been isolated from diseased fish, described as smooth and rough (Pacha 1968; Högfors-Rönnholm and Wiklund 2010; Sundell et al. 2013). Högfors-Rönnholm and Wiklund (2010) showed an irreversible phase variation from smooth to rough phenotype in TYES broth. In general, it has been suggested that phase variation is a result of a change in cell structures, mostly associated with the surface of bacterial cells such as proteins (lectins), as a response to environmental changes (Berne et al. 2015). Phase variation is also a mechanism for evading the immunity of the host (Sharon 1987). Cell protein profiles of smooth and rough phenotypes of *F. psychrophilum* have been shown to be metabolically similar. However, smooth and rough isolates have shown differences in physiological characteristics such as in enzymatic activity (Högfors-Rönnholm and Wiklund 2010). It has been speculated that *F. psychophilum* phase variation plays a significant role in the pathogenesis by providing a biofilm mechanism for the bacterium to temporarily bypass the host's immunity or other undesirable conditions/factors (Högfors-Rönnholm 2014). However, the role of phase variation in adhesion and biofilm formation of *F. psychrophilum* is not well understood yet (**Paper II and Paper III**).

1.4 Adhesion and biofilm formation

Bacterial adherence or bacterial attachment is the typically step in the pathogenesis of biofilm-related infections, since surface is not always a pre-requisite for the formation of biofilm and sessile cells can appear as aggregate clusters in liquid contributing to virulence (Berlanga and Ricardo Guerrero 2016). Bacteria can adhere to surfaces via their proteins and/or polysaccharide appendages (LPS, capsular polysaccharides) depending on species (Katsikogianni and Missirlis 2004). Bacterial biofilm is defined as a transition mechanism in which single (planktonic) cells amass to a multicellular (sessile) community formed gradually over time and characterized by cells attached to biotic and abiotic surfaces and by the production and secretion of extracellular products (ECPs) in a diverse range of natural, industrial and clinical settings (Giaouris et al. 2014). The sessile cells are commonly referred as biofilm phenotypes that allow success of the pathogen and lead to infection that is responsible for many human and foodborne chronic diseases. Biofilm formation also enables pathogens to survive in many adverse and diverse environments using their own defence and communication systems, making their eradication difficult (Bjarnsholt 2013).

Mono- and multi-species biofilms harbour pathogens as reservoir of persisting cells (unlike planktonic cell populations) with tolerance against UV radiation, oxidizing molecules, disinfectants, antibiotics, and phagocytosis (host defences). The slow penetration of agents inside biofilms and micro environmental changes in biofilms are some examples of the resistant mechanisms (Kart et al. 2014). What drives the single planktonic cells to form biofilms is often the response to nutritional and environmental changes like a shift in pH and nutritional state (**Paper III**). This change facilitates cell aggregation and many cellular regulatory networks resulting in different expression of cell surface molecules (phase variation), genes, virulence factors, and a series of other events that reflect adaptation to these conditions (Kostakioti et al. 2013). Horizontal genetic transfer within biofilms generates genetic diversity and subpopulations of cells as small-colony variants with apparently defined tasks and hence sharing labour in the biofilm formation (‘division of labour’) (Bordi and de Bentzmann 2011).

In general, the development of bacterial biofilms can be divided into different stages, including the (a) reversible attachment of bacterial cells to surfaces (conditioning layer); driven by physical forces (van der Waals forces, steric interactions and electrostatic interactions) and specific molecular interactions (pili, flagella, ligand-bindings) (Gupta et al. 2016). The next step is the (b) irreversible attachment of bacterial cells determining cell-cell

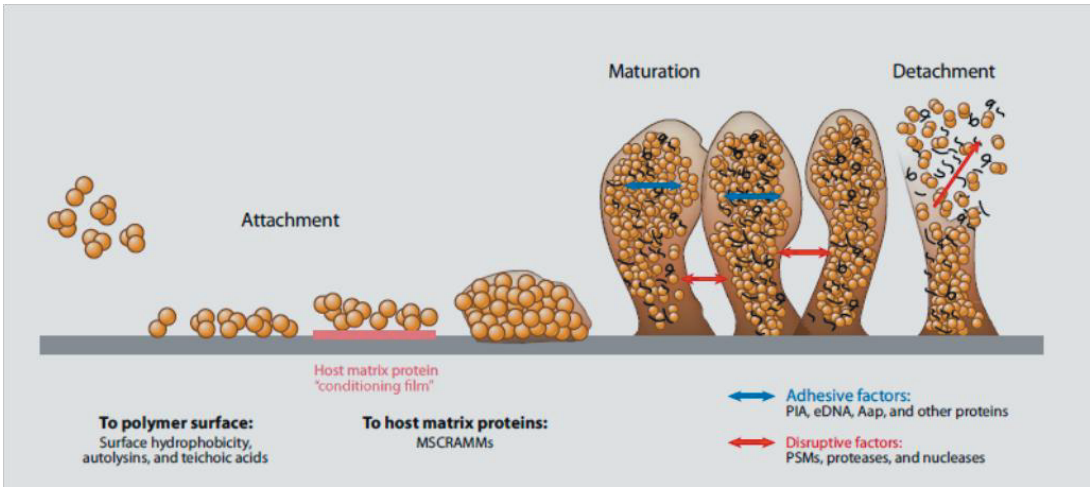
adhesion known as co-adhesion mediated by shear forces, motility and binding adhesins (e.g. flagella proteins, amyloid proteins). Specifically, hydrophobic forces have been shown to play an important role in bacterial adherence (Dufrêne 2015). Different factors such as temperature, ions and iron availability can also greatly modulate the bacterial irreversible adherence. The production of these adhesins influences the cell aggregation mechanisms and contributes to cohesive properties of (c) bacterial biofilm formation (Garrett et al. 2008; Borlee et al. 2010). At this stage, sessile cells are surrounded by a self-produced extracellular polymeric matrix consisting mainly of polysaccharides, eDNA, proteins, lipids, adhesins (carbohydrate-binding proteins) and other materials like dead cells. The matrix composition might be different between and within species. The matrix acts as scavenger for molecules (e.g. nutrients, ions) and assembles cells, supports the formation of signalling networks, binds to antibiotics and shields against predators and phagocytes (Kostakioti et al. 2013).

Then, (d) bacterial biofilms mature. An increasing amount of matrix components and bacteria stimulate the cell-to-cell communication through sensing systems including the cyclic dimeric guanosine monophosphates and quorum-sensing (QS), which results in production of auto-inducers, the N-acyl-L-homoserine lactones for gram-negative bacteria and oligopeptides for gram-positive bacteria (Donlan 2002). QS has a conditional role in biofilm formation via up or down gene regulation that lead to coordinated patterns of gene expression, production of matrix and virulence factors. QS can trigger subpopulations of cells that reversibly enter a slow-growing or dormant cell state under stressful conditions. These events describe the heterogeneous nature of biofilms including mutational events (e.g. physiological characteristics, phenotypic responses, and gene and protein expressions) (Bordi and de Bentzmann 2011). It is also known that cellular signalling mechanisms (e.g. QS) and bacteriophages control cell dispersal and cell death by up-regulating the expression of specific genes e.g. flagella motility proteins or through lateral gene transfer that apparently support the different biology of planktonic living bacteria (Garrett et al. 2008).

The transition from the relatively antibiotic-susceptible planktonic mode of growth and the more resistant biofilm state is reversible. Once the biofilm matures, (e) dispersal becomes an option for some single sessile cells or small sessile clusters to resume a planktonic (free-living) cell form due to alterations in nutrient availability, oxygen fluctuations, increase of toxic products or other stress-inducing conditions (increased amount of carbon and nitrogen). These conditions can cause cell dispersal and cell death which are regulated by sensory systems (e.g. cell-to-cell signalling, nitric-oxide-sensing proteins), ESP-degrading enzymes

(e.g. polysaccharide lysases, D-amino acids), and/or surfactants (e.g. bacteriophage induction) (Donlan 2002; Hochbaum et al. 2011). This dispersal is also known as seeding dispersal, where 'sacrificed' sub-cell populations escape from biofilms or die inside biofilms to offer a nutrient source and place for the surviving cells (Kaplan 2010). These dispersed sub-populations show variation in growth, motility and biofilm formation. This cell differentiation ensures the successful survival and colonization of planktonic bacteria to new surfaces (Gupta et al. 2016). Biofilm dispersal is a programmed process involving multiple genetic determinants and regulatory processes, a beneficial step for bacteria that can spread in environments and re-initiate the process of biofilm formation.

Figure 1. Phases of bacterial biofilm formation on experimental surfaces (Otto 2013)



In aquaculture, especially in recirculating aquaculture systems, beneficial bacterial biofilms maintain water quality e.g. in biofilters (Bentzon-Tilia et al. 2016). However, where nutrients are available, bacterial pathogens adhere to and form biofilms on fish-farming equipments (Cai and Arias 2017), leading to a number of problems. At the moment, intensive labour work is needed to handle these harmful biofilms including cleaning in adequate prolonged exposure time, higher temperature, higher flux, and possibly with the combination of detergents and disinfectants such as Virkon Aquatic, daily check of fish tanks, filtration units, pipelines, and pumps (Yanong and Erlacher-Reid 2012). Effective strategies against these bacterial biofilms need to be reconsidered (**Paper IV**).

So far, there is limited knowledge on the biofilm formation process of *F. psychrophilum*. It has been suggested that the capacity of *F. psychrophilum* to form biofilms serve as a

reservoirs for putative virulence factors (Levipan and Avendaño-Herrera 2017) and that *F. psychrophilum* biofilm formation is a strategy to ensure structural maintenance and metabolic functions (Levipan et al. 2018). It has also been shown that motile behavior cannot be observed in biofilm cells of *F. psychrophilum* (Castillo et al. 2016), and that these bioiflm cells may rapidly develop antibiotic resistance, if they expose to sub-inhibitory concentrations of those compounds (Sundell and Wiklund 2011).

1.5 Strategies for controlling bacterial adhesion and biofilm formation

Bacteria within a biofilm show high levels of resistance to biocides and antibiotics. It can require 1000 times greater concentration of antibiotics to kill the bacteria within biofilms compared to free living planktonic cells (Harper et al. 2014). Alternative strategies have been suggested to inhibit the bacterial adhesion and eliminate the biofilms such as mechanical, physical and chemical methods (**Paper II and Paper IV**).

Figuratively speaking, the biofilm formation can be described as a bacterial growth curve following four phases: The lag phase (adhesion), the exponential or logarithmic (log) phase (early biofilm formation), the stationary phase (mature biofilm), and the decline or death phase (detachment). Since the growth rate of each organism has unique characteristics, the same principles exist for the biofilm formation of each bacterium. Compounds that can combat the bacterial adhesion to, biofilm formation and mature biofilms on surfaces need to be identified. Sub-minimum inhibitory concentrations (sub-MICs) of these compounds are not enough to kill or inhibit the bacterial cells, but to down regulate some of the chemo-physiological activities (Macé et al. 2017). It has been suggested that bacteria cells do not develop resistance against compounds used in sub-MIC concentrations, in contrast to conventional antibiotics, which do (Cortés et al. 2011). The text below indicates four different strategies (i) the use of natural and (ii) synthetic compounds, (iii) antimicrobial surfaces and (iv) bacteriophages against the bacterial adhesion and biofilm formation to surfaces.

1.5.1 Natural compounds

Phytochemicals protect plants from pathogens (Batta 2016). A number of researchers have tested a different plant extracts e.g. extracts from natural products as terrestrial plants (apples, garlic, ginger, and cranberries) and marine plants (algae) at micromolar to millimolar non-bactericide dose/concentrations to control different stages of bacterial biofilms (Lou and Writer 2010; Worthington et al. 2012; **Paper IV**). For example, extracts from cinnamon and

cranberries have decreased the adherence and biofilm formation of bacteria to inert surfaces (Chenia and Chadwick 2013; Ulrey et al. 2014). Extracts from a brown algae, *Fucus vesiculosus,* found in the Baltic Sea, blocked the adherence of bacterial pathogens to surfaces by affecting the quorum sensing system (Goecke et al. 2010; Saha et al. 2011).

1.5.2 Synthetic compounds

A plethora of synthetic compounds such as peptides, enzymes (e.g. proteinase K, DNAse, alginate lyase), amino acids and chelex have shown the potential for bacterial biofilm inhibition by interfering with cell-to-cell interactions and/or up-regulating gene dispersal signals (Gupta et al. 2016). For example, the chelating agent ethylenediaminetetraacetic acid (EDTA) can prevent bacterial adherence to surfaces by binding to ions (e.g. Ca^{2+} and Mg^{2+} ions) in the environments and thus blocking the ionic interactions (Wang et al. 2017).

1.5.3 Antimicrobial surfaces

An antimicrobial surface contains an antimicrobial agent that inhibits the ability of bacteria to grow on the surface of a material, for example modified anti-adhesive coated devices with metals inhibiting the biofilm formation of bacterial pathogens (Aparna and Yadav 2008; Bernstein et al. 2011). For example, toxic heavy metals have been shown to reduce biofilms by triggering phenotypic cell diversification (Harrison et al. 2007). Bamboo surfaces have been suggested as antibacterial and anti-adhesive surfaces to inhibit *F. columnare* (Cai and Arias 2017), probably due to tannins. Another study has shown promising results using continuous direct electrical current at low amperages, intermittent direct current, and combinations of surface materials (teflon or titanium) and electrode compositions (stainless steel, graphite, titanium, or platinum) against biofilms of bacterial pathogens (Schmidt-Malan et al. 2015).

1.5.4 Bacteriophages

Bacteriophages (phages), the viral predators of bacteria, are a natural, specific, non-toxic, feasible approach for controlling bacterial biofilms (Gutiérrez et al. 2010). Phages exist in two forms: lytic and lysogenic. In the lytic life cycle, phages bind to receptor(s) on the bacterial surface and invade the cell. Then, the virus is replicated inside the bacterial cell, and when the viral progeny is released, the host cell usually erupts and dies. During the lysogenic cycle, the genome of phage(s) is injected into the host cell but it will integrate into the chromosome of the bacterium (transduction) for a long time (Clokie et al. 2011; De Paepe et al. 2014). Phage

therapy is nowadays an attractive method that can be used against bacterial biofilms associated with chronic diseases (Simoes et al. 2010; Harper et al. 2014). Phages are generally considered safe, because they do not replicate in eukaryotic cells, and they are only infecting specific bacteria (and often a certain strain) (Drulis-Kawa et al. 2012).

Recent studies show that virulent (lytic) phages can structure or restructure the bacterial biofilm by killing the bacterial cells through cell lysis and digesting the biofilm matrix via EPS-degrading enzymes (e.g. polysaccharide lyase enzyme) (Kostakioti et al. 2013; Abedon 2015). It has been shown that phages can inhibit (at least) the early development of biofilms and reduce the biomass of mature biofilms (Sillankorva et al. 2004; **Paper IV**). Biofilms can possess anti-phage mechanisms, which can be intrinsic to the host cell or acquired after the phage exposure to the host cell e.g. due to host mutations (Moons et al. 2013), impermeability of biofilm matrix (Harper et al. 2014) and development of adaptive immunity interfering CRISPR sequences (Örmälä and Jalasvuori 2013). A phage combination, to broaden the spectra of activity, has been proposed as a successful anti-biofilm strategy targeting different receptors on host cells (Tait et al. 2002). The persistence of *F. psychrophilum* in aquaculture has been attributed to their ability to form biofilms with increased tolerance of disinfectants and antibiotics (Sundell and Wiklund 2011; Tan et al. 2015a), and phages could potentially be used to control bacterial biofilms (**Paper IV**). Few studies have suggested the successful application of phages to reduce different bacterial infections (Nakai et al. 1999; Imbeault et al. 2006), but the effect of phage on biofilm-forming aquatic pathogens need to further studied (**Paper IV**).

2 AIMS OF THE THESIS

The overall aim of this thesis is firstly to understand which factors contribute to *F. psychrophilum* adherence and biofilm formation, and secondly evaluate the potential anti-adhesion and anti-biofilm activity of some selected compounds on *F. psychrophilum*. Although the *in vitro* adherence of smooth and rough colony phenotypes is known, this thesis aims to examine the *in vivo* adherence of these distinct phenotypes and consequently evaluate differences in adherence between the two phenotypes on host tissues (**Paper I**). Cells of *F. psychrophilum* can form biofilms on abiotic surfaces; however, further characterization of the biofilm formation of the two colony phenotypes has not been done before. The present thesis focuses on which environmental conditions and factors are favourable for *F. psychrophilum* to produce biofilms (**Paper III**). The ultimate aim of this thesis has been the understanding of possible mechanisms involved in adhesion and biofilm formation by *F. psychrophilum*. Experiments for the inhibition of *F. psychrophilum* adhesion to (**Paper II**), inhibition of the biofilm formation and reduction of the biomass of the mature biofilms on synthetic surfaces (**Paper IV**) were carried out. Biofilm inhibition strategies were experimentally tested by using compounds isolated from different plants, synthetic compounds and phages. This thesis proposes efficient inhibiting compounds that could target the *F. psychrophilum* adhesion to and biofilm formation on plastic surfaces.

The main objectives of this PhD thesis were to investigate:

1. The *in vivo* adherence of *F. psychrophilum* on mucosal tissues of rainbow trout fry (**Paper I**),
2. The *in vitro* biofilm formation of *F. psychrophilum* under certain environmental conditions, and to verify or reject the hypothesis concerning cell phenotype switch from rough-to-smooth within the biofilms under nutrient deprivation (**Paper III**),
3. The *in vitro* inhibiting effect of selected compounds and phages on the adhesion (**Paper II)** and biofilm formation of *F. psychrophilum* (**Paper IV**).

Specific research hypotheses and questions are presented in the original **Papers I-IV**.

3 SUMMARY OF MATERIAL AND METHODS

The details of the materials and methods used in the experimental work are described in the original publications (**Papers I-IV**). A summary of the materials and methods used is provided below.

3.1. Bacterial isolates (I-IV)

Four *F. psychrophilum* isolates, two smooth (P7-9/10 and P1-10B/10) and two rough (P7-9/2R/10 and P1-10B/2R/10) isolates, were used in all the experiments. The bacteria were routinely cultivated on TYES agar plates (TYES broth+1.1% agar; Holt et al. 1993), and colony phenotypes were confirmed using a stereomicroscope prior to the assays. Isolates were stored at −80°C in TYES broth containing 20% (v/v). Bacterial suspensions were prepared by dissolving bacterial colonies in lake water (LW), in phosphate-buffered saline (PBS) or TYES broth. Bacterial suspensions were routinely measured by spectrophotometric turbidity at 520 nm and adjusted to 0.45 ± 0.005 corresponding to approximately 1.0 x 10^8 CFU/ml and further used in the assays.

3.2 *In vivo* adhesion assay (I)

Sixty rainbow trout fry with a mean weight of 3 ± 0.5 g were divided into five groups (n=12) and kept in separate 8-L polypropylene aquaria with flow-through water. The adhesion of *F. psychrophilum* to mucosal surfaces of rainbow trout fry was evaluated as previously described by Olivares-Fuster et al. (2011). Rainbow trout fry were immersed into bacterial suspensions of *F. psychrophilum* for 40 min at 12 °C, and then moved to fresh water for 20 min for removing non-adhered cells. Control groups of rainbow trout fry were immersed in PBS. All rainbow trout fry were subsequently returned to their aquaria, and three rainbow trout from each group were killed at time points 0, 1, 24 and 48 h post-immersion (40 + 20 min). Samples from fins, gill arches, skin and both eyes were cut off, and homogenized in PBS followed by serially 10-fold dilutions and sub-samples were plated on TYES agar. Concentration of the adhered cells on each tissue was calculated as CFU/g tissue. Swab samples from spleen and kidney were directly streaked on TYES agar plates. Colony phenotypes were confirmed as smooth or rough using a stereomicroscope after 5 days incubation of the agar plates at 15 °C. Yellow-pigmented colonies were counted and the cells identified as *F. psychrophilum* by specific PCR assay (Wiklund et al. 2000) and by showing no growth on blood agar plates.

3.3 *In vitro* bacterial growth in skin mucus (I)

Skin mucus was obtained from healthy rainbow trout (Balebona et al. 1998). Briefly, skin mucus was collected, by gently scraping the surfaces of the whole fish body. After centrifugation to remove debris (i.e. scales and epithelial cells), the supernatant was filtered. A part of the filtered skin mucus was also autoclaved. The protein content of the mucus solutions was determined. The ability of the *F. psychrophilum* isolates to grow in filtered or filtered and autoclaved skin mucus solution as a sole nutrient source was determined. Samples of the bacteria/mucus suspensions were serially 10-fold diluted in sterile PBS and each dilution plated on TYES agar plates at 0, 1, 2, 24 and 48 h post-inoculation by a 6 × 6 drop plate method. The TYES agar plates were incubated at 15 °C and bacterial colonies were counted under a stereomicroscope and the average number of bacteria (CFU/ml) was calculated.

3.4 *In vitro* assay for the inhibition of *F. psychrophilum* adhesion (II)

To determine if the adhesion of smooth and rough *F. psychrophilum* cells to plastic surfaces can be inhibited, a number of different compounds were tested (Table 1; **Paper II**). Compounds were dissolved in lake water and then filter sterilized (0.22 μm). Bacterial cells suspended in sterile lake water were incubated for 1 h at 15 °C with each compound respectively, followed by 1 h adhesion to polystyrene surfaces. Adhered bacteria were stained with crystal violet (Álvarez et al. 2006) and measured spectrophotometrically. The inhibition of adhesion was calculated from the treated adhering bacteria exposed for the compounds in comparison to untreated adhering bacteria using the formula: IA% = [(AB–NC)/(PC–LW)]*100.

IA% is the percentage of adhering bacteria, treated with inhibitor compound, compared to untreated adhering bacteria. AB is the OD_{595} of the stained bacterial cells in the presence of inhibitor compound; NC is the OD_{595} of the negative control containing lake water and inhibitor compound in the absence of bacteria; PC is the OD_{595} of stained adhering bacterial cells in the absence of inhibitor compound; LW is the OD_{595} of lake water without inhibitor and bacteria.

3.5 *F. psychrophilum* biofilm formation (III)

3.5.1 Experimental setup

The ability of *F. psychrophilum* to form biofilms under static conditions was evaluated as previously described by Álvarez et al. (2006). Briefly, 20 µl from 1:100 dilution of the bacterial suspension was inoculated into 180 µl of TYES broth in the wells of 96-well microtiter plates and incubated statically for 1 h at 15 °C. After incubation, non-adhered cells were carefully removed from the wells and fresh growth media indicated in each experiment was added to each well to form biofilms. After incubation for 5 days at 15 °C, the wells were carefully decanted and biofilms were stained with crystal violet solution. Then the wells were filled with ethanol to de-stain the biofilms, and the mean of the OD_{595} values from the four replicates was calculated. To classify the biofilm forming capacity of tested *F. psychrophilum* isolates, we used a mathematic method that describes the propensity of the isolates to form biofilms (BF), and this was calculated using the formula BF = AB⁄CW, where AB = OD_{595} of stained biofilm bacteria and CW = OD_{595} of stained control wells containing bacteria-free medium only.

3.5.2 Growth media and growth condition for the biofilm assays (III)

The ability of *F. psychrophilum* to form biofilms in different growth media or under different growth conditions was tested in different temperatures (10 °C, 15 °C and 20 °C), pHs (6.0 (acid), 7.0 (neutral-control) and 8.0 (alkaline), Ca^{2+} and Mg^{2+} concentrations (0.50 g/L, 0.25 g/L, 0.10 g/L, 0.05 g/L and 0 g/L), nutrient content (lake water, fish feed, TYES broth and nutrient broth), fish mucus, iron, and carbohydrates.

3.5.3 Growth of *F. psychrophilum* in different media (III)

The growth of *F. psychrophilum* in different media and under different conditions was examined in a 96-well microtiter plate with constant agitation at 180 rpm for 3 days at 15 °C and the growth was measured spectrophotometrically at OD_{520}. Some media and conditions were directly included in the biofilm assays if previous studies reported a growth for *F. psychrophilum*, such as the carbohydrates, temperatures, and pHs.

3.5.4 Switch of phenotype (III)

The ability of rough cells to switch in smooth cells within biofilms under nutrient-limited growth conditions was tested in microtitle plate. Cells of the rough P7-9/2R/10 and P1-10B/2R/10 isolates were allowed to form biofilms in TYES broth for up to 23 days at 15 °C.

3.6 Inhibition of *F. psychrophilum* biofilms (IV)

3.6.1 MIC test of the compounds

Minimum inhibitory concentrations (MIC) of compounds (Materials and methods; **Paper IV**) were established using standard technique. Stock solutions were prepared by dissolving the compounds in phosphate buffered saline (PBS) or 96% ethanol (the final ethanol concentration did not exceed 2% v/v). To test the MIC of each compound on bacterial isolates, compounds were serially two-fold diluted in 96-wells microtiter plates with added bacteria as previously described by Rangdale et al. (1997). Plates were incubated at 15 °C at 180 rpm for 48 h. The plates were read by the naked eye over dark background and visible growth in the form of opaque spots on the bottom or turbidity of each well was considered a positive reaction. The lowest concentration of the compounds inhibiting the bacterial growth defined as the MIC value.

3.6.2 *In vitro* assay for biofilm inhibition (IV)

The effect of each compound and phage on two different stages of biofilm development of the smooth and rough *F. psychrophilum* isolates was quantified via two different experiments on biofilm formation and mature biofilms of the *F. psychrophilum* isolates. Biofilm formation was prepared in TYES broth as described above in section 3.1.5. In biofilm formation experiment diluted bacterial suspensions in TYES broth were added in quadruplicate wells, and allowed to adhere for 1h. Following the adherence, the supernatants with non-adhered cells was carefully removed and replaced with fresh TYES broth containing each of the examined compounds (see 3.6.1) separately at three different sub-MIC concentrations (MIC/2, MIC/8 and MIC/32). After that, plates were statically incubated for 5 days. In mature biofilm experiments, bacterial cells were allowed to adhere, and then the supernatant containing unattached cells was discarded and replaced with fresh TYES broth. After incubation at 15 °C for 5 days, the supernatant was removed and fresh TYES broth containing each of the compounds at three sub-MICs was added to wells followed by incubation at 15 °C for 24 h. The percentage of biofilm or adherence inhibition by each of the compounds was calculated according to the following equation:

Biofilm inhibition index (%) = $(A_1–A_2)/A_1\times100$ (Das et al. 2017)

where A_1 is the mean OD_{595} value of the positive control wells and A_2 is the mean OD_{595} value of the biofilms exposed for each of the compound respectively.

3.6.3 Bacteriophages

In this thesis, a number of different phages previously isolated and characterized by Stenholm et al. (2008) was tested. Finally, four phages, two lytic phages (Fpv-9, Fpv-10), two non-lytic phages (Fpv-3, Fpv-21) and one combination of phages (Fpv-9 + Fpv-10), isolated from freshwater of fish farming environments in Denmark were used to examine their effect on the biofilm formation of *F. psychrophilum*. The choice of these phages was based on their potential to be used against the fish pathogen *F. psychrophilum* (Stenholm et al. 2008).

3.7 Data processing and statistical analysis (I-IV)

The data from the experiments on cell adherence and persistence on mucosal surfaces were analyzed using the programming environment R with multiple parameters, and specifically a generalized linear mixed model (GLMM) (Bates et al. 2013; R Core Team 2014). All data from the biofilm assays are expressed as mean values ± standard deviations. The effect of the different growth media and growth conditions on the *F. psychrophilum* biofilm formation was assessed by comparing each growth condition or media indicated in experiments above with each control group using a paired-samples *t*-test (Krzywinski and Altman, 2014) (**Paper III**). The inhibition of adhesion assay, paired comparisons were tested (IBM SPSS Statistics 21). Concentration-response between the sub-MICs values of the examined compounds and the biofilm formation and mature biofilms of *F. psychrophilum* were analysed by linear regression analysis in GraphPad Prism software (Schneider et al. 2010) (**Paper II and Paper IV**). The probability values of equal or less than 0.05 (two-tailed) were considered statistically significant in most cases for the statistical methods used in this thesis (Table 1). Details about the analysis are given in the respective papers.

Table 1. A summary of the statistical methods used in the present thesis.

Statistical analysis	**Paper I**	**Paper II**	**Paper III**	**Paper IV**
GLMM	x			
Paired-samples *t*-test		x	x	x
Linear regression				x

4 MAIN FINDINGS OF THE THESIS

The large data set of statistics (*P* values) is reported in details in original **Papers I-IV**.

4.1 *F. psychrophilum* adherence to rainbow trout tissues (I)

The adherence between the two phenotypes and between isolates of the same phenotype was significantly different. The adherence of rough isolates slightly decreased with time, but the temporal change was steeper for the smooth cells compared to the rough ones (Figure 1; **Paper I**).

The adherence of smooth (P7-9/10) and rough (P7-9/2R/10) cells was onto different tissues for the data from 0 h time point, since cells of these isolates seem to adhere on fish tissues more efficiently than the other (P1-10B/10 and P1-10B/2R/10) isolates. The cells of both isolates exhibited tissue tropism, and fin tissue showing the highest number of adhered cells. The number of smooth cells adhering to gill, skin and eye tissues was significantly higher than the corresponding rough cells, but there was no significant difference in the adhesion to fin tissue for the two phenotypes (Figure 2; **Paper I**).

We analysed the effects of variables "Tissue" and "Time" for the rough phenotype isolate P7-9/2R/10. The number of rough cells adhered on each tissue did not change between tissues during the experiment. The other rough phenotype isolate P1-10B/2R/10 was excluded from this analysis, since the adhered cells had CFU = 0 after 48 h (Figure 3; **Paper I**).

4.2 Growth of *F. psychrophilum* in rainbow trout skin mucus (I)

The average concentration of smooth and rough cells of the four *F. psychrophilum* isolates in filtered or filtered and autoclaved skin mucus increased with time, after an initial lag period, and peaked similar log levels at 48 h post inoculation. No clear differences in growth rate were observed between smooth and rough cells neither in skin mucus, nor in TYES broth (Figure 4; **Paper I**).

4.3 Inhibition of *F. psychrophilum* adhesion *in vitro* (II)

The results of adhesion experiments with the *F. psychrophilum* isolates show that sucrose octaacetate was the only carbohydrate that alone inhibited the adhesion of smooth cells of one examined isolates. Although, single carbohydrates were inefficient inhibitors, the three different combinations of carbohydrates, significantly decreased the adhesion of the smooth

isolates (Figure 1; **Paper II**). L-cystein strongly inhibited the adhesion of smooth cells, while D-leucine, D-methionine and D-tryptophan moderately to strongly inhibited the smooth cells. Inhibitor effects were not observed with D-tyrosine. Unexpectedly, as some compounds inhibited the adhesion of smooth cells, at the same time, they increased the adhesion of rough cells such as L-cysteine and D-tryptophan, but the differences were not statistically significant (Figure 2; **Paper II**). Fucoidan and A-PACs were strong inhibitors of the adhesion of smooth cells, while cinnamaldehyde had a significant, moderate to strong effect on the adhesion of cells. In contrast, *trans*-cinnamaldehyde weakly inhibited the adhesion of smooth cells of only one smooth isolate and one rough isolate (Figure 3; **Paper II**). Both EDTA and proteinase K strongly reduced the adhesion of smooth cells and weakly reduced the adhesion of rough cells of both examined isolates (Figure 4; **Paper II**).

4.4 *In vitro* biofilm formation of *F. psychrophilum* (III)

The results from the biofilm assays showed that both smooth isolates, P7-9/10 and P1-10B/10, displayed high ability to form biofilms in TYES broth at 15 °C, and the isolates were classified as strong biofilm producers. One of the rough isolates (P7-9/2R/10) was classified as a weak biofilm producer and the other one (P1-10B/2R/10) as a negative biofilm. The results of the growth assay using different media showed that the examined *F. psychrophilum* isolates replicated in NB with or without iron, FF, RT mucus medium, and TYES broth but not in LW (Table 1; **Paper III**).

4.4.1 The effect of growth media and conditions on *F. psychrophilum* biofilm formation (III)

Temperature

There was a significant decrease in the ability of the smooth isolates to form biofilms at 10 °C and 20 °C compared to 15 °C. In contrast, rough isolates showed a significantly increased biofilm formation both at 10 °C and 20 °C compared to 15 °C (Table 2; thesis).

pH

The biofilm produced by the smooth isolate P7-9/10 at pH 6 was comparably increased compared to that produced at pH 7, while biofilm formation of the other smooth isolate P1-10B/10 was not affected. On the other hand, biofilm formation of the rough isolates at pH 6

was significantly decreased compared to that at pH 7. Biofilm formation of the smooth and rough isolates was significantly reduced at pH 8 in comparison to pH 7 (Table 2; thesis).

Ca^{2+} and/or Mg^{2+}

The results showed that smooth isolates formed biofilms in TYES broth with both Ca^{2+} and Mg^{2+} at all concentrations. None of the tested *F. psychrophilum* isolates was able to form biofilm in TYES media without Ca^{2+} and Mg^{2+}. Different concentrations of only Ca^{2+} in TYES broth did not generally affect the biofilms, but different concentrations of only Mg^{2+} in TYES broth significantly decreased the biofilm formation of both smooth and rough isolates. None of the examined isolates were able to produce biofilms neither in LW nor in NB medium with Ca^{2+} and Mg^{2+} at any concentrations (Table 2; thesis).

Nutrients, fish mucus, iron

Biofilm formation of both smooth and rough isolates was significantly increased in FF and TYES broth compared to LW. None of the *F. psychrophilum* isolates was able to form biofilms in RT mucus similarly to LW, while both smooth isolates formed biofilms in diluted TYES broth, compared to LW. Neither the cells of smooth nor rough isolates were able to form biofilms in NB with or without the supplementation of iron ($FeCl_3$) (Table 2; thesis).

Carbohydrates

L-rhamnose, D-galactose, D-mannose and D-glucose significantly reduced the biofilm formation of both smooth isolates. D-galactose, D-mannose and D-glucose (but not L-rhamnose) significantly decreased the biofilm formation of the rough isolate P7-9/2R/10 in TYES broth. Biofilm formation of the rough isolate P1-10B/2R/10 was not affected (Table 2; thesis).

4.4.2 Switch of phenotype (III)

Nutrient deprivation did not trigger phenotype switching from rough to smooth for the two examined isolates within 23 days.

Table 2. A summary of the effect of growth media and conditions on biofilm formation of *F. psychrophilum* is presented. The symbols (+) and (-) represent increased or stable biofilm and reduced or no biofilm compared to positive control, respectively (see 4.4.1).

	Biofilm formation			
	Smooth cells		Rough cells	
Isolates	P7-9/10	P1-10B/10	P7-9/2R/10	P1-10B/2R/10
Environmental factors				
Temperature				
10 °C	-	-	+	+
15 °C	+	+	+	+
20 °C	-	-	+	+
pH				
pH 6	+	+	-	-
pH 7	+	+	+	+
pH 8	-	-	-	-
Divalent ions				
Ca^{2+} and Mg^{2+}	+	+	+	+
Ca^{2+}	+	+	+	+
Mg^{2+}	-	-	-	-
Ion absence	-	-	-	-
Nutrients				
Fish feed	+	+	+	+
Trout mucus	-	-	-	-
Lake water	-	-	-	-
Iron ($FeCl_3$)	-	-	-	-
Carbohydrates				
L-rhamnose	-	-	+	+
D-galactose	-	-	-	+
D-mannose	-	-	-	+
D-glucose	-	-	-	+

4.5 Inhibition of *F. psychrophilum* biofilms *in vitro* (IV)

Each compound was examined for the MIC value, and three sub-MIC concentrations (MIC/2, MIC/8 and MIC/32) were subsequently calculated. The effect of each compound was evaluated against the smooth and rough isolates of *F. psychrophilum* biofilm formation and mature biofilms using a linear regression plot with fitted lines derived from the sub-MICs and their biofilm inhibition index.

4.5.1 Effects of compounds on biofilm formation (IV)

A strong positive linear concentration-response was obtained between the sub-MICs of 2-AI and the biofilm inhibition index of all isolates. The fitted line plots of both smooth isolates showed nearly identical results (Figure 1a; **Paper IV**). A positive linear concentration-response was obtained between the sub-MICs of emodin and L-alliin and the biofilm inhibition index of both smooth isolates and one rough (P1-10B/2R/10) isolate (Figure 1 b and d). A non-linear concentration-response with horizontal fitted lines was obtained between the sub-MICs of PTL and D-leucine and the biofilm inhibition index of all tested isolates (Figure c and e). A non-linear concentration-response was obtained between the sub-MICs of fucoidan and the biofilm inhibition index of the smooth isolates, while a positive linear concentration-responsewas obtained between the sub-MICs of fucoidan and the biofilm inhibition index of the rough isolates (Figure 1f). A non-linear concentration-response was obtained between the sub-MICs of EDTA and the biofilm inhibition index of the smooth (P7-9/10 and P1-10B/10) isolates, while a positive linear concentration-response was obtained between the sub-MICs of EDTA and the biofilm inhibition index of the rough isolates (Figure 1g). A positive linear concentration-response was obtained between the sub-MICs of A-PACs and the biofilm inhibition index of all tested isolates (Figure 1h).

4.5.2 Effects of compounds on mature biofilms (IV)

A positive linear concentration-response was obtained between the sub-MICs of 2-AI and the biofilm inhibition index of smooth isolates and one rough (P1-10B/2R/10) isolate, while a non-linear concentration-response was shown for the other rough (P7-9/2R/10) isolate (Figure 2a; **Paper IV**). A positive linear concentration-response was obtained between the sub-MICs of emodin, L-alliin and fucoidan and the biofilm inhibition index of all tested isolates (Figure 2b, d and f). A positive linear concentration-response was obtained between the sub-MICs of PTL and the biofilm inhibition index of the smooth isolates, while in contrast a negative linear

concentration-response was obtained for the rough isolates (Figure 2c). A positive linear concentration-response was obtained between the sub-MICs of D-leucine and the biofilm inhibition index of all tested isolates (Figure 2e). A positive linear concentration-response was obtained between the sub-MICs of EDTA and the biofilm inhibition index of one smooth (P1-10B/10) and one rough (P7-9/2R/10) isolate (Figure 2g). A positive linear concentration-response was obtained between the sub-MICs of A-PACs and the biofilm inhibition index of all tested isolates (Figure 2h).

4.5.3 Effect of bacteriophages on biofilms (IV)

The lytic phages (Fpv-9, Fpv-10), and the combination of lytic phages (Fpv-9 + Fpv-10) inhibited the biofilm formation of all *F. psychrophilum* isolates, while the non-lytic phages (Fpv-3, Fpv-21) partly reduced their biofilm formation of all *F. psychrophilum* isolates tested. The combination of lytic phages (Fpv-9 + Fpv-10) reduced the biomass of the mature biofilms of smooth and rough isolates after 1 h and 24 h exposure, while these phage combination only affected the mature biofilms of the rough P7-9/2R/10 isolate 12 h after exposure.

The lytic phages (Fpv-9, Fpv-10) reduced the biomass of mature biofilms of both smooth and rough *F. psychrophilum* isolates after 1 h exposure, while but they did not completely eradicate it. The non-lytic phages (Fpv-3, Fpv-21) reduced the biomass of the mature biofilms of two smooth and one rough isolates 1 h after exposure, but not for the rough P1-10B/2R/10 isolate. The addition of non-lytic phages (Fpv-3, Fpv-21) slightly to weak reduce the biomass of the mature biofilms of *F. psychrophilum*, while they reduced the biomass of the mature biofilms mainly of one smooth isolate (P1-10B/10) after 12 h and 24 h exposure respectively (Table 3; **Paper IV**).

Table 3. A summary of the effect of compounds on adhesion and biofilm formation of *F. psychrophilum* is presented. The 'Yes' describes the inhibition of adhesion to and/or biofilm formation of *F. psychrophilum* on inert surfaces, while the 'No' describes the bacterial tolerance against the compound(s) during the adhesion to and/or biofilm formation of *F. psychrophilum* on inert surfaces compared to positive controls (see 4.3 and 4.5.1).

Compounds	Chemical formula	Inhibition							
		adhesion				biofilm formation			
		Isolates							
		Smooth cells		Rough cells		Smooth cells		Rough cells	
		P7-9/10	P1-10B/10	P7-9/2R/10	P1-10B/2R/10	P7-9/10	P1-10B/10	P7-9/2R/10	P1-10B/2R/10
D-fucose	$C_6H_{12}O_5$	No	No	No	No				
D-maltose monohydrate	$C_{12}H_{22}O_{11} \cdot H_2O$	No	No	No	No				
β-lactose	$C_{12}H_{22}O_{11}$	No	No	No	No				
3-sialyllactose	$C_{23}H_{39}NO_{19}$	No	No	No	No				
Sucrose octaacetate	$C_{28}H_{38}O_{19}$	No	Yes	No	Yes				
D-(glucose; galactose; mannose; mannitol)	$C_6H_{12}O_6$; $C_6H_{12}O_6$; $C_6H_{12}O_6$; $C_6H_{14}O_6$	Yes	Yes	Yes	No				
D-(fructose; arabinose; methyl-*α*-D-mannopyranoside; fucose)	$C_6H_{12}O_6$; $C_5H_{10}O_5$; $C_7H_{14}O_6$; $C_6H_{12}O_5$	Yes	Yes	Yes	No				
N-acetyl-D-(galactosamine; glucosamine)	$C_8H_{15}NO_6$; $C_8H_{15}NO_6$	Yes	Yes	Yes	No				
L-cysteine	$HSCH_2CH(NH_2)CO_2H$	Yes	Yes	No	No				
D-leucine	$(CH_3)_2CHCH_2CH(NH_2)CO_2H$	Yes	Yes	No	No	Yes	Yes	Yes	Yes

D-methionine	$C_5H_{11}NO_2S$	Yes	No	Yes	No				
D-tryptophan	$C_{11}H_{12}N_2O_2$	Yes	No	Yes	Yes				
D-tyrosine	4-(HO)C6H4CH2CH(NH2)CO2H	No	No	No	No				
Fucoidan	$C_6H_{12}O_5$	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
Cinnamaldehyde	$C_6H_5CH=CHCHO$	Yes	No	Yes	No				
Trans-cinnamaldehyde	$C_6H_5CH=CHCHO$	No	Yes	Yes	No				
A-PACs		Yes	Yes	No	No	Yes	Yes	Yes	Yes
EDTA	$(HO_2CCH_2)_2NCH_2CH_2N(CH_2CO_2H)_2$	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
Proteinase K		Yes	Yes	Yes	Yes				
L-rhamnose	$C_6H_{12}O_5 \cdot H_2O$					Yes	Yes	No	No
D-galactose	$C_6H_{12}O_6$					Yes	Yes	Yes	No
D-mannose	$C_6H_{12}O_6$					Yes	Yes	Yes	No
D-glucose	$C_6H_{12}O_6$					Yes	Yes	Yes	No
2-aminoimidazole	$C_3H_5N_3 \cdot 0.5H_2SO_4$					Yes	Yes	Yes	Yes
emodin	$C_{15}H_{10}O_5$					Yes	Yes	No	Yes
parthenolide	$C_{15}H_{20}O_3$					Yes	Yes	Yes	Yes
L-alliin	$C_6H_{11}NO_3S$					Yes	Yes	Yes	Yes

5 DISCUSSION

The thesis characterizes the *in vivo* adhesion and *in vitro* biofilm formation of both cells, smooth and rough colony phenotypes, of *F. psychrophilum* and evaluates the effects of different compounds and phages on adhesion and biofilms *in vitro*. The results contribute to an increased knowledge of the adherence and biofilm properties of *F. psychrophilum* on surfaces under specific conditions, providing potentially useful information for the prevention and reduction of adhesion and biofilms on surfaces associated with aquaculture equipments in the future.

5.1 Adhesion of *Flavobacterium psycrhophilum* to epithelial tissues

Bacterial adhesion to living surfaces, in which adhesins (often glycoproteins or lipoproteins) bind to receptors on host cell (mostly polysaccharides) has been extensively studied over the past decades (Ofek et al. 2003). The bacterial adherence to host surfaces has been suggested to be the prerequisite step in the pathogenesis of many infectious diseases caused by aquatic pathogens including the *Vibrio anguillarum*, *Aeromonas hydrophila* and *F. psychrophilum* (Horne and Baxendale 1983; Vatsos et al. 2001; Saidi et al. 2011).

The mucus layer provides a niche for bacterial colonization because it contains attachment sites and it is also a carbon source for bacterial growth. Specific lectins on the surface of both *F. psychrophilum* and *F. columnare* bind to host cells probably through to components exist in host cell receptors e.g. carbohydrates present in mucus (Decostere et al. 1999). The mucus layer also prevents pathogens from reaching and persisting on the epithelial surfaces and thereby is a major component of innate immunity. It is constantly renewed and acts as a trap for commensal residents and pathogens, preventing their access to the epithelia. The fish mucus contains different glycolipids, polysaccharides and glycoproteins (Romo et al. 2016). *F. columnare* (Shoemaker and LaFrentz 2015) and *F. psychrophilum* (**Paper I**) are able to use these nutrients and grow in fish mucus. Both filtered, and filtered and autoclaved mucus supported the *F. psychrophilum* growth, suggesting that the presence of possible humoral immune components in only filtered mucus did not affect the growth of *F. psychrophilum* (**Paper I**). This suggests that cells of *F. psychrophilum* at fish farms can use mucus to their advantage to promote their growth (as an energy source) and adhere on epithelial mucosal tissues.

This thesis showed that fins and gills (and, to a lesser degree, skin and eye) are targets for *F. psychrophilum*, suggesting these as main entrance ports after successful bacterial adhesion on these epithelial tissues (**Paper I**). Smooth cells adhered greater on host tissues than rough cells. It seems that different adhesins express on smooth cells than on rough cells, giving smooth phenotype the adhering advantage and invasion since few smooth and rough colonies were isolated from internal organs (spleen and kidney) (**Paper I**). Although it seems that smooth cells adhered greater than the rough cells, this adherence was inversely proportional with time (**Paper I**). It is known that the mucus layer has the capability to trap and slough the pathogens, since profuse secretion of mucus detach pathogens from host (Magnadóttir 2006). In this thesis, *F. psychrophilum* smooth cells adhered on host surface, but then they gradually disappeared indicating that adhered smooth cells were captured by either the humoral skin mucosal immune responses or by mechanical clearance responses from the host (**Paper I**).

However, one of the rough (P7-9/2R/10) isolates persisted on rainbow trout tissues 48h post-exposure suggesting that the adherence of rough cells to mucosal surfaces might be isolate dependent, similarly to a previous study of *F. columnare* where a few cells of one isolate of two tested were able to persist at high numbers and for long time (Olivares-Fuster et al. 2011). Bacterial pathogenesis is the process; bacteria infect and cause disease in a host. Although, the external defenses of the fish are bypassed, the persistence of rough cells on mucus surface of fish could not end up in BCWD, if those fish were maintained for a longer time (more than 48 h). The reason is that the probability of infection of rough isolates was also decreasing with time but slower in comparison to smooth isolates (**Paper I**; Figure 1). It is possible that a stressful factor e.g. removal of adipose fin and disruption of dermal layer, treatment with formalin, or a high fish stocking density in each tank might enhance the possibilities for mortalities in *F. psychrophilum* bath challenges (Madsen and Dalsgaard 1999; Madetoja et al. 2000; Long et al. 2014).

F. psychrophilum has an outer layer comprised of carbohydrates as well as several proteins, which degrade actin and myosin, which are elements of the fish muscular system (Otis 1984; Bertolini et al. 1994; Secades et al. 2001). In this thesis, few smooth and rough *F. psychrophilium* colonies were isolated from the internal organs of few trout during the adhesion experiment (**Paper I**), suggesting that *F. psychrophilum* has evolved mechanisms to go through to the mucus layer in order to reach epithelial cells for example by extracellular proteases presented on outer membrane that directly target host mucins. The question then arose as to which conditions during the adhesion of *F. psychrophilum* to mucosal tissues

contribute to invasion, and thus to BCWD outbreaks. It is known that environmental factors influence the adhesion of *Flavobacterium columnare* to epithelial surfaces (Decostere 1999; Nematollahi et al. 2003a) and stress factors which are related to poor water quality (nitrite and organic contents), temperature fluctuation, and handling procedures of the fish have been shown to be associated with the adherence of *F. psychrophilum* to surfaces (Pérez-Pascual et al. 2009; Barnes and Brown 2011). It can be speculated that an increase of mucus production in water by stress factors such as the fish handling (Morgan et al. 2009), which can cause a further decrease in immunity to pathogens, may further facilitate the growth of *F. psychrophilum* in fish tanks resulting in increased bacterial load in the water, increasing the probability of *F. psychrophilum* cells to adhere on mucosal tissues, and subsequently facilitating higher risk of BCWD outbreaks.

Even though mucus can possess specific and non-specific antimicrobial factors secreted by epithelial cells, such as antimicrobial peptides (AMP), antibodies targeting specific microbial antigens (agglutinins, immunoglobulins), proteins (lectins) and enzymes (lysozymes), pathogens can still be isolated from mucosal surfaces (Dongari-Bagtzoglou 2008). *F. psychrophilum* was able to grow in mucus *in vitro* and adhere to mucus surfaces *in vivo* (**Paper I**), but *F. psychrophilum* did not form biofilms in mucus (**Paper III**). In this thesis, the concentration of *F. psychrophilum* cells decreased with time on mucosal tissues (**Paper I**), which suggests that fish mucus can prevent the biofilm formation of *F. psychrophilum* in order to avert infections. Mucin proteins and biopolymers can influence the expression of genes implicated in biofilms, preventing the adhesion and biofilm formation of the gram negative bacteria *Pseudomonas aeruginosa* (Caldara et al. 2012; Haley et al. 2014). Motility genes have been shown to be downregulated in biofilm state were compared to the planktonic state of *F. psychrophilium* isolates (Levipan and Avendaño-Herrera 2017). Despite this, little is known about bacterial biofilm of aquatic pathogens in the mucus layer in general.

5.2 Inhibition of *F. psychrophilum* adhesion to inert surfaces

Adherence is an advantageous step for bacterial survival and a key step in pathogenesis. Anti-adhesion strategies aim to reduce the contact between host tissues and bacterial pathogens by preventing the adherence of the bacteria (Ofek et al. 2003). To effectively achieve adherence to host surfaces, many bacteria produce multiple adherence factors called adhesins. It is known that bacterial adhesion to surfaces is mediated by cell hydrophobicity, proteins and surface charge (Donlan 2002). The involvement of adhesins in the *F. psychrophilum* genome

as virulence factors has been tentatively suggested (Duchaud et al. 2007). In the present thesis, the inhibition of the adhesion of mainly smooth cells of *F. psychrophilum* by different types of compounds indicates that the outer surface of smooth cells consists of a large number of bacterial adhesins with distinct receptor specificity, since some compounds were markedly stronger inhibitors of *F. psychrophilum* adhesion than others (**Paper II**).

Carbohydrates seem to be the main target for the *F. psychrophilum* adhesins like the sialic-acid lectin, which is a carbohydrate-binding protein of non-specific immune system (lectins), and capable of interrupting the agglutination of cells (Møller et al. 2003; Högfors-Rönnholm et al. 2014). In this thesis, treatment of *F. psychrophilum* cells with sucrose octaacetate and a combination of carbohydrates inhibited the adhesion of cells, indicating that carbohydrate-binding proteins (lectins) exist on bacterial cells and they are involved in the adherence of the *F. psychrophilum* to surfaces (**Paper II**), similarly to *F. columnare* adherence (Klesius et al. 2010). It is possible that the inhibition of *F. psychrophilum* adherence by multivalent carbohydrates can imply the presence of different receptor sites on *F. psychrophilum* cell surfaces, or that different carbohydrates can be recognized by one receptor mediating cell-to-cell adhesion.

Exposure of cells to D-amino acids, cranberries A-PACs and cinnamaldehyde resulted in reduction of *F. psychrophilum* adhesion possibly by decreasing the cell hydrophobicity and blocking the cell-to-cell interactions of smooth cells in a concentration-dependent manner (**Paper II)**; as has been shown in previous studies for different bacterial species (Feldman et al. 2012; Nostro et al. 2012; Xing et al. 2015). Fucoidan and proteinase K inhibited the adhesion to and biofilm formation of *F. psychrophilum* (**Paper III** and **Paper IV**). This suggests that proteins seem to be involved in the adhesion of *F. psychrophilum* to surfaces, similar to previous studies describing that proteinase K causes a decrease in *F. psychrophilum* hemagglutination (Møller et al. 2003; Högfors-Rönnholm and Wiklund 2010). Recently, Kunttu et al. (2011) and Chenia and Chadwick (2013) observed a significant decrease in the adhesion of cells of *F. columnare* and *F. johnsoniae*-like isolates, respectively, following proteinase K treatment. Clearly, the adhesion of different *Flavobacterium* sp. to abiotic surfaces is at least partly mediated by protein structures on the cell surface or in the capsular material. In this thesis, it is suggested that fucoidan and proteinase K might interact with cell membrane proteins, thus blocking cell-to-cell interactions of the smooth cells (**Paper II**). It can be concluded that surface hydrophobicity and adhesins such as proteins are responsible for the cell-to-cell contact and thus strong adherence of smooth cells to surfaces. The present

thesis suggest that *F. psychrophilum* has the potential to adhere on plastic surfaces present in fish farms, however this environment is rather complex involving different bacteria species and fungi competing with *F. psychrophilum* for space and nutrients.

The development of *F. psychrophilum* biofilms to inert surfaces (**Paper III**) was dependent mainly on Ca^{2+}, and EDTA inhibited the adhesion of *F. psychrophilum* to surfaces (**Paper II**) indicating that Ca^{2+} and Mg^{2+} play an important role in adherence of *F. psychrophilum*. This suggests the possibility that Ca^{2+} is necessary for the adhesion and subsequently the biofilm formation of the bacterial cells to inert surfaces.

Rough cells exhibit some degree of resistance to most inhibiting compounds during the adherence on plastic surfaces, except for the EDTA and proteinase K. Although smooth and rough cells show clear differences in adherence capacity, which is associated with the expression of different adhesins or/and with surface hydrophobicity of cells (Högfors-Rönnholm et al. 2014; **Paper II**), it is also suggested that a common complex of ions and proteins is responsible for the adherence of cells of both phenotypes on inert surfaces (**Paper II**).

5.3 Influence of environmental factors on *F. psychrophilum* biofilms

The smooth phenotype has an advantage over the rough phenotype during the cell adhesion to inert surfaces (**Paper II**), and clearly, smooth isolates have the capacity to produce stronger biofilms than the rough isolates (**Paper III**). One rough isolate (P7-9/2R/10) produced weak biofilms and the same isolate has been shown to persist on host epithelial tissues (**Paper I**). It seems that smooth cells represent the sessile cell form in *F. psychrophlum* biofilms and the results of the present thesis that *F. psychrophilum* produces biofilms on inert surfaces are consistent with previous findings (Levipan and Avendano-Herrera 2017; Pérez-Pascual et al. 2017).

Once attached, bacterial cells can start to proliferate, leading to biofilm formation (Schroeder et al. 2017). This formation is not a random mechanism, since most bacterial pathogens prefer certain sites over others due to particular local environmental conditions (Huang et al. 2011). Environmental factors and conditions influence the *F. psychrophilum* biofilm formation (**Paper III**). Although the ability of *F. psychrophilum* to adhere to surfaces in lake water (**Paper II)** could explain the survival of the bacterium under adverse conditions like starvation. In the present thesis *F. psychrophilum* isolates formed biofilms only in nutrient-

rich environments *in vitro* like in fish feed media but not in low nutrient environments e.g. lake water (**Paper III**). This suggests that *F. psychrophilum* reservoirs may be hidden in biofilms attached to synthetic or plastic surfaces in aquaculture environments.

It has been suggested that BCWD occurs from 4–10 °C, but the disease can be less severe at 15 °C (Cipriano and Holt 2005). The optimum temperature for bacteria is associated with an increase in nutrient intake, resulting in a rapid formation of biofilm (Garrett et al. 2008), and optimum growth temperature for the *F. psychrophilum* has been suggested to be at 15°C (Holt et al. 1993; Duchaud et al. 2007). *F. psychrophilum* formed greater biofilms at 15 °C compared to 20°C and 10°C (**Paper III**), which suggests that a great uptake of nutrients by the bacterial cells at 15 °C favour the biofilm production. At the low and high temperatures, the physiological functions of psychrophilic bacteria are impaired to a lesser degree. It is known that biofilm cells adjust the activity and synthesis of proteins during pH variations (Olsen 1993). The enhanced biofilms produced by smooth isolates under acidic growth conditions (pH 6) suggest the presence of slightly acid-tolerant sessile cells (**Paper III**). It is common in freshwater recirculating aquaculture systems to use nitrifying bioiflm bacteria in biofilters to lower the pH (Hüpeden et al. 2016; Bartelme et al. 2017); however this strategy could enhance the production of *F. psychrophilum* biofilms increasing the bacterial load in fish-tank water.

It has been suggested that *F. psychrophilum* is capable of enhancing their iron uptake through siderophore production (Møller et al. 2005). In this thesis, the iron in NB was not the limiting factor for the lack of biofilm formation, but it is possible that the nutrient-rich environment repressed the expression of certain genes essential for biofilm similar to previous studies with the *Pseudomonas aeruginosa* (Musk et al. 2005) thus acting as a repressor for the biofilm formation of *F. psychrophilum* (**Paper III**).

F. psychrophilum cells form biofilms if a sufficient level of Ca^{2+} and Mg^{2+} is present (at least 0.05 g/L corresponding to 6°dH hardness), although biofilms seem to be mainly Ca^{2+}-dependent (**Paper III**). This seems to be consistent with the role of Ca^{2+} in biofilms formed by *F. columnare* (Staroscik and Hunnicutt 2007; Cai et al. 2013; Cai and Arias 2017). Ca^{2+} binding molecules are involved in the initial stages of bacterial biofilm formation increasing the cell-to-cell aggregation (Rose 2000). It is possible that *F. psychrophilum* uses these divalent ions for its adhesion (**Paper II**) and biofilm formation (**Paper III**) to plastic surfaces by enhancing the aggregation of cells. Notably, the level of 6°dH hardness with the presence

of nutrients is equal to the hardness of the water in Finnish rainbow trout farms (Tapio Kiuru; personal communication), suggesting that the water environment is suitable for *F. psychrophilum* to form biofilms on aquaculture surfaces.

5.4 Phase variation in biofilms

It is considered that biofilms begins with the adhesion of planktonic cells to a surface, and then a phenotypic switch between planktonic to sessile growth occurs. This switch may occur under different conditions (Karimi et al. 2015). The heterogeneity of phenotypes developed within biofilms can be translated by the need of biofilm cells to face challenges (Sousa and Pereira 2014), and nutrient limitation in the environment can drive phenotypic heterogeneity in bacteria (Schreiber et al. 2016). In this thesis, the hypothesis that rough cells respond to nutrient limitation altering colony phenotype (to smooth) was rejected (**Paper III**). It is possible that a switch from rough to smooth phenotype of *F. psychrophilum* may occur under host conditions for avoidance of the host immune system contributing to the cell resistance during disease outbreaks as have been observed in *Mycoplasma pulmonis* (Denison et al. 2005). Otherwise, it is possible that genetic rearrangements via mobile genetic elements via phages could alter the phenotype of the bacterium during the lysogenic cycle (Clokie et al. 2011). This need to be studied further for the phase variation of *F. psychrophilum*.

5.5 Inhibition of *F. psychrophilum* biofilms

Recent studies have shown that natural compounds such as plant extracts can inhibit the biofilm formation of bacterial pathogens and their use can lead to decrease of likelihood of development of resistant cells as those observed within bacterial biofilms when antibiotics are used (Miquel et al. 2016; Rabin et al. 2015). In this thesis, the compounds 2-AI, emodin, PTL and D-leucine were displaying clear effects on both prevention and reduction of biofilms formed by mainly smooth *F. psychrophilum* isolates (**Paper IV**), while 2-AI, emodin, PTL were ineffective against the mature biofilms formed mainly by rough isolates suggesting a tolerance to these compounds or a persistence within the biofilms. Our data also revealed the potential of 2-AI, D-leucine, EDTA, emodin and fucoidan to reduce the biomass of mature biofilms of the smooth *F. psychrophilum* probably due to their ability to cause biofilm dispersal, as have been reported by other studies with different bacterial pathogens (Raad et al. 2003; Kolodkin-Gal et al. 2010; Frei et al. 2012; Elbi et al. 2017; Yan et al. 2017). It is known that bacteria that detach from the EPS matrix can re-adhere and re-form biofilms (López et al. 2010). By causing cell dispersal, an increased number of cells will be released in

water, increasing the bacterial load on the fish followed by an increased risk of BCWD infections.

PTL and D-leucine showed a high potential to reduce the *F. psychrophilum* biofilms in a concentration-independent manner, and that a linear concentration-response relationship may occur in lower concentrations (**Paper IV**). The use of different compounds has shown that they are more likely to prevent the biofilm formation of different bacterial pathogens than to reduce the biomass of mature biofilms due to higher thickness, complicated cell-to-cell interactions and the presence of persistent cells (Kostakioti et al. 2013). In the present thesis, the fucoidan, EDTA and A-PACs inhibited the biofilm formation of *F. psychrophilum* on inert surfaces but totally efficient biofilm removal was not achieved (**Paper IV**). This weak inhibition could reflect the inability of fucoidan, EDTA and A-PACs to disturb the biofilm matrix of *F. psychrophilum* isolates at the sub-MIC concentrations used in this thesis. Interestingly, biofilms formed by rough *F. psychrophilum* isolates were not affected by sub-MICs and some of them may represent the tolerant or persister cells within the biofilms. If, in the future, dispersal compounds are introduced in fish farm environments, these compounds should be used in combination with antimicrobials agents such as disinfectants or phages, which will kill the bacterial cells. However, further studies need to evaluate whether *F. psychrophilum* cells produce biofilms on aquaculture settings and the fish host cell toxicity of these compounds.

5.6 Bacteriophages against *F. psychrophilum* biofilms

The use of phages to control bacterial infections in aquaculture has gained attention, and constitutes a realistic alternative to the use of antibiotics (Richards et al. 2014). Fish bacterial pathogens such as *Aeromonas salmonicida* (Imbeault et al. 2006), *Vibrio harveyi* (Vinod et al. 2006), *V. anguillarum* (Tan et al. 2015b), *Pseudomonas plecoglossicida* (Park and Nakai 2003), *F. columnare* (Laanto et al. 2015), and *F. psychrophilum* (Kim et al. 2010; Castillo et al. 2012; Castillo et al. 2015; Christiansen et al. 2016) have been shown to be susceptible to phages. The addressed stability of phages in fresh water fish farms and the reduction in trout mortalities after phage therapy have been proven to be a promising control weapon against BCWD outbreaks (Madsen et al. 2013).

In this thesis, lytic phages and a combination of lytic phages inhibited the biofilm formation of *F. psychrophilum*, while the non-lytic phages partly reduced the biofilm formation of the bacterium. It is assumed that lytic phages had the capacity to replicate in the smooth and

rough *F. psychrophilum* cells causing cell lysis of the adhered cells. In future, the use of lytic phages may prevent the *F. psychrophilum* biofilms on aquaculture settings (**Paper IV**). The partial inhibition of *F. psychrophilum* biofilm formation caused by non-lytic phages might be associated with biofilm inhibitor molecules like matrix degrading enzymes (Chan and Abedon 2015; Pires et al. 2016), or dispersal mechanisms (Feiner et al. 2015).

In this thesis, both lytic and non-lytic phages incompletely reduced mature *F. psychrophilum* biofilms in a time-dependent manner (**Paper IV**). Fu et al. (2009) suggested that the presence of subpopulations with distinct phenotypes in biofilms of *Pseudomonas aeruginosa* conferred resistance to phages. The presence of similar sub-populations may explain the persistence of some *F. psychrophilum* cells to phages within the biofilms (**Paper IV**), e.g. metabolically inactive cells (Abedon 2017). The mechanism(s) mediating resistance in *F. psychrophilum* biofilms against phages is unknown, although spontaneous mutations of *F. psychrophilum* have been linked to phage resistance (Castillo et al. 2015) or temperate (lysogenic) phages (Castillo et al. 2016).

Bacteria that become resistant to a certain phage can still be infected by other phages. For this reason, cocktails of phages with overlapping host ranges, or the use of polyvalent phages with a wide host range can diminish the probability of development of phage resistance (Wittebole et al. 2014). In this thesis, a combination of lytic phages had greater effect on the biomass of mature biofilms than the use of individual phages, suggesting the potential therapeutic use phage cocktails against *F. psychrophilum* biofilms at fish farms (**Paper IV**). It is worthwhile to mention that the broad host range Fpv-9 has been previously suggested for phage therapy in aquaculture (Stenholm et al. 2008; Christiansen et al. 2016), and its combination with the Fpv-10 in this thesis, can be recommended as a future treatment against biofilms of *F. psychrophilum* on fish-farming surfaces.

6 CONCLUSIONS

The results of the present thesis show that the adhesion of *F. psychrophilum* cells of mainly smooth isolates, to inert surfaces was clearly inhibited by most of the tested compounds. These results encourage the introduction of surface materials with molecular composition resembling those used in this thesis to reduce the *F. psychrophilum* adhesion to aquaculture surfaces. However, biochemical and molecular experiments are needed to better understand how these inhibitors affect the adhesion of *F. psychrophilum* to surfaces.

Bacterial adhesion to epithelial tissues is the first step of the disease process in the host, enhancing the entry of the cells into the host. In the present thesis, it was shown that mucus supports the growth and the adhesion of *F. psychrophilum* facilitating the bacterial invasion. However, a strong adhesion to fish mucus could support the expelling of the bacterial cells together with the mucus from the fish surface. The results show that the bacteria were not able to form biofilms on the mucus layer *in vitro*, and the presence of large number of bacteria in the surface layer of fish exposed for *F. psychrophilum* could not be documented, suggesting that biofilms are not formed in the mucosal tissue *in vivo*. However, rough cells were still present on host tissues after 24 h indicating that rough cells might have adhesins on the outer membrane important for the protection of cells from the exclusion and host defense. The adherence of cells to mucosal surfaces may be a crucial step in the pathogenesis of *F. psychrophilum* although how the bacterial invasion can lead to an infection and then to a disease is unknown.

The results allowed us to gain a better understanding of the development of *F. psychrophilum* biofilms and help us to develop strategies for the control of these biofilms. The results of this thesis suggest that smooth cells represent the sessile cell form that adhere to surfaces, form biofilms and thereby protect cells inside the biofilms. Biofilms were formed *in vitro*, by *F. psychrophilum* cells in nutrient (fish feed)-rich media with a certain amount of divalent ions suggesting that *F. psychrophilum* has the capacity to produce biofilms on fish-farming surfaces.

Although, it is not explicitly known if *F. psychrophilum* forms biofilms in fish-farming environments, biofilms have been produced and examined under laboratory conditions. A good knowledge on how the biofilm is formed by *F. psychrophilum* in aquaculture facilities is needed. An open system, where dispersed and dead cells are constantly moved with the fresh

medium, can give more information about the *F. psychrophilum* biofilms. Under fish-farming conditions, multispecies biofilms can exist, where antagonism, commensalism, parasitism, and mutualism may occur between the different microorganisms.

Additionally anti-biofilm methods need to be examined and developed. Ca^{2+} and Mg^{2+} are important component for the development of *F. psychrophilm* biofilms, however, to keep the level of these divalent ions low in fish tanks in order to reduce the biofilms would mean that farmers could not achieve the required level of ions for healthy fish growth. During the last few years, there has been a large pharmaceutical interest in natural products for the control and/or prevention of bacterial biofilms claiming that there are unexplored sources of such potential anti-biofilm compounds in nature. In the present thesis, a number of selected compounds e.g. fucoidan from *Fucus vesiculosus*, inhibited the biofilm formation of *F. psychrophilum*, while the biomass of the mature biofilms of smooth isolates were reduced. This suggest that the biofilms produced by *F. psychrophilum* can be inhibited and reduced and future research should focus on finding an effective anti-biofilm compound with no side effects on the host.

The use of lytic phages to prevent the biofilm formation of *F. psychrophilum* cells on inert surfaces may be an advantageous strategy due to low cost for the farmers. Future studies are needed to evaluate how the mature biofilms formed by *F. psychrophilum* can be disrupted. It may be worthwhile to test a combination of inhibiting compounds and phages against the *F. psychrophilum* biofilms.

The resistance of rough isolates to some compounds indicates that these cells comprise a bacterial subpopulation that have a tolerance to agents and might reach this state undergoing phenotypic change in order to disperse and re-colonize new niches/surfaces for propagation and self-renewal of the community. The ability of the rough cells to remain in the mucosa of fish skin and sustain the immune components, to form weak biofilms and tolerate anti-adhesion and anti-biofilm compounds may suggest the ability of these cells to spread in the aquatic environment or inside the host representing the tolerant cells in *F. psychrophilum* biofilm. Future detailed studies to elucidate this tolerance may give important information about the role of these rough cells in infections caused by *F. psychrophilum*.

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Eureka (Archimedes c. 287 BC - c.212 BC)

‘‘I do not care if it is my fifth bath today, it is science time and I am so, so dirty’’-Archimedes on his methods.

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Anna Papadopoulou

Flavobacterium psychrophilum adhesion and biofilm formation

Flavobacterium psychrophilum, the etiological agent of bacterial cold water disease, causes significant mortalities in trout and salmon populations worldwide, including Finland. Phase variation occurs in *F. psychrophilum* with two different morphological colony phenotypes, smooth and rough. The focus of this thesis has been on the adherence between the host and both phenotypes; the factors favour the bacterial biofilm formation on inert surfaces and the anti-adhesion and anti-biofilm ability of compounds-derived from natural products and bacteriophages. The work shows that growth of *F. psychrophilum* in mucus facilitates bacterial adhesion to mucosal surfaces and subsequently the invasion into host. Nutrients are vital to aquaculture, but the propensity of F. *psychrophilum* to form biofilms in nutrient (fish feed)-rich environments poses additional problems in disease-management programs. The use of anti-adhesion and anti-biofilm compounds and cocktails of bacteriophages may be effective approaches to reducing re-occurrence of the *F. psychrophilum* infections in farmed fish in near future.

Cover picture:
Minoan fresco-man with fish ca 1650 BC (Bronze age excavation, Akrotiri, Thira, modern Santorini, Greece). Cycladic civilization

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