Norwegian University of Life Sciences
Faculty of Chemistry, Biotechnology and Food Science

Philosophiae Doctor (PhD)
Thesis 2018:34

The farmed Atlantic salmon *(Salmo salar)* skin-mucus microbiome - bacterial diversity, mucus degrading potential and putative virulence factors

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Giusi Minniti

Ås, April 2018

SUMMARY

Norway is the main producer and exporter of Atlantic salmon worldwide, representing one of the pillars of the Norwegian economy. Today, about 1.2 million tons of salmonids are produced in Norway and a five-fold increase is estimated for the year 2050. The large-scale production of fish is continuously challenged by maladaptive conditions, which often cause diseases that manifest themselves in the form of economic losses and reduced animal welfare. Skin disorders are one of the problems associated with bacterial diseases and seem to be influenced by the combination of several factors such as the fish health status, the bacteria present and the environmental conditions. Indeed, it is recognized that exposure of fish to stress increases their vulnerability to infectious diseases. In the last two decades, the use of vaccines has drastically diminished disease outbreaks caused by bacteria, nonetheless infectious diseases are still occurring in aquaculture. It is therefore important to increase our understanding of the skin, its associate microbial flora and the interaction between the microbiota and the host. In the present work, several aspects of this complex interaction have been investigated, which are reported in three research papers.

In paper I, 16S rRNA gene amplicon sequencing was employed to investigate the composition of the farmed Atlantic salmon skin microbiome before and after netting and transport, which represent common aquaculture practices. A rapid community shift was observed after only 24 hours, suggesting susceptibility of the skin-mucus microbiome to external events within a short period of time. The composition of the bacterial community present in the rearing water was also investigated, showing low correlation with the mucus microbiome. Thus, despite the skin-mucus microbiome is in direct contact with the surrounding environment, it seems to be specifically adapted to the mucosal surface.

In paper II, label-free quantitative mass spectrometry was used to study the skin-mucus exoproteome associated with both the host (farmed Atlantic salmon) and its bacterial community. The host skin-mucus exoproteome was dominated by host proteins, where many were related to antibacterial activities. By allowing the resident skin-mucus bacteria to grow in the mucus over a period of 9 days, we were able to monitor the temporal proteome dynamics and to evaluate the bacterial capacity to utilize mucus components as nutrient source. Host proteins were gradually replaced by bacterial proteins predominantly related to

protein degradation and iron acquisition. The majority of the bacterial proteins detected in the present study belonged to the genus *Vibrio*.

In Paper III, *Aliivibrio salmonicida* enzymes putatively involved in chitin degradation (*As*LPMO10A, *As*LPMO10B and *As*Chi18A) were characterized in order to evaluate their possible role as virulence factors in Cold Water Vibriosis, a disease primarily associated with farmed salmon and rainbow trout. The chitinase *As*Chi18A showed activity towards chitin, although its capacity to depolymerize this substrate was substantially lower compared to the well-studied chitinases from *Serratia marcescens* and *Cellvibrio japonicus*. This result suggests that the *Aliivibrio salmonicida* chitinase may have evolved activity towards other substrates than chitin. The two lytic polysaccharide monooxygenases *As*LPMO10A and *As*LPMO10B also showed activity towards chitin. All enzymes bound to Atlantic salmon scales, and in particular the chitinase showed strong binding. Nevertheless, none of the enzymes indicated activity towards scales and salmon skin-mucus.

In conclusion, the studies described in this PhD project cover a broad experimental palette that provides new insights into the complex host-microbiota interactions on the fish skin. The work described also pinpoints some of the aspects that must be further investigated to better understand the interactions existing between environment, host and microbial community in the salmon skin-mucus in order to improve fish welfare in aquaculture.

SAMMENDRAG

Norge er verdens største produsent av oppdrettslaks og oppdrettsnæringen representerer en av bærebjelkene i norsk økonomi. I 2017 ble det produsert omkring 1,2 millioner tonn oppdrettslaks i Norge og det forventes en femdobling av produksjonen mot år 2050. Storskala produksjon av oppdrettslaks er utfordrende, da betingelsene rundt oppdrett kan forårsake sykdom hos laksen og derav gi betydelige økonomiske tap og redusert dyrevelferd. Hudsykdommer representerer ett av problemene som kan forekomme innen lakseoppdrett og utbrudd påvirkes av mange ytre faktorer som for eksempel hvilke mikroorganismene som er tilstede på huden og stressnivået laksen er utsatt for. I løpet av de siste tiårene har utvikling og bruk av vaksiner redusert forekomsten av bakterieinfeksjoner betydelig, men problemet er fremdeles forekommende. Det er derfor viktig å forbedre vår forståelse av hudbarrieren, bakteriefloraen assosiert med huden og interaksjonen mellom verten og bakteriene. I arbeidene presentert i dette doktorgradsprosjektet har flere aspekter innen relasjonen mellom vert og bakterier blitt belyst. Prosjektet har gitt opphav til tre vitenskapelig studier.

I den første studien ble 16S rRNA amplicon sekvensering benyttet til å undersøke sammensetningen av bakteriefloraen på laksehuden før og etter hoving og transport, to håndteringsmetoder som er vanlig i bruk innen lakseoppdrett. Analyse av dataene viste at sammensetningen av mikrobiotaen endret seg betydelig i løpet av kun 24 timer etter håndteringen av fisken. Sammensetningen av bakterier i karvannet ble også undersøkt og viste lav korrelasjon med hudbakteriefloraen. Det kan derfor virke som bakteriefloraen på huden er spesifikt tilpasset mukusoverflaten.

Den andre studien benyttet merkefri kvantitativ proteomikk til å undersøke eksoproteomet assosiert med hudmukus hos både verten (Atlanterhavslaks) og bakteriefloraen. Eskoproteomet var dominert av proteiner fra verten, hvorav mange var relatert til antimikrobiell aktivitet. Ved å la bakteriefloraen i hudmukusen vokse over ni dager kunne vi også undersøke hvordan eksoproteomet utviklet seg og hvilke strategier bakterien brukte for å benytte mukus som næring for vekst. Proteinene fra verten forsvant gradvis og ble erstattet av bakterielle proteiner som først og fremst var knyttet til proteinnedbrytning og anskaffelse av jern. Majoriteten av proteinene observert tilhørte genuset *Vibrio*.

I den tredje studien gikk nærmere inn på en spesifikk sykdomsbakterie, *Aliivibrio salmonicida,* som forårsaker kaldtvannsvibriose hos laks. Tre potensielle virulensfaktorer (*As*LPMO10A, *As*LPMO10B og *As*Chi18A) med mulig aktivitet mot kitin ble karakterisert biokjemisk. Kitinasen *As*Chi18A viste aktivitet mot kitin, men var opp til 50 ganger mindre aktiv enn velkjente kitinaser fra jordbakterier som *Serratia marcescens* og *Cellvibrio janonicus*. Dette kan tyde på at *As*Chi18A har utviklet aktivitet mot andre substrater i tillegg til kitin. De to lytisk polysakkarid monooksygenasene (*As*LPMO10A og *As*LPMO10B) viste også aktivitet mot kitin. Alle enzymene bandt til lakseskjell og spesielt kitinasen bandt sterkt. Ingen av enzymene viste aktivitet mot komplekse substrater som laksehudmukus eller lakseskjell.

Alt i alt dekker dette doktorgradsprosjektet et bredt eksperimentelt arbeid som gir innsyn flere forskjellige fasetter av forholdet mellom lakshuden og bakteriene som finnes der. Arbeidet belyser også en rekke områder som krever dypere forskning for å øke forståelsen av interaksjonen mellom miljø, vert og det mikrobiota på fiskehuden, hvilket er viktig for forbedring av fiskehelse og fiskevelferd innen akvakultur.

ABBREVIATIONS

AA	Auxiliary Activity
AMPs	Antimicrobial Peptides
CAZy	Carbohydrate Active EnZyme
CBMs	Carbohydrate binding modules
CBP	Chitin binding protein
CID	Conserved insertion domain
CWV	Cold Water Vibriosis
GalNAc	*N*-acetylgalactosamine
GHs	Glycoside hydrolases
GlcN	Glucosamine
GlcNAc	*N*-acetylglucosamine
$(GlcNAc)_2$	Chitobiose
$(GlcNAc)_3$	Chitotriose
GlcNAc1A	*N*-acetylglucosamine aldonic acid
GO	Gene Ontology
LPMOs	Lytic polysaccharide monooxygenases
OTUs	Operational Taxonomic Units

LIST OF PAPERS

Paper I

Giusi Minniti, Live H. Hagen, Davide Porcellato, Sven M. Jørgensen, Phillip B. Pope and Gustav Vaaje-Kolstad, 2017, The skin-mucus microbial community of farmed Atlantic salmon (*Salmo salar*), Frontiers in Microbiology, 8, 2043.

Paper II

Giusi Minniti, Simen R. Sandve, János T. Padra, Sara Lindén, Phillip B. Pope, Magnus Ø. Arntzen and Gustav Vaaje-Kolstad, 2018, The farmed Atlantic salmon (*Salmo salar*) skin-mucus proteome and its nutrient potential for the resident bacterial community, Submitted to Applied and Environmental Microbiology.

Paper III

Giusi Minniti, Jennifer S. M. Loose, Sophanit Mekasha, Bastien Bissaro and Gustav Vaaje-Kolstad, Characterization of putative virulence factors with chitinolytic activity from *Aliivibrio salmonicida*, Manuscript.

Co-authorship of other papers not included in the thesis

Giusi Minniti, Benoit J. Kunath, Live H. Hagen, Gustav Vaaje-Kolstad, Vincent G.H. Eijsink, 2018, Metaproteomics: Sample Preparation and Methodological Considerations, Exp Med Biol, In press.

* The authors contributed equally to this work

1 INTRODUCTION

1.1 Aquaculture of Atlantic salmon (*Salmo salar*)

The Norwegian aquaculture of Atlantic salmon started during the 1970s but became only commercially viable at the beginning of the 1980s (Asche and Bjorndal, 2011). The annual production of farmed fish increased from less than 1000 tons in 1970s to 8000 tons in 1980s (Hovland, 2014). The early successes in Norway prompted the growth of the Atlantic salmon aquaculture in other European countries such as Scotland, Ireland, Faroe Island and with minor production in France and Spain. It developed as well in Canada, North Eastern seaboard of the USA, Chile and Australia (FIMA, 2004-2012). In 2009, the production of farmed Atlantic salmon reached a total of 1.5 million tons, where Norway represented the largest producer and exporter (65%), followed by the United Kingdom (10%), Chile (9%), Canada (8%), Faroe Island (3%), Australia (2%) and United States (1%) (Torrissen et al., 2011). Recently, the Norsk statistisk sentralbyrå (SSB) reported a production of farmed *Salmo salar* in Norway of about 1.2 million tons (2015-2016) and a five-fold increase is estimated for the year 2050 by the Ministry of Trade, Industry and Fisheries based on the report «Verdiskaping basert påproduktive hav I 2050».

Successful farming of Atlantic salmon is strictly dependent on the health management of the fish. Unfortunately, large-scale production is continuously challenged by stressful conditions (e.g. high stocking, netting, sorting and transport), deterioration of the environmental conditions and infectious diseases, often causing economic losses in the aquaculture sector (Balcázar et al., 2006). Skin disorders represent one of the reasons of fish mortality. Indeed, it is estimated that 1.1%-2.5% of fish in aquaculture die due to skin ulcers (Karlsen et al., 2017). The aetiology of skin ulcers is complex, however it is clear that the main factors associated with these disorders are mechanical injuries, environmental conditions, nutrition and infectious agents (Takle et al., 2015). Among the long list of bacterial fish pathogens associated with skin ulcers and necrosis, *Moritella viscosa* (Winter-Ulcer Disease) (Lunder et al., 1995; Austin et al., 2012; Karlsen et al., 2017), *Aeromonas salmonicida* (Furunculosis) (Austin et al., 2012) and *Aliivibrio salmonicida* (Cold Water Vibriosis) (Austin et al., 2012; Kashulin et al., 2017) represented the main causes of mortality in farmed Atlantic salmon. Fortunately, the introduction of prophylactic procedures, aiming to avoid fish infections such as antibiotics and vaccines, had successfully reduced mortality (Lillehaug et al., 2003). Indeed, since the development of vaccines against

Cold Water Vibriosis, Furunculosis and Winter Ulcer Disease, these infections do not represent the main problem in aquaculture. Nowadays, the major concerns regarding salmon farming are associated with salmon lice and viral diseases.

Despite the significant reduction of bacterial disease outbreaks, Winter Ulcer Disease is periodically observed along the Norwegian coast line e.g. a slight increase in the number of outbreaks was registered during the year 2011, 2012 and 2013 (Hjeltnes, 2014; Hjeltnes, 2017). The presence of bacterial epidemics in vaccinated salmon highlights the importance of a major knowledge on fish immunology and biology of fish pathogens in order to prevent future losses in aquaculture.

1.2 The skin of teleost and its bacterial community

1.2.1 Teleost skin

Atlantic salmon (*Salmo salar*) belongs to the infra class teleost (Greek: teleios, "complete" + osteon, "bone"), which constitutes the richest group among vertebrates with more than 30000 species found in all the aquatic environments (Weitzman, 2016). The skin of teleost is composed by a squamous stratified epithelium called epidermis and by the dermis, which is constituted of loosely organized collagen fibers and vasculature section (hypodermis) and a highly ordered region of orthogonally arranged collagen (innermost layer) (Hawkes, 1974; Ángeles Esteban, 2012). The surface of the epidermis is covered by a complex, viscous and hydrated secretion called mucus, which allows osmo- and ion regulation (Negus, 1967), gas exchange (Park et al., 2003) and the reduction of fluid friction (Rosen and Cornford, 1971). In addition, mucus is considered as the first barrier of defense from the external environment, exhibiting immune functions (Salinas et al., 2011). Mucus is mainly produced by three different cell types: goblet cells, club cells and sacciform cells (Brinchmann, 2016). The main components of mucus are mucins, which are highly glycosylated proteins consisting of a NH_2-terminal-, a large central- and a COOH-terminal peptide domain (**Figure1**). The two terminal regions are very lightly glycosylated, but rich in cysteines, which are involved in establishing disulfide linkages within mucin monomers. The central peptide domain consists of multiple tandem repeats (10 to 80 residues), where each contains mainly the amino acids, serine and threonine. These amino acids carry a large number of O-linked glycans, which protect mucin from proteolytic cleavage. N-linked glycans are as well observed in mucin but in lower abundance. Glycan chains can be composed of 1 to 20

monomeric sugars, which are mainly represented by *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), fucose, galactose and *N*-acetylneuraminic acid (sialic acid) (Bansil and Turner, 2006; Linden et al., 2008). Transcriptomic analysis of skin samples of Atlantic salmon revealed that fish mucins are similar to MUC2 and MUC5 mucins described in higher vertebrates (Micallef et al., 2012). Additionally, Padra et al., showed that the mucins present on the Atlantic salmon skin-mucus were characterized by shorter glycans and a lower level of the sialic acid *N*-acetylneuraminic acid compared to the mucins of the fish intestinal regions (Padra et al., 2014). Mucins play a key role in accommodating bacterial residents and limiting the adhesion of pathogens (Linden et al., 2008; Padra et al., 2014).

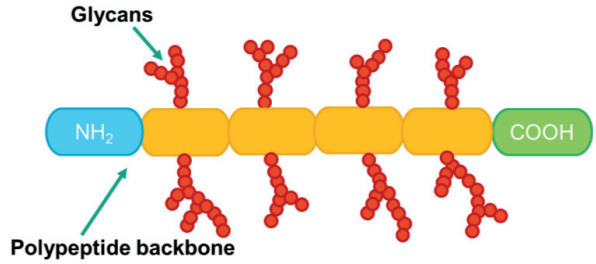

Figure 1. Simplified representation of mucin composition. The following components are shown: NH_2-terminal peptide domain (blue), central peptide domain (orange), COOH-terminal peptide domain (green) and the O-linked glycans containing various monomeric sugar components (red).

In addition to mucins, fish mucus contains proteins, salts and lipids. The latter are believed to provide protection against bacterial and fungal attack (Lewis, 1970). The majority of mucus proteins play an important role in the fish defense against pathogens (**Figure 2**) (Johansson et al., 2008; Brinchmann, 2016). The specific role for some of these proteins such as lysozymes, proteases, lectins, heat shock proteins, complement factors, antimicrobial peptides (AMPs), iron binding proteins, apolipoproteins A1, calmodulins and antibodies, will be briefly discussed in the following text.

Lysozymes are important enzymes of the innate immune system that possess bacteriolytic activity against Gram-positive and Gram-negative bacteria by hydrolysis of the cell wall peptidoglycan (Chipman et al., 1968). Lysozymes can also trigger the activation of the complement system, leading to an enhanced recruitment of antibodies and phagocytic cells in order to ensure effective elimination of infectious invaders. The activity of lysozyme can be influenced by multiple factors such as sex, age, size, season, temperature of the water,

pH, toxicants, infections and stress (Saurabh and Sahoo, 2008; Ángeles Esteban, 2012). Proteases are involved in fish resistance to infections with the potential to kill pathogens either directly through protein cleavage or to modify the mucus consistence in order to remove pathogens from the surface (Aranishi et al., 1998). Lectins are carbohydrate binding proteins, which have been shown to be produced in fish skin-mucus during infections (Easy and Ross, 2009; Provan et al., 2013). The antimicrobial activity of lectins is believed to be associated with their capacity to stimulate aggregation and precipitation of glyco-conjugates (Ángeles Esteban, 2012). For instance, mannose binding lectins (MBLs) and galectins (β-galactoside binding proteins) can bind to glycopeptides on the pathogen surfaces, activate the complement system and thereby mediate the killing of microbes (Rajan et al., 2013a; Rajan et al., 2013b; Stowell et al., 2014). Heat shock proteins, which are often detected in fish skin-mucus (Provan et al., 2013; Cordero et al., 2015; Jurado et al., 2015; Sanahuja and Ibarz, 2015), are believed to possess both immunostimulatory and immunosuppressive functions (Pockley et al., 2008). Complement factors are proteins primarily found in blood that represent an important part of the innate immune system due to their ability to enhance/complement an immune response (complement system). The activity of complement factors may result in bacterial lysis, chemotaxis of immune cells and phagocytosis. The complement factor C3 has been found in fish skin-mucus (Easy and Ross, 2009; Cordero et al., 2015; Sanahuja and Ibarz, 2015). AMPs derive either from precursor molecules encoded by dedicated genes or from proteolysis of larger proteins. It is believed that some AMPs kill the pathogenic cells by membrane permeabilization (Oren and Shai, 1998). An example of an AMP detected in fish is the histone H1 peptide, which was identified as the dominant antimicrobial peptide in the Atlantic salmon skin-mucus (Lüders et al., 2005). Iron binding proteins such as transferrin, which can limit bacterial growth by reducing the availability of iron in the environment, have also been found in Atlantic salmon skin-mucus (Easy and Ross, 2009). Apolipoprotein A1 was identified in fish mucus where it is believed to perform antimicrobial activities (Concha et al., 2003). Specifically, this protein was upregulated in the skin-mucus of sea lice infected Atlantic salmon (Easy and Ross, 2009) and *Vibrio anguillarum* infected Atlantic cod (Rajan et al., 2013b), highlighting its protective role. Calmodulin is a calcium dependent activator of various enzymes in eukaryotic cells and its presence in mucus suggests an enzymatic control of the integumental permeability (Flik et al., 1984). In addition, several antibodies have been identified in the mucus of teleost such as IgM, IgD and IgT/IgZ, whereof IgM is the predominant isotype (Hordvik et al., 1999; Salinas et al., 2011). Antibodies are responsible for the recognition of

specific molecules present on the microbes, called antigens. After specific binding of antibodies to antigens, components of the immune system are activated, leading to the elimination of potential pathogens through phagocytosis. Moreover, the DNA present in skin-mucus may be involved in trapping of pathogens (Brinkmann et al., 2004; Brinchmann, 2016). Proteins related to cellular function such as actin, collagen, ribosomal subunits and proteins related to metabolic pathways have been as well observed on the fish mucosal surface (Easy and Ross, 2009; Rajan et al., 2011; Jurado et al., 2015). It is not known whether such proteins originate from dead or living cells, or if they are truly secreted to fulfill specific biochemical functions. However, it has been shown that actin, which is involved in phagocytosis and cell motility, has antibacterial activity in insects (Sandiford et al., 2015).

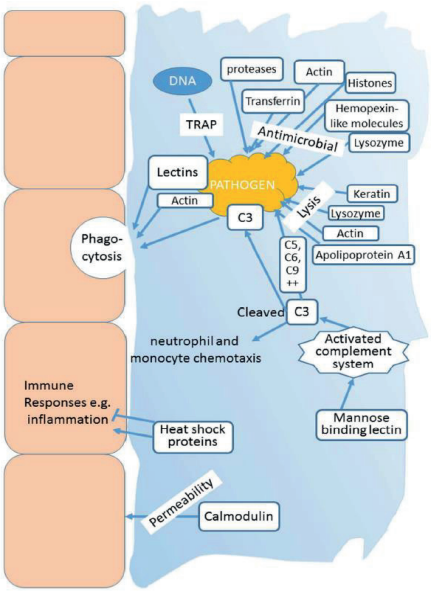

Figure 2. Immune relevant proteins identified in fish skin-mucus. Schematic representation of the fish skin showing epithelial cells as orange boxes and the mucus as a blue surface. The proteins associated to fish mucus that are thought to play an important role in the fish defense against pathogens (orange colored cloud-like shape) are indicated in white boxes. The figure is adapted from (Brinchmann, 2016).

1.2.2 The fish skin-mucus microbiome

The fish skin-mucus, as all the mucosal surfaces, is colonized by different bacterial species, which play an important role in the development of the fish immune system (Lee and

Mazmanian, 2010). The immune system is capable to recognize commensal from pathogenic bacteria, allowing them to live in the external mucosal surfaces. At the same time, commensal bacteria are provided with a rich source of nutrients in the form of mucins that promotes sustainability and reduces opportunism. Colonization of the mucosal surfaces by microorganisms also offer physiological, metabolic and immunological benefits to the host. For instance, microorganisms harvest nutrients from food, provide essential vitamins and produce biofilms, which hamper the entrance of pathogens (Maynard et al., 2012; Gomez et al., 2013). Potentially pathogenic bacteria are also part of the community living on the mucus and under particular conditions (discussed below) they can become infectious (Austin et al., 2012).

The evaluation of the composition and abundance associated with the fish-skin mucus microbiome is challenging due to issues related to sample contamination occurring either from the surrounding water (Minniti et al., 2017) or during the sampling procedures (Beck and Peatman, 2015). In addition, the microbial composition seems to be influenced by environmental variables such as season, salinity and temperature (Cahill, 1990). Variability has been reported as well among species, individuals and different body parts (Cahill, 1990; Beck and Peatman, 2015; Chiarello et al., 2015). Despite high variability at the genus level, *Proteobacteria* seems to be the most abundant phylum observed in the skin-mucus microbiome of fish (Llewellyn et al., 2014; Chiarello et al., 2015; Lowrey et al., 2015; Lokesh and Kiron, 2016; Kearns et al., 2017; Minniti et al., 2017).

Many recent studies on the fish skin-mucus microbiome have investigated the interaction existing between stress conditions and the host microbial community. The high demand of farmed fish on the food market have had a strong impact on the fish welfare in the last decade. Farmed fish are often exposed to conditions that are detrimental to the animal health (e.g. high stocking density, diet, feeding techniques, netting, sorting, transport), which cause a stress response (Ashley, 2007) that manifest itself in the form of changes in the fish microbiomes. In general, a well-regulated and stable microbiome is characterized by a high bacterial diversity and typically includes a relative low number of opportunists (**Figure 3**). This provides a strong resistance against colonization by potential pathogens, which therefore do not reach the dominance needed to develop infections. On the contrary, in a disrupted microbial community (e.g. caused by stress) the microbial diversity decrease promotes growth of pathogenic bacteria, which along with commensals, can cause

infections. Indeed, changes in the ecological factors can modify the existing relationship between commensals and hosts, evolving to a pathogenic relation with the host (Takle et al., 2015). A study by Boutin et al. showed that the microbiome present on the mucus of Brook charr (*Salvelinus fontinalis*), exposed to high density and hypoxia, was different compared to the one of healthy fish (Boutin et al., 2013). *Psichrobacter*, *Steroidobacter*, *Pseudomonas*, *Acinetobacter* and *Aeromonas* were mostly observed in stressed and dead fish, whereas *Sphingomonas*, *Methylobacterium*, *Propionibacter* and *Thiobacter* on healthy fish. Furthermore, Sylvain and colleagues showed that a drop in the pH influenced the structure of the microbiome associated with *Colossoma cropomum* (Tambiqui, a fresh-water fish native to tropical South America) (Sylvain et al., 2016). In this study, four potential stress specific taxonomic microbial biomarkers have been assessed: the class *Betaproteobacteria* and the genera *Flavobacterium*, *Duganella* and *Undibacterium*. The susceptibility of the skin-mucus microbiome to change upon stress events highlights the need of promoting a controlled bacterial community in (and on) farmed fish.

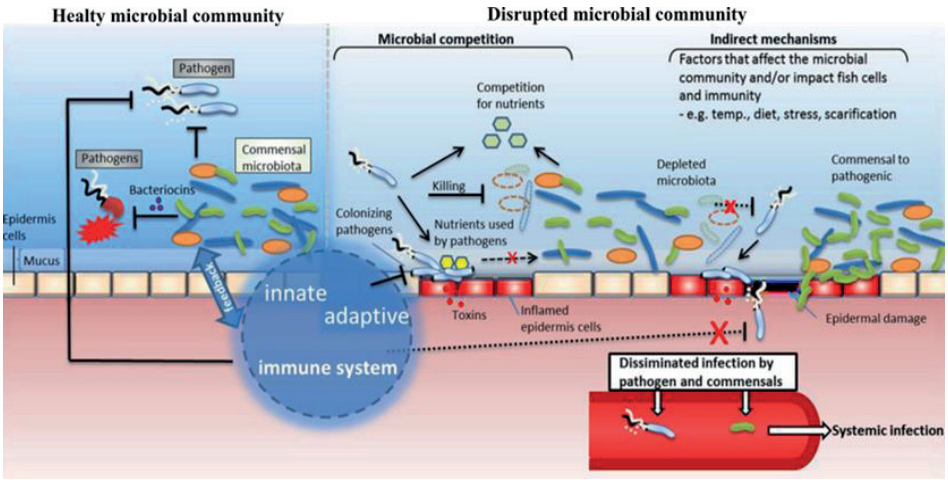

Figure 3. The role of the skin-mucus microbial community in healthy and diseased conditions. The figure is adapted from (Takle et al., 2015).

1.2.3 The study of complex microbial communities by omics-techniques

In the past, culture-dependent techniques such as sequential dilutions, used to reduce a dense cell culture to a more usable diluted concentration, in combination with spread-plating have mainly been used to characterize and classify the fish microbiomes. The cultivation of microorganisms depends strongly on the cultivation media, the incubation conditions and

the sampling time (hours or days can pass between the sample collection and examination). Indeed, it is well known, that such culture-dependent techniques do not allow sufficient detection of the whole microbial community, since the cultivation of some populations is simply not feasible under laboratory conditions (Austin, 2006).

In the last decades, high sensitive culture-independent molecular approaches have been developed for in-depth investigation of the host associated microbiome. The "human genome project" (Turnbaugh et al., 2007) has been the major driving force for the development of these technologies together with the dramatic decline of their relative cost (Muir et al., 2016). A brief explanation of these methods, some are schematically represented in **Figure 4**, is given below.

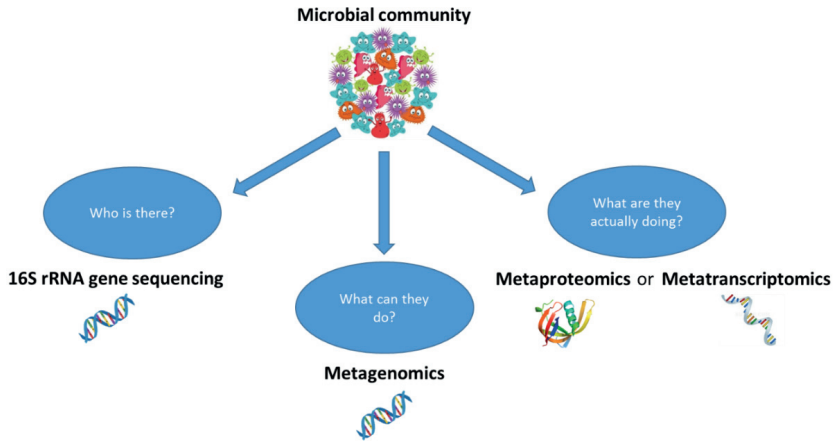

Figure 4. Overview of some of the multi-omics methods to analyze complex microbial communities. Each information level, based on DNA, RNA and proteins, provides a different level of characterization of the microbial community.

High throughput sequencing of conserved marker genes such as 16S rRNA has long been used to phylogenetically identify complex microbiomes (Weisburg et al., 1991). The higher sensitivity of this method in respect to culture-dependent techniques has clearly been demonstrated in a comparative study that investigated the intestinal microflora of rainbow trout (Spanggaard et al., 2000). Since then, 16S rRNA gene sequencing has often been used to characterize the microbiomes present on the skin-mucus and gut of different fish species in the last years (Askarian et al., 2012; Boutin et al., 2013; Larsen et al., 2013; Song et al., 2016; Karlsen et al., 2017; Minniti et al., 2017; Pimentel et al., 2017). While 16S rRNA gene

sequencing analysis can provide information regarding "who is there and "in which amount", metagenomic studies provide insight into the possible functions associated with the bacteria (Handelsman et al., 1998; Qin et al., 2010; Tarnecki et al., 2017). In metagenomics, the all genomes present in an environmental sample (e.g. fish mucosal surfaces) are randomly sequenced and analyzed, providing information about the potential metabolic functions of the community (Xing et al., 2013). In addition, metaproteomic and metatranscriptomic approaches provide even more complementary information about the "expressed" function within a community. Metaproteomic analysis allow the detection of expressed proteins and their abundances in the community (Wilmes and Bond, 2004; Wilmes et al., 2015). Metaproteomics in combination with 16S rRNA gene sequencing and metagenomic analysis (see Paper II and (Hagen et al., 2017)) represents a powerful tool that allows a more precise assessment of the expressed proteins and of the functions performed by the individual members of a community. Using bioinformatics, the different genomes of a microbiome can be reconstructed, taxonomically identified and annotated by the combination of 16S rRNA gene sequencing and metagenomics, providing a sample-specific database for the metaproteomic analysis that drastically improve the protein identification rate. Metatranscriptomics is based on the analysis of mRNA and thus provides information about the identity and abundance of functionally expressed genes within the whole community. Despite the similar informational output of metaproteomics and metatranscriptomics, a substantial difference exists. While metaproteomics defines specific cellular proteotypes, giving the actual functions of a community, the mRNA, which is used as molecular template for protein expression, is characterized by a very short lifespan and as so, provides a more accurate snapshot of the expression profile at a given time point. Additionally, microbial communities can be characterized by metabolomics, which is used to analyze the metabolites produced by the organisms (Aguiar-Pulido et al., 2016)

Although the combination of these different meta-omics based techniques represents a powerful tool for characterizing the members of a microbial community and determining their interactions and functions, these techniques are still prone to several problems. 16S rRNA genes sequencing analysis is influenced by PCR bias, reference database and short reads (microbial identification up to genus level) (Schloss et al., 2011; Klindworth et al., 2013). In addition, a variable number of 16S rRNA gene copies is present in the different bacterial species, causing either an over- or underestimation of the investigated microbial community. At the same time, metaproteomics is still facing issues mainly associated with

protein extraction from difficult matrices, sequence unavailability, large database and low sensitivity of search engine (Minniti et al., 2018).

1.3 Bacterial pathogens in aquaculture

Bacterial infections are the result of an interplay between the bacterium, the host and the environment. It is well known that pathogenic bacteria are capable to cause epidemics when a large number of population is susceptible to the pathogen and when hosts are exposed to pathogens for a sufficient time for the infection to occur. Secondary, the disease transmission takes place when susceptible hosts come into close distance and for a long enough period of time with the infected individuals (Scott, 1994). In aquaculture, fish live in close contact with each other and they are characterized by a low genetic diversity (Zeinab et al., 2014; Glover et al., 2017). These conditions make them more susceptible to diseases compared to wild fish. As a result, the use of vaccines against bacterial pathogens is a routine practice in aquaculture. Despite good control of bacterial diseases in the last years, microbial outbreaks have been still registered in aquaculture (Hjeltnes, 2014; Hjeltnes, 2017). It is difficult to assess the reasons associated with these outbreaks. Possible factors include the modality of vaccination (oral, immersion or injection) (Hjeltnes et al., 1989), the physiological state of the fish during vaccination and the environmental conditions (Kashulin et al., 2017). In addition, the lack of knowledge regarding the interaction existing between pathogens and the host makes even more complicated to understand the causes leading to these epidemics. Thus, an increase of the knowledge concerning microbial infections associated with farmed fish (**Table 1**) is fundamental for the identification of future preventive measures aimed to improve the fish welfare. In the current thesis we focus our attention on Cold Water Vibriosis (CWV), which is a well-known disease associated with farmed Atlantic salmon.

Table 1. Bacterial fish diseases affecting marine fish cultures. This table was adapted from (Toranzo et al., 2005).

Agent	Disease	Main marine hosts
Listonella anguillarum (formerly *Vibrio anguillarum*)	Vibriosis	Salmonids, turbot, seabass, striped bass, eel, ayu, cod, red seabream
Vibrio ordalii	Vibriosis	Salmonids
Vibrio salmonicida (currently *Aliivivrio salmonicida*)	Cold Water Vibriosis	Atlantic salmon, cod
Vibrio vulnificus	Vibriosis	Eels
Moritella viscosa (formerly Vibrio viscosus)	Winter ulcer	Atlantic salmon
Photobacterium damselae (formerly *Pasteurella piscicida*)	Photobacteriosis (Pasteurellosis)	Seabream, seabass, sole, striped bass, yellowtail
Pasteurella skyensis	Pasteurellosis	Atlantic salmon
Aeromonas salmonicida	Furunculosis	Salmonids turbot
Tenacibaculum maritimum (formerly Flexibacter maritimus)	Flexibacteriosis	Turbot, salmonids, sole, seabass, gilthead seabream, red seabream, flounder
Pseudomonas anguilliseptica	Winter disease	Seabream, eel, turbot, ayu
Lactococcus garvieae (formerly *Enterococcus seriolicida*)	Streptococcosis/lactococcosis	Yellowtail, eel
Streptococcus iniae	Streptococcosis	Yellowtail, flounder, seabass, barramundi
Streptococcus parauberis	Streptococcosis	Turbot
Streptococcus phocae	Streptococcosis	Atlantic salmon
Renibacterium salmoninarum	BKD	Salmonids
Mycobacterium marinum	Mycobacteriosis	Seabass, turbot, Atlantic salmon
Piscirickettsia salmonis	Piscirickettsiosis	Salmonids

1.3.1 Cold Water Vibriosis (Epidemiology, Symptoms and Pathogenesis)

CWV appeared for the first time in Norway in 1977. In 1979 and 1980, it caused one of largest loss of farmed Atlantic salmon recorded, around the Hitra Island, which is located south of Trondheim in Norway (Egidius et al., 1981; Holm, 1985; Evensen et al., 1991; Kashulin et al., 2017). In 1983, the disease appeared also in Stavanger and in particular, a high number of fish farms in the region of Bergen have been infected (Austin et al., 2012). CWV has also been registered in Scotland, Iceland, Faroe Island, Canada and USA (Egidius et al., 1981; Dalsgaard et al., 1988; O'Halloran et al., 1993; Hastefnl et al., 2005). Moreover, this disease has also been detected in rainbow trout (*Oncorhyncus mykiss*) and Atlantic cod (*Gadusmorhua*), but they are less susceptible than Atlantic salmon (Egidius et al., 1986; Jørgensen et al., 1989; Schrøder et al., 1992; Hastefnl et al., 2005). CWV occurs mainly during the late autumn to early spring, when the water temperature is below 10°C (Egidius et al., 1986). In addition, the disease is often associated with stress events caused by moving, marking, sorting or feed changes, and it seems to target the fast growing and fattest fish (Poppe et al., 1983). During the first stages of the disease, fish usually display problems in swimming, tend to stay close to the surface, appear lethargic, stop eating and exhibit hemorrhages on the skin surface, especially around the abdomen, fin basis and anal region (**Figure 5**). In addition, the skin appears dark colored. Internally, there are evidences of anemia and extensive hemorrhages are detected in the organs (Bruno, 1996).

Figure 5. External signs of *Aliivibrio salmonicida* in Atlantic salmon. This figure shows the typical hemorrhages observed on the ventral surface of the skin of diseased salmon. The picture has been adapted from (Bruno, 1996).

The skin, gills and the gastrointestinal tract have been suggested as the port of entry of *Aliivibrio salmonicida* (Hjeltnes et al., 1987; Hoff, 1989a; Onarheim et al., 1994; Bjelland et al., 2012a). However, recently it was established that the skin seems to be the main portal of CWV infection (Kashulin and Sørum, 2014). Furthermore, the same study indicated that the initial stage of infection occurs in a short invasion time (3 minutes) and the bacterium entrance rate was up to 1 × 106 CFU × ml^{-1} of blood after the 3 minutes of exposure. Thus,

it seems that the bacterium from the skin rapidly enters into the blood system of Atlantic salmon. In a previous challenging experiment, the structural changes caused by CWV in Atlantic salmon have been described by using light and electron microscopy (Totland et al., 1988). Initially the bacterium was found inside the vascular system but there was no sign of structural cell damage. The first target of the bacterium seemed to be the endothelium cell of capillaries and blood cells. Those cells appeared to be disintegrated by the pathogen with the development of the diseases. The tissue with the richest blood supply like heart, red muscles fibres and the lamina propria of the intestine were the most damaged tissues of the fish.

CWV is transmitted horizontally through the fish population (Bruno, 1996) between salmonids in the same farm or neighboring locations. Transmission between salmon and cod has been also described (Sørum et al., 1990). The origin of CWV may be correlated with the presence of *Aliivibrio salmonicida* in the water (Bruno, 1996), which may be transferred by water currents (Enger et al., 1991). *Aliivibrio salmonicida,* is believed to have a free-living and a facultative pathogenic phase (Reidl and Klose, 2002). Indeed, the bacterium is able to live in unsupplemented water for more than 1 year (Hoff, 1989b). Analysis of the total bacterium count in the water of the fish farming surrounding, showed seasonal variation between 4×10^4 and 9×10^5 bacteria/ml, and the lowest concentration was observed in the winter. However, despite its presence during all the year, fish susceptibility occurs only at low temperature, which may be associated with stress and a weaker immune defense at temperatures below 10°C (Enger et al., 1991). In addition, it has been suggested that the bacterium harbors an asymptomatic carrier stage, where healthy individuals carry *Aliivibrio salmonicida* without having any symptoms of disease. This could explain as well the presence of the bacterium in the farm surrounding water during all the year (Enger et al., 1991). Finally, the origin of this infection has been associated with wild fish. Bruno et al., suggested that the pathogen may have appeared from wild fish cohabiting with the salmon in the sea cages or derived from the feeding of unpasteurized wild fish (Bruno, 1996).

1.3.2 Aliivibrio salmonicida

The genus *Vibrio* constitutes a large group of gram-negative bacteria, which mainly live in freshwater, seawater and sediment (Colwell and Grimes, 1984), although they have been recovered from the surface of freshwater and seawater fish (Austin, 2006). The majority of *Vibrio* sp. are not pathogenic, many are commensal or mutualistic and some have beneficial

functions. For instance, *Vibrio fischeri* provides squids with biolumination through a symbiotic relationship (Jones and Nishiguchi, 2004). However, this genus is as well associated with pathogenesis in humans (West, 1989; Spagnuolo et al., 2011; Ramamurthy et al., 2014; Phillips and Satchell, 2017) and animals like fish (Benediktsdottir et al., 1998; Strom and Paranjpye, 2000; Frans et al., 2011). The most known *Vibrio* pathogens in fish are *Vibrio anguillorum* (*Listonella anguillarum*), *Vibrio salmonicida* (*Allivibrio salmonicida*), *Vibrio ordalii*, *Vibrio harvei*, *Vibrio damsela* and *Vibrio vulnificus* (Jun and Woo, 2003). In December 2007, the genus *Vibrio* was split into two different genera and *Vibrio salmonicida* was transferred to the genus *Aliivibrio* (Urbanczyk et al., 2007).

Aliivibrio salmonicida is a curved rod, slightly halophilic and facultative anaerobic bacterium (**Figure 6**). The optimal growth salinity is at 1.5% NaCl and the optimal growth temperature at 15°C (Egidius et al., 1986). Specifically, the optimal growth temperature is observed at 15°C on solid media and 10°C in liquid media (Colquhoun et al., 2002). Analysis of 341 isolates of *Aliivibrio salmonicida* from CWV outbreaks in Norway demonstrated that all the microbes contain plasmids and the eleven identified plasmids had the same profile over the 6 years of sampling (Sørum et al., 1988). However, none of these plasmids seem to be related to virulence (Wiik et al., 1989). The genome of *Aliivibrio salmonicida* strain LFI1238 with a size of 4.6 Mb was sequenced in 2008. The genome comprises two chromosomes: chromosome I encodes the essential genes, while chromosome II encodes the accessory genes. Additionally, four plasmids have been observed. In total 4286 predicted proteins were identified in *Aliivibrio salmonicida* (Hjerde et al., 2008).

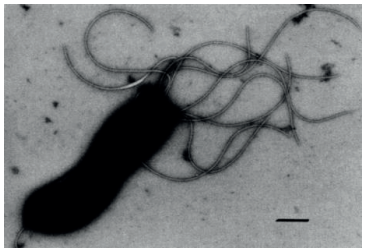

Figure 6. Scanning electron microscopy of *Aliivibrio salmonicida*. The image is adapted from (Egidius et al., 1986).

1.3.3 *Aliivibrio salmonicida* virulence factors

Despite the decades of experimentation with *Aliivibrio salmonicida*, its virulence factors are largely unknown. In this section, the available data related to the bacterial virulence are briefly discussed. *Aliivibrio salmonicida* seems to be incapable of producing capsules in vivo

(Colquhoun and Sørum, 1998). It is a poor producer of proteases and hemolysine, and it seems not to release extracellular toxins involved in virulence (Holm et al., 1985; Toranzo and Barja, 1993; Bjelland et al., 2012a). The antigen surface protein (VS-P1) of *Aliivibrio salmonicida* has been suggested to mediate resistance against the immune system. These molecules are expressed on the bacterial surface as well as released into the tissues. Probably, the microorganisms defend themselves from the immune system by their liberation that acts as a decoy (Espelid et al., 1987). Bacterial virulence has also been associated with a mutation in the lux operon (Nelson et al., 2007), which usually encodes genes for the production of luminescent proteins. *Aliivibrio salmonicida* is a cryptic bioluminescent (Fidopiastis et al., 1999) that harbors the genes for luciferase, even though they do not produce detectible light. Thus, it is speculated, that their presence might be related to pathogenesis. Furthermore, temperature-dependent iron sequestration was suggested to play a key role in the pathogenicity of the bacterium (Winkelmann et al., 2002). Several genes for the production of siderophores, three TonB systems and one heme system are annotated in its genome (Hjerde et al., 2008). Production of the major siderophores was observed at low temperature (below 10°C), confirming their potential association with bacterial virulence (Winkelmann et al., 2002). Also quorum sensing (QS), motility and production of hydrogen peroxidases are considered as possible virulence factors (Bjelland et al., 2012a): For instance, it is believed that QS plays an important role in the adaptation from a stationary stage (biofilm) to a planktonic living mood that make it suitable for causing infections (Bjelland et al., 2012b). In addition, it has been shown that the virulence of the bacterium is dependent on motility during the invasion stage. On the contrary, motility is not essential after the invasion, although the absence of normal flagellation delayed the disease development (Bjelland et al., 2012a; Nørstebø et al., 2017). The expression of flagellin increases in response of fish mucus (Colquhoun and Sørum, 1998) and is correlated with salinity and temperature (Karlsen et al., 2008). Moreover, *Aliivibrio salmonicida* seems to mute the general expression of its genes to suppress the fish immune system during the first stages of infection (Bjelland et al., 2012a). Alternatively, it is believed that the bacterium modify its microbe associated molecular patterns (MAMPs) to be ignored by the Toll-like receptors (TLRs), which play a key role in the host innate immune system (Ausubel, 2005; Rumbo et al., 2006; Kashulin et al., 2017). Finally, as it was mentioned before, Atlantic salmon (*Salmo salar*) is more susceptible to CWV compared to rainbow trout (*Oncorhynchus mykiss*) (Egidius et al., 1986). The reasons for these diverse susceptibilities associated with the two fish species are poorly known. However, it may be correlated with the presence of chitin in the scales of

Salmo salar (Wagner et al., 1993; Tang et al., 2015; Kashulin et al., 2017). Indeed, genes involved in chitin degradation are conserved among the family *Vibrionaceae* (Hunt et al., 2008). Thus, it is justified to speculate that chitinases may represent another potential virulent factor associated with *Aliivibrio salmonicida*.

1.4 Chitin degradation by bacteria

Degradation of chitin by bacteria has been investigated since the early 1930s. The first chitin degrading bacterium, isolated from infected lobster shells, was called *Chitinovorus bacterium*, which was later renamed as *Vibrio parahemolyticus* (Benton, 1935; Cobb and Phillips, 2012). Since then, microbial chitin degradation has intensively been studied, highlighting its occurrence in both aquatic and terrestrial environments (Keyhani and Roseman, 1999; Kielak et al., 2013; Bai et al., 2016). The most common chitin degrading bacteria belong to the genera *Aeromonas*, *Actinomycetes*, *Enterobacter*, *Serratia*, *Bacillus*, *Erwinia* and *Vibrio* (Gooday, 1990a; Brzezinska et al., 2014). These bacteria produce a vast array of enzymes, collectivity termed as chitinases. These enzymes are able to degrade chitin selectively into its smallest building blocks, the monomeric sugar GlcNAc, which is in turn used as an energy source by the bacterium. Chitinases act independently from each other, but in a mixture they may have complementary and synergistic modes of actions.

1.4.1 Chitin

Chitin is the second most abundant polysaccharide in nature and consists exclusively of β-1,4 linked GlcNAc units (**Figure 7**; panel A). According to the orientation of the adjacent chitin chains, chitin can be classified into three different allomorphs: α-, β- and γ-chitin (**Figure 7**; panel B) (Rudall, 1963; Rinaudo, 2006). In α-chitin, the linear polysaccharide chains are arranged anti-parallel to each other and are held together via inter- and intra-chain hydrogen bonds, resulting in a densely packed material. The β-allomorph is characterized by a parallel arrangement of chains, but is in contrast to α-chitin, less densely packed due to a low number of intramolecular hydrogen bonds. Finally, γ-chitin is a mixture of parallel and antiparallel arrangements of the chitin chains.

Chitin serves as a structural element in many organisms (Gooday, 1990a; b) and additionally, its robust structure offers protection towards external agents (Gardner and Blackwell, 1975; Gooday, 1990b). Most of chitin is found as α-chitin in the exoskeletons of marine

crustaceans, insects and arachnids, in the shells, radulae and cuttlebone of molluscs, in the calyx of hydrozoa, in the cell walls of protists and in the inner layer of fungal cells walls (Gooday, 1990b; Rinaudo, 2006; Bai et al., 2016). β-chitin is found in the shell of squid pen, in the tubes of some annelids and in the outer protective lorica of some protists (Gupta, 2010), while γ-chitin is found in the stomach of the squid Loligo and in the cocoon fibers of *Ptinusbeetle* (Jang et al., 2004). Previously, it was believed that chitin was absent in vertebrates, although more recently it was identified in fish. Specifically, chitin was detected in the epithelium of fish scales, in the gut of zebrafish and in the appendages of salamander larvae (Wagner et al., 1993; Tang et al., 2015). The presence of chitin on the fish surface may be correlated with a protective function.

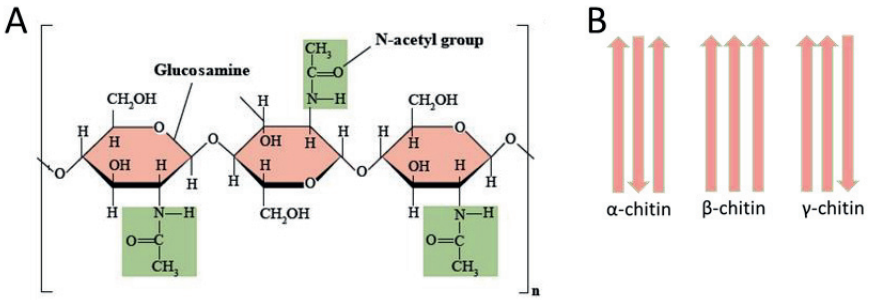

Figure 7. Structure and organization of chitin. Panel A shows the chemical structure of a chitin single chain made of β-1,4 linked GlcNAc units, while panel B displays the three different polymorphic forms of chitin.

1.4.2 Chitinases

Chitinases are glycoside hydrolases (GHs) that are capable of cleaving the β-1,4 glycosidic bonds in chitin chains via a hydrolytic mechanism, leading to the generation of soluble oligomeric products. Based on their amino acid sequence, chitinases are classified in the Carbohydrate Active Enzyme (CAZy) database (Cantarel et al., 2008) into two families, GH18 and GH19. Since the latter seems to not be involved in bacterial virulence, this section will focus only on the family GH18. In terms of structure, GH18s consist of alternating α-helices and β-sheets, forming a $(\beta/\alpha)_8$ barrel structure (Perrakis et al., 1994) also known as a TIM-barrel, named after the first protein solved with this fold; triosephosphate isomerase (**Figure 8;** panel A). The active site is located in the loop connecting the β-sheet 4 and α-helix 4, harboring the characteristic sequence motif DxxDxDxE, where the glutamate (E)

represents the catalytic acid/base (**Figure 8**; panel B and C**)**. Catalysis of chitin hydrolysis by GH18 chitinases proceeds through a mechanism that invokes substrate assistance (**Figure 9**). In short, protonation of the interglycosidic oxygen of the substrate by the catalytic acid, and concomitant nucleophilic attack of the N-acetyl group on the anomeric carbon (i.e. substrate assistance) leads to the formation of an oxazolinium intermediate and departure of the leaving group. Then, the catalytic acid acts as a general base, abstracting a proton from a water molecule, which performs a nucleophilic attack on the anomeric carbon, completing thus the reaction mechanism, and resulting in a retained stereochemistry of the anomeric carbon (Vaaje-Kolstad et al., 2013).

Chitinases can be classified into exo- (exo-chitinase) or endo- (endo-chitinase) acting enzymes based on their ability to catalyze consecutive reactions without releasing the substrate, a property known as processivity. While exo-chitinases processively cleave off chitobiose units $(GlcNAc)_2$ from the reducing or non-reducing chain ends of chitin chains, endo-chitinases cleave the glycosidic bonds at random positions within a chitin chain, resulting in the production of mainly $(GlcNAc)_2$ and chitotriose $(GlcNAc)_3$ (Gooday, 1990b; Horn et al., 2006). Structural variations in the substrate binding clefts are observed when comparing endo-chitinases and exo-chitinases (**Figure 8**; panel D): Exo-chitinases are characterized by a deep substrate binding cleft, which seems to be correlated with the presence of a conserved insertion domain (CID) in the barrel structure (**Figure 8**; panels C and D; red colored structural motif), while the endo-chitinases assume a shallow substrate-binding cleft (Horn et al., 2006). In addition to the catalytic domain, bacterial chitinases often contain accessory domains known as carbohydrate-binding modules (CBMs) (**Figure 8**; panels C and D; part of the structures colored in blue). The role of CBMs is to recognize the target substrate and to improve the enzymatic activity towards chitin by anchoring the enzymes to the substrates, which may potentiate catalysis (Boraston et al., 2004; Guillén et al., 2010).

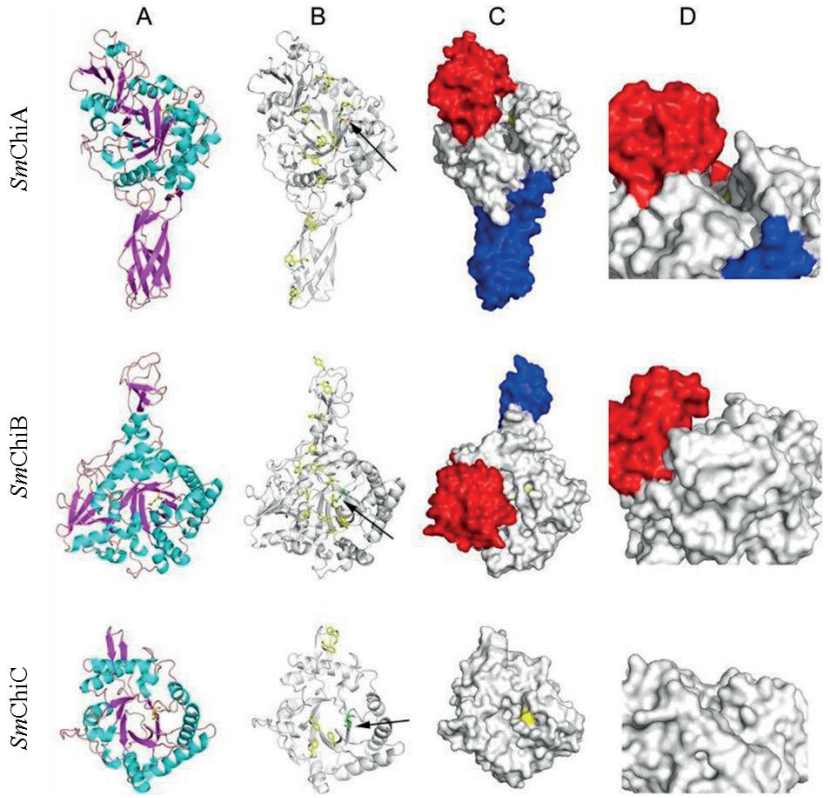

Figure 8. Structures of GH18 chitinases from *Serratia marcescens*. The chitinolytic system of the soil bacterium *Serratia marcescens* contains three GH18 chitinases: the exo-chitinases *Sm*ChiA (PDB ID 1CTN) and *Sm*ChiB (PDB ID 1E15) and the endo-chitinase *Sm*ChiC (PDB ID 4AXN). Panel A shows the TIM-barrel fold typical for GH18s. Panel B highlights the exposed aromatic amino acids in the substrate binding region (yellow) and the catalytic glutamic acid (green) indicated by an arrow. Panel C shows a surface representation of chitinases, where the CID (red), the CBM (blue) and the catalytic glutamic acid (yellow) are visualized. Panel D shows the substrate binding clefts of the different chitinases demonstrating the deep cleft of the exo-chitinases (*Sm*ChiA and -B) and the shallow cleft of the endo-chitinase (*Sm*ChiC). Figures were made using PyMol (DeLano and Lam, 2005).

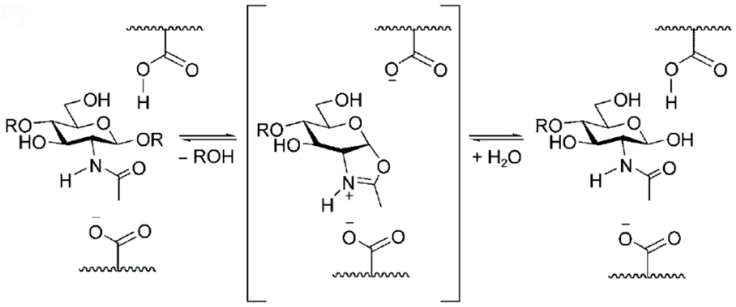

Figure 9. Substrate-assisted mechanisms used by GH18 chitinases. The top amino acid represents the catalytic acid which acts alternatively as a general acid (protonation of the interglycosidic oxygen) and as general base (activation of a water molecule by proton abstraction). The bottom amino acid aids the reaction by stabilizing the oxazolinium intermediate. The "R" represents β-1,4 linked GlcNAc residues. The figure is adapted from (Macdonald et al., 2010).

1.4.3 Lytic polysaccharide monooxygenases (LPMOs)

LPMOs are copper dependent redox-enzymes that cleave glycosidic bonds in the crystalline regions of polysaccharides by an oxidative mechanism (Vaaje-Kolstad et al., 2010; Phillips et al., 2011; Quinlan et al., 2011). The activity of these enzymes was first shown on β-chitin (Vaaje-Kolstad et al., 2010), later to be followed by α-chitin (Vaaje-Kolstad et al., 2012), cellulose (Forsberg et al., 2011), hemicelluloses containing a backbone of β-1,4 linked glucose units (Agger et al., 2014), starch (Vu et al., 2014) and xylan (Frommhagen et al., 2015). Some LPMOs are also able to cleave soluble, short substrates like e.g. cellooligosaccharides (Isaksen et al., 2014). The reaction carried out by LPMOs was initially proposed to involve two electrons (provided by an external electron donor, e.g. a reducing agent), two protons and dioxygen, harnessed by the copper-bound enzyme to yield cleavage of the glycosidic bond through oxidation of the C1 carbon (**Figure 10**) (Vaaje-Kolstad et al., 2010; Phillips et al., 2011; Quinlan et al., 2011). The inclusion of one oxygen atom in the oxidized product indicated that the enzyme activity could be characterized as a monooxygenase, hence the name lytic polysaccharide monooxygenase. However, in a recent study, the role of O_2 in the reaction was questioned, suggesting that rather hydrogen peroxide (H_2O_2) is the co-substrate of LPMOs (Bissaro et al., 2017). Initially, it was thought that oxidation of polysaccharides by LPMOs was restricted to the C1 carbon, but subsequent studies on cellulose revealed that some LPMOs also are able to oxidize the C4 carbon or

both (Beeson et al., 2012; Forsberg et al., 2014; Isaksen et al., 2014). For chitin-active LPMOs only C1 oxidation has been demonstrated.

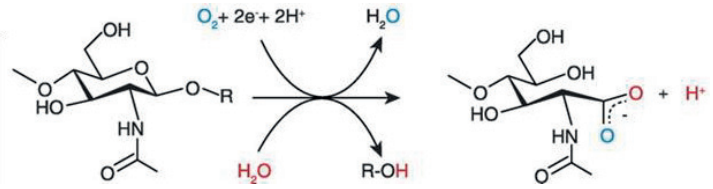

Figure 10. Oxidative cleavage of chitin by LPMOs. The oxidized product resulting from LPMO catalyzed cleavage of chitin is an aldonic acid (right). The figure is adapted from (Vaaje-Kolstad et al., 2010).

The activity of LPMOs is dependent on a copper ion (Cu^{2+} in resting state) present in the active site, coordinated by two conserved histidine residues and the N-terminal amino group (NH_2) of one of these histidines (**Figure 11**). By the recruitment of one electron, the LPMO is reduced from the Cu^{2+} state into the catalytically active Cu^{1+} state, which enables initiation of the catalytic cycle through activation of O_2 or H_2O_2. Although the details of the catalytic cycle have been discussed and hypothesized by several (Beeson et al., 2015; Walton and Davies, 2016; Bissaro et al., 2017), many aspects of the reaction mechanism remains unclear. Nevertheless, it is general consensus that LPMOs utilize a reactive oxygen species to mediate cleavage of the C-H bond of either the C1 or the C4 carbon of the glycosidic bond, followed by hydroxylation of the same carbon that ultimately leads to cleavage of the glycosidic bond itself. By oxidation of the C1 carbon, the resulting product is a 1,5-δ-lactone that spontaneously hydrates to an aldonic acid, whereas oxidation of the C4 carbon yields a ketoaldose that can hydrate to a geminal diol (**Figure 12**).

LPMOs were originally classified as carbohydrate binding modules (CBM33) or glycoside hydrolases (GH61), but after the discovery of their enzyme activity, they were reclassified into the auxiliary activity (AA) families of the CAZy database (Levasseur et al., 2013). At the writing of this thesis, five families of LPMOs exist: AA9, AA10, AA11, AA13 and AA14. Families AA9, AA11, AA13 and AA14 contain mainly fungal LPMOs, while family AA10 is found in all domains of life (archaea, bacteria and eukaryota). The latter family also has representatives in viruses that target insects (baculovirus and entomopoxvirus).

Despite the large diversity of the LPMOs, all three dimensional structures so far solved (more than 30) show that these enzymes share an immunoglobin-like core, consisting of antiparallel β-strands, which are organized in a β-sandwich (**Figure 11**) (Vaaje-Kolstad et al., 2005b; Li et al., 2012; Wu et al., 2013; Hemsworth et al., 2014; Leggio et al., 2015). The β-strands are linked to each other by loops, which often contain a varying number of short α-helices that are involved in forming the flat substrate-binding surface which also contains the active site. The relatively flat substrate-binding surface is believed to enable the enzyme to act on the crystalline substrates (Horn et al., 2012).

Chitin active LPMOs are so far found in AA10 and AA11 families. The capability of these enzymes to oxidize crystalline chitin manifest itself in the form of yielding a more accessible substrate to the chitinases, speeding up the overall degradation process. Indeed, a clear synergistic effect, meaning that the combination of two enzymes results in a higher activity than the sum of actions by the two individual enzymes, has been observed between LPMOs and GH18s (Vaaje-Kolstad et al., 2010; Vaaje-Kolstad et al., 2012; Nakagawa et al., 2015).

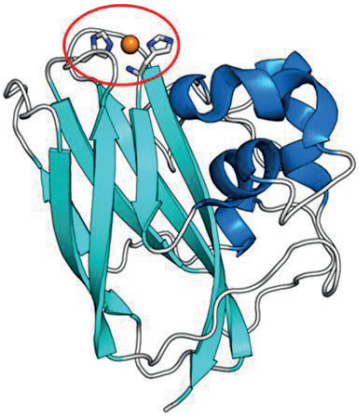

Figure 11. Tertiary structure of *Sm*CBP21 (PDB ID 2BEM). The copper (golden sphere), the two conserved histidine residues and the N-terminal amino group of the N-terminal histidine, constituting the catalytic center, are outlined by a red circle. The figure was made using PyMol (DeLano and Lam, 2005).

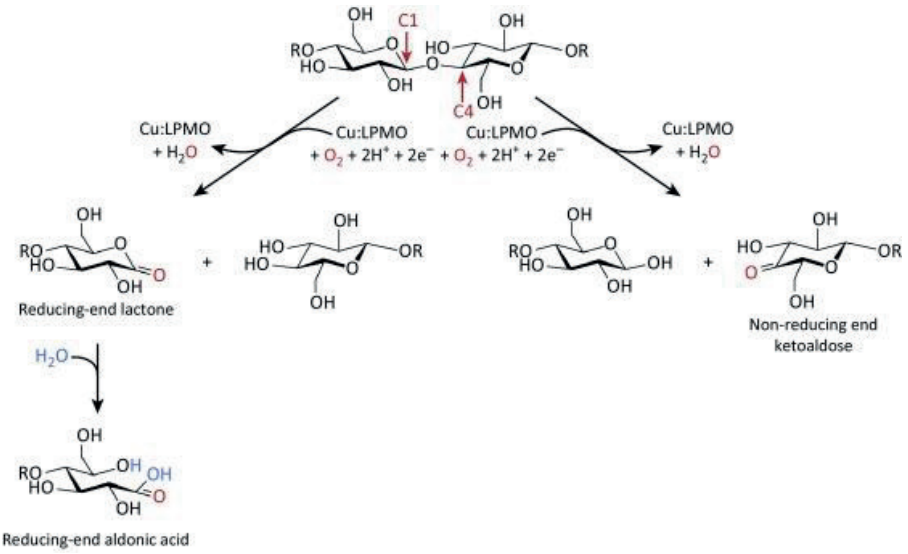

Figure 12. LPMOs oxidation within a polysaccharide chain. Oxidation at C1 results in the formation of a lactone, which is hydrated to become a reducing-end aldonic acid. C4 oxidation leads to the formation of a ketoaldose at the non-reducing end. The figure is adapted from (Hemsworth et al., 2015)

1.4.4 Chitinolytic systems

One of the most studied chitinolytic machineries is that of the soil bacterium *Serratia marcescens* (**Figure 13**). It consists of two exo-chitinases (*Sm*ChiA, *Sm*ChiB), one endo-chitinase (*Sm*ChiC), one lytic polysaccharide monooxygenase (CBP21; see section 1.4.3 for more details) and one *N*-acetylhexosaminidase (also known as "chitobiase"). The latter converts the $(GlcNAc)_2$ (and also longer chitooligosaccharides) released from the chitinases to the monomeric sugar GlcNAc (Vaaje-Kolstad et al., 2013). The exo-chitinases exhibit, beside their catalytic domain, an additional module, an N-terminal Fibronectin type-III-like module (such as in *Sm*ChiA) or a CBM5 chitin binding module (such as in *Sm*ChiB), which facilitate substrate binding (Van Aalten et al., 2000; Uchiyama et al., 2001; Vaaje-Kolstad et al., 2013). The genome of *Serratia marcescens* also encodes a fourth GH18 chitinase, known as *Sm*ChiD, whose expression is not induced by chitin and most likely has a function that is not related to chitin degradation (Tuveng et al., 2017). Moreover, a protein known as CBP21 ("CBP" stands for "chitin binding protein") is also part of the chitinolytic system. The protein was discovered in 2005 and was shown to assist GHs in their degradation of chitin. Initially, it was believed that this protein did not exhibit enzymatic activity *per se*, but

was rather causing structural changes on the surface of crystalline chitin, leading to an increase in its accessibility to GHs (Vaaje-Kolstad et al., 2005a). However, in 2010 it was shown that this protein is an LPMO (Vaaje-Kolstad et al., 2010).

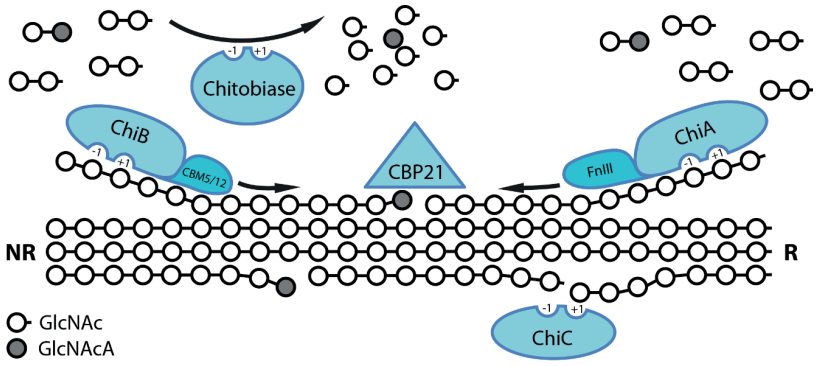

Figure 13. The chitinolytic machinery of *Serratia marcescens*. *Sm*ChiC (named "ChiC" in the figure) is an endo-chitinase, which generates random cuts within the chains in amorphous (non-crystalline) regions of chitin, thereby forming new chains ends for the exo-chitinases *Sm*ChiA and *Sm*ChiB. These exo-chitinases degrade chitin from the reducing (labelled as R) and non-reducing (labelled as NR) chain ends, respectively, producing mainly $(GlcNAc)_2$. CBP21, a chitin-active LPMO, introduces cuts in the highly ordered crystalline regions of chitin via an oxidative mechanism, producing aldonic acids and new chain ends for the exo-chitinases. Finally, native and oxidized chitobiose is converted to their monomers, native GlcNAc or oxidized *N*-acetylglucosamine (GlcNAc1A) by the action of chitobiase (Vaaje-Kolstad et al., 2013).

In nature, microorganisms have evolved different strategies to utilize and degrade chitin. For instance, a substantial difference has been observed between aquatic and terrestrial bacteria, which may be correlated with an adaptation to the prevalent resources present in the surrounding environment (Bai et al., 2016). In the latter study, a higher number of genes encoding for chitinases and LPMOs, along with a higher diversity of associated CBMs was observed in terrestrial bacteria compared to the aquatic ones. However, these differences may be due to a bias in the availability of annotated bacterial genomes. Indeed, it is well known that the chitin utilization pathway of marine bacteria such as *Vibrio* sp. is complex and involves a high number of characterized and uncharacterized enzymes, chitin binding proteins and transport proteins (Hunt et al., 2008; Jung et al., 2008; Aunkham et al., 2018).

1.4.5 Chitin degrading enzymes as virulence factors

Many studies in literature describe the secretion of LPMOs (called "CBPs" before 2010) and chitinases in relation to infection. For instance, several works reported the contribution of bacterial chitinases to pathogenesis in mammals, which are known to not produce chitin (Joshi et al., 2005; DebRoy et al., 2006; Chaudhuri et al., 2010; Mondal et al., 2014). Proteins that have evolved new roles without losing their original function are called "moonlighting proteins" and are common in many organisms including plants, animals, fungi and bacteria (Huberts and van der Klei, 2010). For instance, Mondal and colleagues showed that an extracellular chitinase from *Vibrio cholerae* called ChiA2, which is essential for chitin degradation in the aquatic stage of the bacterium, is involved in pathogenesis during host infection (Mondal et al., 2014). Specifically, ChiA2 can degrade intestinal mucins to release GlcNAc that is probably utilized in turn as a nutrient source. Furthermore, this enzyme seems to be able to decrease the viscous property of intestinal mucus to promote the infection. In another study, Kirn and colleagues demonstrated that the *Vibrio cholerae* protein called GbpA, which is a multi-domain LPMO that can oxidatively cleave chitin (Loose et al., 2014), enhances bacterial colonization of the host intestine through mucin binding (Kirn et al., 2005). The moonlighting property as chitin degrader and virulence factors in mammals has also been shown for an LPMO and two chitinases from the pathogenic bacteria *Listeria monocytogenes* (Chaudhuri et al., 2010). All the chitinases and LPMOs indicated as virulence factors in mammalian pathogens are summarized in **Table 2** and many of these enzymes seem to have a common affinity or activity towards mucin.

Most pathogens attacking Atlantic salmon infect the fish by penetration of the skin-mucus barrier. Not much is known about the infective mechanisms used by these pathogens, but it is well established that infection of healthy fish requires the bacterium to pass through the skin-mucus layer. As mentioned previously, the fish skin-mucus inhibits invasion and proliferation of most pathogenic microorganisms and prevents colonization of the epidermis (Shephard, 1994). Indeed, in challenge experiments with bacteria, removal of mucus/epidermal cells increased the cumulative mortality in salmonids, compared to undamaged fish (Svendsen and Bogwald, 1997; Madetoja et al., 2000). Thus, successful pathogens seem to have developed enzymatic tools that allow them to infect salmon via the skin. Such pathogens also include *Moritella viscosa* and *Vibrio anguillarum*. These bacteria are documented to adhere to the Atlantic salmon and rainbow trout skin and to have the ability to degrade salmon mucin (Weber et al., 2010). In addition, *Aeromonas salmonicida*

has been subjected to a comprehensive secretome analysis (virulent vs. non-virulent) and this study identified all chitinases and the LPMO as potential virulence factors (Vanden Bergh et al., 2013). Interestingly, all pathogenic bacteria reported as important threats to Atlantic salmon contain a chitinase, an LPMO, or both in their genomes (**Table 3**).

Aliivibrio salmonicida is a remarkably interesting fish pathogen. The sequence of its genome revealed evidence of extensive gene decay and the most affected pathway was the chitin metabolism, where ~50% of its genes were disrupted (Hjerde et al., 2008). Intriguingly, genes representing one GH18 chitinase and two LPMOs remain intact in the genome, indicating a function other than that of chitin deconstruction. Thus, since many of the virulence related chitinases and LPMOs from mammalian pathogens show affinity or activity towards mucins, it may also be that the activity of chitinases and LPMO in fish pathogens as *Aliivibrio salmonicida* is related to mucin containing substrates.

Table 2. Examples of pathogenic bacteria harboring genes representing chitinases (GH18s) or LPMOs (AA10s) in their genomes (the number of genes are indicated). Observations relating these proteins to virulence is indicated in the included references.

Bacterium	GH18	LPMO	Observations, proposed role and references
Pseudomonas aeruginosa	1	1	Heavily secreted upon infection. The bacterium cannot metabolize chitin. LPMO suggested to aid in adhesion (Folders et al., 2000; Hanna et al., 2000; Wagner et al., 2003; Sriramulu et al., 2005; Manos et al., 2009). Chitinase induced by mucus (Funget al., 2010).
Vibrio cholerae	4	2	Protein adheres to cell surfaces containing mucin; promotes bacterial adhesion. Suggested to mediate bacterial adhesion to cell surfaces (Kirn et al., 2005; Bhowmick et al., 2008; Jude et al., 2009; Wong et al., 2012).
Yersinia enterocolitica	0	1	LPMO is secreted by type II secretion system. Suggested to be involved in cell adhesion, pathogenicity (Shutinoski et al., 2010).
Listeria monocytogenes	2	1	LPMO enables virulence via bloodstream infection. Proposed to aid infection, host cell surface alteration (Chaudhuri et al., 2010). Chitinase suggested to suppress host immune system (Chaudhuri et al., 2013).
Enterococcus faecalis	3	1	Upregulated when bacterium grows in virulence inducing conditions (blood and urine), but function is unknown (Vebo et al., 2009; Vebo et al., 2010).
Legionella pneumophila	2	1	Chitinases are secreted by type II secretion system and promote bacterial persistence in lungs (DebRoy et al., 2006).
Escherichia coli	1	0	Chitinase mediates binding of bacterium to epithelial cells (Tran et al., 2011).

Table 3. Examples of Atlantic salmon potential pathogenic bacteria harboring genes representing GH18 chitinases or AA10 LPMOs in their genomes (the number of genes are indicated). Observations relating these proteins to virulence are indicated, including their references.

Bacterium	GH18	LPMO	Observations, proposed role and references
Aliivibrio salmonicida	1	2	Identified by genome sequencing, function unknown. Virulent strain cannot metabolize chitin (Hjerde et al., 2008).
Aeromonas salmonicida	1	1	All chitinases and the LPMO were detected as major secreted proteins from a virulent strain and suggested to be virulence factors (Vanden Bergh et al., 2013).
Vibrio anguillarum	5	1	Identified by genome sequencing, chitinases are secreted (Takiguchi and Shimahara, 1988).
Moritella viscosa	0	1	Identified by genome sequencing, function unknown
Aliivibrio wodanis	2	1	Identified by genome sequencing, function unknown
Yersinia ruckeri	1	2	Identified by genome sequencing, function unknown

2 OUTLINE AND AIMS OF THE THESIS

Minimizing the loss of farmed fish in aquaculture is essential for a successful production. Today, large-scale production exposes fish to detrimental conditions (e.g. high stocking, netting, sorting, transport, deterioration of the environmental conditions), which in turn causes stress. It is well known that stress is harmful to the fish welfare, especially for farmed fish that are more susceptible to diseases compared to wild fish. Skin disorders are one of the problems associated with fish mortality in aquaculture. Its aetiology is complex, but infectious agents seem to play a central role, in particular during specific conditions such as stress. Some pathogens such as *Aliivibrio salmonicida* seem to attack Atlantic salmon by penetration of the skin-mucus barrier. Despite of the occurrence of few bacterial disease outbreaks during the last years, infectious diseases are still happening in aquaculture. Within this frame, the identification of the relationship existing between environment, microbiome and host represents the key factor for understanding and preventing infectious diseases in aquaculture. This thesis is based on three research papers.

In Paper I, 16S rRNA gene sequencing analysis was utilized to investigate the microbial community present on the skin-mucus of farmed *Salmo salar*. Specifically, the role of stress on the skin-mucus microbiome was evaluated by comparing the community before and after fish handling. In addition, the composition of the bacterial community present in the rearing water was monitored in order to identify its influence on the skin-mucus microbiome.

In Paper II, label free quantitative mass spectrometry was employed to study the skin-mucus proteins associated with farmed Atlantic salmon and its microbiome. In particular, the temporal proteome dynamics during 9 days of mucus incubation was performed to evaluate the capability of microbes to utilize mucus component as nutrient source to grow in this environment.

In Paper III, putative virulence factors from *Aliivibrio salmonicida* related to chitin degradation were characterized with respect to activity towards chitin and other relevant substrates like salmon mucus and scales. The aim of this study was to evaluate the possible involvement of these enzymes in Cold Water Vibriosis, which is a well-known salmon disease.

3 MAIN RESULTS AND DISCUSSION

3.1 The Skin-Mucus Microbiome of *Salmo salar* (Paper I)

The fish skin-mucus represents an important defense layer that protects the fish towards the external environment. Similar to all mucosal surfaces, fish skin-mucus is colonized by bacteria that are generally not harmful for the fish. However, when the host is exposed to particular conditions (e.g. stress), they can be a source of infection. Unfortunately, farmed fish are often exposed to stressful conditions. Thus, understanding the composition of the skin-mucus microbial community of farmed Atlantic salmon and the role of stress on this community can be of importance in aquaculture to better understand and ultimately prevent fish diseases.

The microbial community present on the skin-mucus of 45 post-smolt farmed Atlantic salmon, together with the community living in the surrounding water, was investigated using 16S rRNA gene sequencing (**Figure 1**; Paper I). A total number of 14 mucus samples did not generate PCR products and were thus excluded from the analysis. The problematic amplification of the 16S rRNA genes associated with the mucosal microbes may reflect the presence of a low number of bacteria as suggested in literature (Austin, 2006). Indeed, quantitative analysis of 16S rRNA gene copies by Droplet Digital PCR (**Figure 3**; Paper I) confirmed low bacterial biomass in several mucus samples. Notably, samples with the lowest bacterial biomass revealed a similar microbial profile as the rearing water, suggesting detection of the water microbiome in mucus samples (**Figure 5**; Paper I). Low biomass may be caused by the presence of specific cell types on the skin that are able to phagocyte bacteria to ensure a clean surface. For instance, the keratocytes migratory activity and capacity to internalize particles (Ángeles Esteban, 2012; Karlsen et al., 2017) may be used to maintain a clean fish surface. In addition, the continuous production and shedding of mucus may contribute to the removal of microbes, suggesting the presence of a loosely associated bacterial community. This may explain the high variability of bacterial biomass observed in the different mucus samples (**Figure 3**; Paper I).

The analysis of salmon mucus at "T0" (pre-handling time point), performed in Paper I, showed mainly the presence of *Proteobacteria*-affiliated phylotypes, which is in agreement with previous studies (Llewellyn et al., 2014; Chiarello et al., 2015; Lowrey et al., 2015; Lokesh and Kiron, 2016; Kearns et al., 2017). Individual variation was detected among the

biological replicates (**Figure 3** "T0") and was as well reported in other studies (Larsen et al., 2013; Apprill et al., 2014). It may be partly associated with the surrounding environment and partly with the nutrient potential or antimicrobial components of fish mucus. In this particular case, all fish have been exposed to the same environmental conditions. Thus the differences detected may be correlated with the sampling procedure (e.g. removal of mucus and of its bacterial community by netting), the health status of the fish or intrinsic physiological factors that are unknown. However, due to the limited number of biological replicates in the present analysis, we cannot conclusively address our observation. Moreover, comparing the microbial profiles shown in **Figure 14** and **Figure 3** ("T0"; Paper I), which both have been performed at the same fish research center but at different periods of the year, clear differences at the genus level were detected. This observation may be correlated with (and not limited to) the sampling period or the fish feeding. Though, since a large number of factors can influence the fish skin-mucus microbiome, it is difficult to assign the presence of a specific core community.

The remaining 31 fish were exposed to stress conditions (netting and transportation) before mucus sampling, which was performed after 3 hours and 24 hours post-stress (**Figure 1** "T3 and T24"; Paper I). It is well known that the common aquaculture practices such as netting, sorting, transport, high stocking, feeding and poor water quality cause stress to the fish, which may manifest itself in the form of change in the microbiome profile (dysbiosis) as observed in mammals (Myers, 2004; Jones et al., 2014; Tomasello et al., 2016). In Paper I, the comparison of the host microbiome before and after fish handling showed a shift in the composition after 24 hours (**Figure 3**; Paper I), highlighting its susceptibility to significant changes within a short time period. However, this shift cannot be completely addressed to stress due to the lack of a negative control (fish not exposed to handling). Unfortunately, it is not possible to sample fish without causing stress and fish cannot be sampled more than once, making it complicated to have a proper control. Thus, other factors such as individual variability, natural temporal changes or fish's acclimation to the microbial properties of the rearing water may cause the observed variations. In this case, it is tempting to exclude the latter because the same water was used in all tanks and significant differences were observed between the water and the mucus microbiomes (**Figure 5**; Paper I). The large microbial diversity detected between these two environments suggests the presence of a specifically adapted community in mucus, despite its direct contact with the surrounding water.

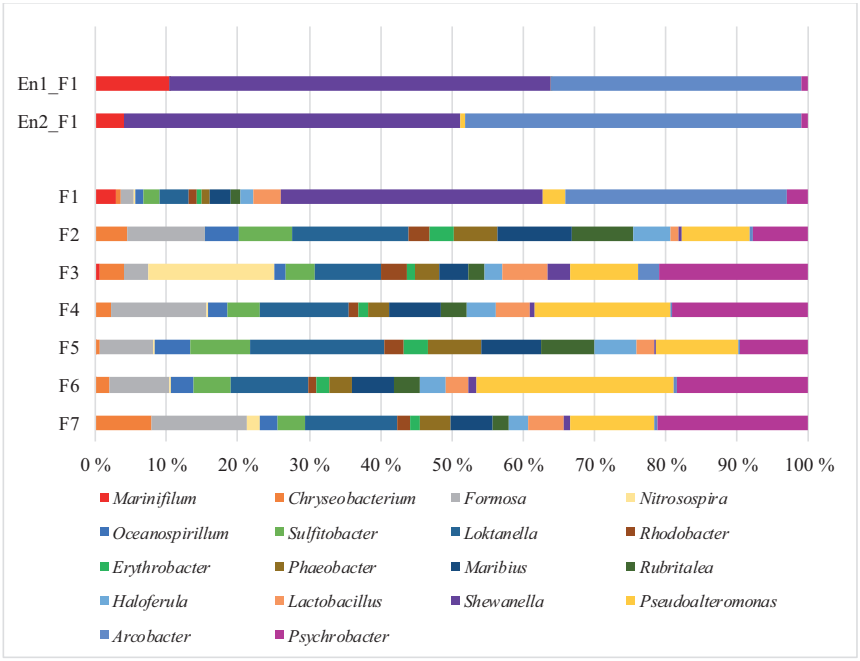

Figure 14. 16S rRNA amplicon sequencing analysis of farmed Atlantic salmon skin-mucus. All fish were sampled from the same tank and are indicated as "F". Additional mucus from F1 was collected and diluted 1-fold "En1_F1" and "En2_F1" with sterile-filtered rearing water and incubated at 10°C for 2 weeks to enrich mucolytic bacteria. The bar chart shows the Operational Taxonomic Units (OTUs) with a relative abundance above 1% of the total sequences. Methods of sample collection, DNA extraction, sequencing and downstream bioinformatic analysis were performed as described in Paper I.

While performing the experimental work for Paper I, we had the opportunity to sample diseased Atlantic salmon smolts (skin ulcers were observed). The skin-mucus sampled from these fish was dominated by *Tenacibaculum* sp., (**Figure 15**; unpublished results), a notorious fish pathogen that causes tenacibaculosis (Avendaño-Herrera et al., 2006). During the sampling procedure the mucus was collected only from the skin with a healthy appearance. Thus, its presence suggests that the bacterium colonizes all the fish surface. This is most likely caused by a failure of the fish defenses, which allows opportunists like *Tenacibaculum* sp. to proliferate in mucus. It should be noted that also the tank water, where the ulcerated fish were hosted, contained a microbial profile similar to what was found on the fish skin (**Figure 15**). This suggests that the bacterium may dislodge from the fish into the surrounding, possibly increasing the chances to infect other fish. However, the presence

of the genus *Tenacibaculum* in water may be as well caused by mucus shedding. A recent study on the host-associated microbiome of seawater-farmed Atlantic salmon showed the presence and dominance of *Tenacibaculum* sp. on the skin of both healthy and ulcerated fish (Karlsen et al., 2017), illustrating the constant threat of this bacterium in fish farming. The latter study did not sample wild Atlantic salmon, thus it may be that the stressful life of farmed fish influences the composition of the microbial community on the skin, as suggested in Paper I, causing diseases.

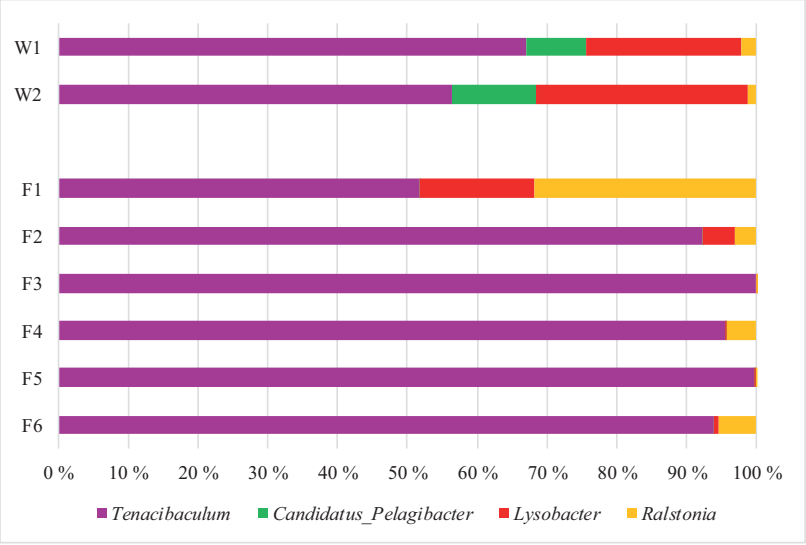

Figure 15. 16S rRNA amplicon sequencing analysis of skin-mucus of ulcerated farmed Atlantic salmon. All fish were sampled from the same tank and are indicated as "F". In addition, the tank water was collected, which is indicated as "W". The bar chart shows the Operational Taxonomic Units (OTUs) with a relative abundance above 1% of the total sequences. Methods of sample collection, DNA extraction, sequencing and downstream bioinformatic analysis were performed as described in Paper I.

3.2 The exoproteome associated with *Salmo salar* skin-mucus and its microbiome (Paper II)

Fish skin-mucus contains mucins (heavily O-glycosylated proteins) and an array of antimicrobial compounds like immunoglobulins, antimicrobial peptides and various enzymes, specialized in protecting the fish from the external environment (Ángeles Esteban, 2012). Despite the protective role of skin-mucus, some microorganisms seem to be able to colonize it. Indeed, mucins seem to play an important role in accommodating commensal

microbes and limiting the adhesion of pathogens (Linden et al., 2008). However, some pathogenic microorganisms have developed efficient strategies to bypass mucosal surfaces (Budiarti and Mubarik, 2007; Szabady et al., 2011). Despite the importance of this topic for the aquaculture industry, little is known about it. Thus, in paper II, label-free quantitative mass spectrometry was used to investigate the exoproteome associated with both farmed Atlantic salmon and its bacterial community.

By quantitative proteomic analysis of *Salmo salar* skin-mucus, we detected 3583 host proteins that were mainly identified in the beginning of the incubation period (**Figure 2** and **Figure 3**; Paper II). The Gene Ontology (GO) analysis revealed that the majority of these proteins were involved in biological regulation, transport, metabolic and cellular processes (**Supplementary Table S1**; Paper II). As expected, Mucin-5B-like and Mucin-5AC-like were detected in large abundance in the mucus proteome, together with proteins associated with protective functions such as lysozymes, lectins, calmodulins, galectins, histones, ribosomal proteins and complement related proteins. Furthermore, structural proteins (actin, keratin, tubulin, confilin-2 and filamin-A) were identified, although their functions in the mucus are not clear. Some studies suggested that actin and keratin have a role in the fish immune response (Molle et al., 2008; Easy and Ross, 2009). Hemoglobin was as well detected in the salmon exoproteome and its presence was previously observed in the epidermal mucus of stressed fish (Lebedeva et al., 2002). In Paper II, salmon were rapidly killed after sampling, but during this short time period, they may exhibit a short stress response. As highlighted in Paper I, it is not possible to sample fish without causing stress, which in turn can cause fish hemorrhages (Selye, 2013). Thus, its detection may simply be correlated with blood contamination. However, hemoglobin-derived peptides have been shown to have antimicrobial activities in vertebrates (Liepke et al., 2003), representing another potential explanation of its presence in mucus. Notably, only 15% of the total salmon proteins were predicted to be actively secreted. Large amounts of intracellular proteins have also been observed in other proteomic studies (Easy and Ross, 2009; Jurado et al., 2015). Their presence may be correlated with secretion through non-classical mechanisms or cell lysis during sampling. Unfortunately, there is no appropriate way to avoid collection of epithelial cells together with mucus due to the close interaction existing between them. It seems that contamination of host cells associated with mucus is not only a problem associated with the skin. In a study performed by Rudi et al., gut samples collected from Atlantic salmon contained large amounts of host DNA, indicating the presence of host cells

(Rudi et al., 2018). In Paper II, we attempted to reduce the contamination of epithelial cells by collecting mucus as gently as possible using a rounding plastic spatula. However, the detection of secreted proteins in the present study suggests a not completely successful sampling procedure, but as already noted, their presence may also arise from unconventional secretion mechanisms. It is worth noting that most of the other studies reporting proteomic investigation of fish skin-mucus have not addressed the potential problem of cell contamination. Proteomic data arising from analysis of mucus must therefore be interpreted with great care.

The presence of a bacterial community on the fish-skin mucus implies the detection of bacterial proteins involved in different life sustainable functions. In paper II, the exoproteome associated with the bacterial community present on the mucus was investigated for 9 days in order to identify the temporal dynamics and the metabolic strategies used by bacteria to grow in this environment. Performing a metaproteomic analysis on a sample of unknown composition can be challenging as a broad range of species must be included in the search database. Indeed, the use of metagenomics to generate a sample database can greatly improve the protein identification rate, but it is a large undertaking. Unfortunately, it was outside the timeframe of this study. As the composition of the community was known by 16S rRNA sequencing analysis, this information was used to filter public sequence repositories at the genus level to create a community database for metaproteomics. This allowed us to obtain a database with a good coverage of the community. However, a larger database is produced with this approach compared to the use of metagenomics (the actual species in the sample are sequenced and then assembled into a protein database) and proteins belonging to unknown organisms might be missed if no homologues are present in the public repositories.

Since Paper I highlights an overall low bacterial biomass in the *Salmo salar* skin-mucus, our strategy was to collect bacterial DNA from the last experimental time point (day 9) for 16S rRNA sequencing analysis in order to ensure a good quality bacterial DNA. One could argue that this strategy may prevent identification of proteins from bacteria that disappear during the cultivation period. However, as observed in the preliminary analysis (**Figure 14**), it seems that the dominant genera at the time of sampling (see sample "F1" in **Figure 14**) also represent the dominant genera after incubating the mucus for 14 days at 10°C (see samples En1_F1 and En2_F1 in **Figure 14**), strengthening the validity of our choice. Indeed, the

proteomic analysis at day 0 displayed the presence of proteins mainly associated with *Vibrio*, which is the dominant genus at day 9 (see below). A total number of 249070 reads, distributed among 23 OTUs and assigned to 18 genera, was identified by 16S rRNA sequencing analysis of the salmon-mucus microbiome. In overall, *Vibrio* was the most abundant genus (**Figure 1**; Paper II). Differences at genus level were observed among the three biological replicates, especially in the third replicate (F3). As mentioned in Paper I, the microbial diversity observed in fish, living in the same environmental conditions, may be caused by several factors such as individual fish variability, health status of the fish or unknown intrinsic physiological factors. Furthermore, a high variation was surprisingly detected among the three technical replicates of F3. It is tempting to speculate that this variation is caused by additional not-resident casual bacteria that adhere to the mucus components and grew after 9 days. However, the experimental procedure used in the present study limited the possibility of contamination. Comparing the microbiome detected in Paper II with the enrichment samples of the microbial profile in **Figure 14**, clear differences are identified at genus level. This observation confirms the difficulty in assigning the presence of a core community on the salmon skin-mucus as mentioned in Paper I. In addition, it highlights that different bacterial genera have the capability to grow on mucus, utilizing its components as nutrient source.

The analysis of the *Salmo salar* skin-mucus microbial exoproteome identified 4563 proteins over 9 days of incubation (**Table 1**; Paper II). Most of these proteins were detected after 5 days (**Figure 2**; Paper II) and were belonging to *Vibrio*. This genus was often associated with pathogenesis in fish (Benediktsdottir et al., 1998; Strom and Paranjpye, 2000; Frans et al., 2011), highlighting its capacity to proliferate and bypass mucosal surfaces. This may explain its dominance after 9 days of mucus incubation. The GO analysis of the predicted secreted *Vibrio* proteins showed mainly proteins correlated with transport and metabolic processes (**Figure 4**; panel A and B; Paper II). Specifically, proteins assigned to siderophore transport and proteolytic activity were abundant (**Figure 4**; panel C and D; Paper II). Their detection is not surprising due to their association with virulence (Biosca et al., 1996; Lantz, 1997; Lamont et al., 2002). In particular, the latter study suggested that siderophores act as signaling compounds for production of virulence factors such as proteases. Proteases may mediate direct damage of the host cells and proteins or indirectly mediate destruction by activating for instance metalloproteases (Sorsa et al., 1992). Indeed, *Salmo salar* proteins drastically decreased after a few days of mucus incubation (**Figure 2**; Paper II). Surprisingly,

a low amount of bacterial carbohydrate active enzymes were detected in the present proteomic dataset, despite the high abundance of mucins in mucus (**Table 2**; Paper II). Only one bacterial enzyme predicted to cleave off sialic acid moieties of mucin (sialidase from family 33 glycoside hydrolases) was detected. The lack of proteins associated with degradation of carbohydrates may be correlated with the size of the database used for proteomics, which makes protein detection less sensitive, or with the experimental time points investigated. Perhaps, these enzymes play an important role during the first hours of incubation. Furthermore, it may be as well possible that the carbohydrate active enzymes were localized in the solid fraction of mucus, which was not analyzed in this study.

3.3 Characterization of putative virulence factors with chitinolytic activity from *Aliivibrio salmonicida*

The two first studies of the thesis were focused on the composition of the skin-mucus microbial community of farmed Atlantic salmon and their interaction with the host. As already noted, prior to the experiments performed for Paper I, a preliminary study of the skin microbiome and its ability to grow in skin-mucus was conducted (**Figure 14**). The exoproteome of samples En1_F1 and En2_F1 from this study was determined in addition to the 16S rRNA gene sequencing analysis. This proteomic dataset was not published, but an intriguing finding was made, namely the identification of glycoside hydrolase (GH) family 18 chitinases. As a verification of this preliminary finding, the exoproteome analyzed in Paper II also revealed the presence of several GH18 chitinases (from the genus *Vibrio*). This discovery sparked our curiosity since the skin-mucus of Atlantic salmon does not intuitively contain chitin. By closer inspection of the literature, there exist a relatively recent study that claims, with convincing data, the presence of chitin in the scales of Atlantic salmon (Tang et al., 2015). Thus, a putative substrate for the bacterial chitinases identified in the skin-mucus may be located in the scale structure. The question is, can bacteria access to chitin when they are attached to the skin or are such enzymes only activated during an infection process? On the other hand, it may be that the target substrate for these putative chitinases is not chitin, but another glycan structure that contains GlcNAc (monosaccharide that constitutes the building block of chitin chains). For instance, ChiA2 from *Vibrio cholerae* has been shown to cleave mucin glycans (Mondal et al., 2014), whereas GH18 chitinases from *Listeria monocytogenes* and *Salmonella typhimurium* have been shown to hydrolyze other non-chitin carbohydrates (Frederiksen et al., 2015). In order to investigate the

biochemical properties of chitinases and other potential chitin targeting enzymes present in the genomes of pathogenic bacteria related to Atlantic salmon, we cloned and characterized the GH18 chitinase and family "auxiliary activity" 10 (AA10) lytic polysaccharide monooxygenases (LPMOs) from *Aliivibrio salmonicida*. This bacterium is the causative agent of Cold Water Vibriosis, well-known disease in aquaculture. We included the LPMOs in the present study since their domain structure indicated a role in chitin degradation and because this family of enzymes plays a role in virulence (Frederiksen et al., 2013).

All the enzymes characterized in Paper III showed activity on chitin (**Figure 3** and **Figure 4**; Paper III). However, *As*Chi18A was barely active compared to the chitinases from *Serratia marcescens* and *Cellvibrio japonicus* (**Figure 6**; Paper III), two soil bacteria well known for their efficiency in chitin degradation (Monreal and Reese, 1969; Tuveng et al., 2016). This outcome suggests that chitin may not be the biologically relevant substrate for *As*Chi18A. In addition, activity towards more complex and potentially natural substrates such as *Salmo salar* skin mucus or scales was not detected for any of the enzymes, although, the chitinase bound strongly to the fish scales (**Figure 5**; Paper III).

It is beyond a doubt that the chitin degrading enzymes of *Aliivibrio salmonicida* are capable of depolymerizing insoluble, crystalline chitin forms such as α- and β-chitin. The efficiencies of these enzymes combined does not seem to be adapted to a lifestyle of the bacterium where chitin is a primary source of nutrients. However, if their role is not in metabolism, but rather as a tool to penetrate the chitin layer related to the Atlantic salmon scales, the activity shown by the enzyme may be sufficient. Further study are required to verify our hypothesis.

4 CONCLUSION AND FUTURE PERSPECTIVES

The fish mucosal surface represents an important physical and immunological barrier that protect the fish from external agents. However, under particular conditions (e.g. stress), fish seem to be more susceptible to bacterial diseases. The mechanisms underlying a healthy versus unhealthy host-microbiome interaction is not well known and its understanding represent a key factor for a successful aquaculture production. Thus, in the present study several aspects of the complex interaction existing between the environment, the host and its microbiome have been investigated.

In paper I, the analysis of the skin-mucus microbiome of farmed *Salmo salar* highlighted the presence of a variable bacterial biomass on the mucus, which seems to be rapidly influenced by external factors such as stress. Moreover, significant differences were observed between the water and mucus microbiome, suggesting the colonization by a specifically adapted bacterial community on the skin-mucus.

In paper II, the analyses of the Atlantic salmon skin-mucus exoproteome revealed a large diversity of proteins mapping to a variety of different biological processes. In particular, antimicrobial proteins and enzymes were detected, confirming the protective role associated with the skin-mucus. Despite its protective function, bacterial proteins, mainly belonging to the genus *Vibrio*, were identified in the present work. The analysis of bacterial proteins and their dynamics over time demonstrated their capacity to secrete proteases, which are probably used to degrade salmon proteins.

In paper III, the putative chitinolytic enzymes from *Aliivibrio salmonicida* (*As*Chi18A, *As*LPMO10A and *As*LPMO10B) were characterized in order to investigate their potential roles as virulence factors. All enzymes showed activity towards α- and β-chitin. Comparison of the *Aliivibrio salmonicida* chitinase with the well characterized chitinases from *Serratia marcescens and Cellvibrio japonicus*, revealed a 50-fold lower activity for *As*Chi18A, suggesting that chitin may not be the primary substrate of this enzyme. Finally, all the studied enzymes seem to be not active on complex substrates like Atlantic salmon skin-mucus or scales, although the chitinase bound strongly to the fish scales.

The present thesis provides new findings on the interactions between bacteria associated with the skin of Atlantic salmon and the skin-mucus. This is a topic that has until recently received little attention and progress in this field is important from a fundamental point of view and from the applied perspective. Indeed, farmed Atlantic salmon is of great importance for the supply of protein and lipid-rich food to the world. Importantly, we have also revealed several challenges in the experimental investigation of the fish skin-mucus and its bacterial microbiome, which must be considered during skin-mucus analysis of water dwelling animals in order to ensure a correct interpretation of the results. Issues related to sample contamination occurring either from the surrounding water or during the sampling procedures seem to be unavoidable, and results from such analysis must be interpreted with great care in order to avoid artefacts. The work performed in Paper II also highlighted another challenge, namely the high abundance of putative intracellular proteins both from the host and bacterial community. Whether this represents the actual *in vivo* situation or rather is caused by cell lysis during sample preparation is not known and should be investigated in more detail in future studies.

All in all, this work brings us a step further in understanding the interactions existing between the environment, the host and the microbial community in the skin-mucus of Atlantic salmon. Such knowledge is crucial to improve fish welfare in aquaculture. However, further studies are needed to enhance our understanding and make use of this knowledge to improve fish production. For instance, by comparison of the microbiome of healthy and diseased fish would be possible to identify the main bacteria present on the skin-mucus during skin disorders. Furthermore, the role of stress could be investigated by exposing stressed and unstressed fish to potential pathogens and evaluate their susceptibility to skin disorders. Moreover, the role of *As*Chi18A and *As*LPMO10A and -B as virulence factors could be asses by the ability of a wild type strain to infect fish in comparison to a knockout strain, which lacks of the functional activity of these enzymes.

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Paper I

The skin-mucus microbial community of farmed Atlantic salmon (*Salmo salar*)

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The Skin-Mucus Microbial Community of Farmed Atlantic Salmon (*Salmo salar*)

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The skin of the teleost is a flexible and scaled structure that protects the fish toward the external environment. The outermost surface of the skin is coated with mucus, which is believed to be colonized by a diverse bacterial community (commensal and/or opportunistic). Little is known about such communities and their role in fish welfare. In aquaculture, fish seem to be more susceptible to pathogens compared to wild fish. Indeed common fish farming practices may play important roles in promoting their vulnerability, possibly by causing changes to their microbiomes. In the present study, 16S rRNA gene amplicon sequencing was employed to analyze the composition of the farmed *Salmo salar* skin-mucus microbiome before and after netting and transfer. The composition of the bacterial community present in the rearing water was also investigated in order to evaluate its correlation with the community present on the fish skin. Our results reveal variability of the skin-mucus microbiome among the biological replicates before fish handling. On the contrary, after fish handling, the skin-mucus community exhibited structural similarity among the biological replicates and significant changes were observed in the bacterial composition compared to the fish analyzed prior to netting and transfer. Limited correlation was revealed between the skin-mucus microbiome and the bacterial community present in the rearing water. Finally, analysis of skin-mucus bacterial biomasses indicated low abundance for some samples, highlighting the need of caution when interpreting community data due to the possible contamination of water-residing bacteria.

Keywords: skin, mucus, teleost, microbiome, stress, aquaculture, *Salmo salar*

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INTRODUCTION

The body surface of vertebrate animals represents a physical barrier between the environment and the animal host. Skin protects the host from the entry of pathogenic organisms or allergens, but also from the leakage of water, solutes or nutrient (Ángeles Esteban, 2012). The skin of teleost is different from that of mammals because it secretes mucus, which exhibits immune functions (Salinas et al., 2011). Mucus contains mucins (heavily *O*-glycosylated proteins) (Barchi, 2013), and an array of antimicrobial compounds like immunoglobulins, antimicrobial peptides and different enzymes (Ángeles Esteban, 2012). The presence of a mucosal tissue on fish skin represents an evolutionary adaption to the water environment (Xu et al., 2013), which is populated by a large

number of potentially harmful organisms (Magnadottir, 2010). Indeed, mucus represents the first barrier against infectious pathogens (Tort et al., 2003). All the mucosal surfaces of humans and animals are colonized by different bacterial species (commensal and/or opportunistic), which play a key role in the development of the host immune system (Cebra, 1999; Lee and Mazmanian, 2010; Maynard et al., 2012). In contrast to the well-studied human skin microbiome (Schommer and Gallo, 2013; Dorrestein et al., 2016; Gallo, 2017) only a limited number of studies have focused on the complexity of the bacterial community associated with the fish skin-mucus (Llewellyn et al., 2014; Chiarello et al., 2015; Lowrey et al., 2015; Lokesh and Kiron, 2016; Kearns et al., 2017). Interestingly, even though the majority of bacteria detected in the skin-mucosal surfaces belong to the phylum *Proteobacteria*, high variations at the species levels have been observed in the abovementioned studies. For instance, Chiarello et al. (2015) reported variability of the skin-associated community between host species, individuals and as well among different external body parts. Moreover, when comparing the microbial community of skin, gills, olfactory rosettes and anterior and posterior gut tissues from rainbow trout, Lowrey et al. (2015) observed that the highest microbial diversity was found in the external mucosal sites of fish.

The ability of fish to maintain a healthy balance between commensal and opportunistic bacteria in their skin-mucus is suggested to represent a key factor to preserve fish health (Gómez and Balcázar, 2007). Unfortunately, the healthy balance of the microbiome can be altered by disturbance factors such as stress or antibiotics (Boutin et al., 2013; Carlson et al., 2015). In aquaculture of salmonids, stressful events include (and are not limited to) netting, sorting and transport (Iversen and Eliassen, 2009). These practices could potentially affect the balance of the fish-skin microbiome and reduce the bacterial biodiversity, promoting proliferation of opportunistic bacteria, a process well documented in mammals (Myers, 2004; Jones et al., 2014; Tomasello et al., 2016). Moreover, a previous study (Iguchi et al., 2003) evaluated the effect of stress on the immune system of fish and reveled an increase of disease susceptibility due to immunosuppression, confirming the close relation existing among stress, immune system responses and pathogens. Stress in aquaculture is considered maladaptive when disturbances cause a prolonged stress response, which is harmful to the fish welfare (Barton, 2002; Llewellyn et al., 2014). Recent studies on the bacterial taxa living in the skin-mucosal surfaces of fish reported a shift in the microbiome as a consequence of exposure to prolonged stress, enhancing the growth of potentially pathogenic bacteria (Boutin et al., 2013; Sylvain et al., 2016). Farmed aquatic animals are often exposed to maladaptive conditions and diseases. Skin disorders represent one of the problems associated with fish mortality in aquaculture. It is estimated that 1.1–2.5% of farmed fish die due to ulceration (Karlsen et al., 2017). Therefore, understanding the composition of the skin-mucus microbiome of farmed fish may represents a step toward improving the welfare of species such as *Salmo salar*. Despite the importance of this topic, little is known regarding the host-associated bacterial population present in the skin-mucus.

In the current work, 16S rRNA sequencing analysis has been used to study the microbial community present in the skin-mucus of farmed *Salmo salar* and the potential influence of common aquaculture practices, such as fish netting and transfer, on its composition. In addition, the bacterial community present in the rearing water was also monitored during all experiments to compare its similarity with the salmon skin-mucus microbiome.

MATERIALS AND METHODS

Fish and Sampling Procedure

Forty five seawater-adapted post-smolt *Salmo salar* (±300 g each) from the Nofima research center NCRA in Sunndalsøra, Norway were randomly selected for this study. A schematic overview of the experimental sampling plan is illustrated in **Figure 1**. At the time of sampling, salmon had been kept in Tank_1 for 6 months, and the total biomass of the tank was approximately 96 kg/3.3m^3. Fish were fed with Ewos Opal 200, following a feeding regime of 6 times/hour, with 8 s feeding/time. The source of the water utilized in the experiment was seawater from a depth of 40 m mixed with fresh ground water, following filtration and UV disinfection (32 ppt salinity and temperature around 10°C). The tank based-system was a Recirculation Water System (RAS). Fifteen of the forty five fish were sampled directly from Tank_1, representing the pre-handling time point (T0), killed with an overdose of MS-222 and immediately transferred to the lab. Mucus samples were taken from the right side of the fish, over the entire side, using sterilized swabs (Plain swab sterile wooden applicator cotton tipped, Copan, Italy) and stored at −80°C until further analysis. The remaining 30 fish were transferred to a small tank containing the same water as Tank_1, lifted up simultaneously with a sterilized net, kept in air for 30 s and back in water to recover; the process was repeated three times. After netting, fish (15 fish per tank) were transferred into Tank_2 and Tank_3, which served as technical replicates. All the tanks used in the experiment had a flow through system. The inlet water to each single tank was the same but the water was not shared among them. The fish feeding was interrupted after fish handling to avoid microbial contamination from unconsumed food as it is observed that fish tend to fast after stressful events. Fish were sampled from Tank_2 and Tank_3 after 3 h (T3) and 24 h (T24) post-handling (15 fish each time), using the same sampling and mucus processing procedure described previously. Furthermore, 50 ml of water was collected from all the tanks, at all experimental time points, using sterile 0.2 μm hollow fiber syringe filters (Dyna Gard, Microgon Inc., Laguna Hills, CA, United States) to retain the bacteria present in the water (3 replicates per tank). Filters were stored at −80°C until further analysis. Samples were entitled according to the source of the sample (water; "W" or mucus; "M"), time of collection (pre-handling ; "T0," 3 h post-handling; "T3" or 24 h post-handling; "T24") and sample tank (Tank 1–3), e.g., sample M2-T3-3 represents mucus sample number 2 collected from Tank_3 at 3 h post-handling . The animal experiment was approved and done according to laws and regulations of the Norwegian Food Safety Authority and the 'European Convention

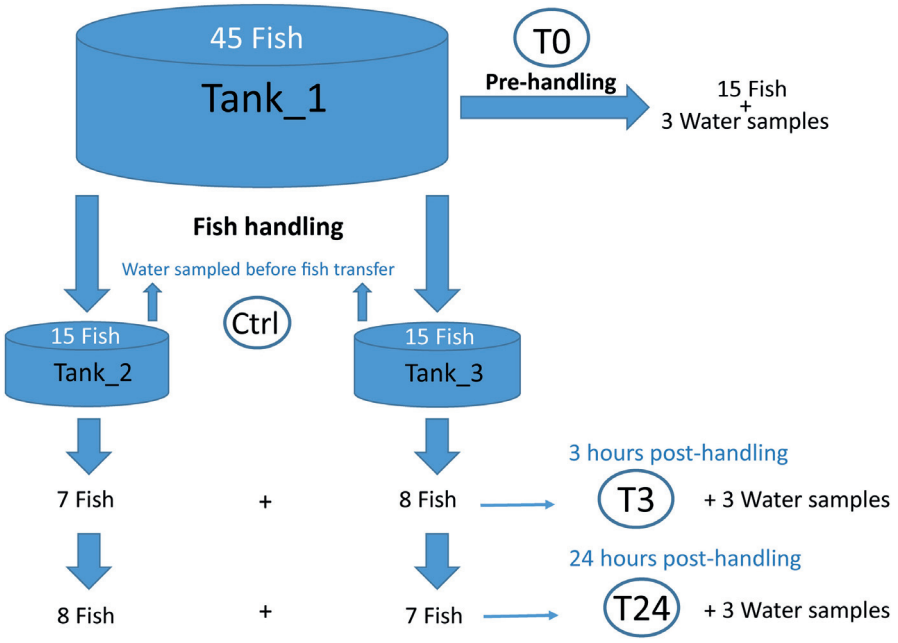

FIGURE 1 | Schematic illustration of the experimental design. A total of 45 fish were used in this experiment. Fifteen fish were sampled directly from the main tank (Tank_1) and skin mucus samples were collected (T0). The resting 30 fish, after netting, were transferred into Tank_2 and Tank_3, and successively mucus samples were collected at 3 h (T3) and 24 h (T24) post-handling. In parallel, water samples were collected at different time points, with 3 replicates taken per tank for each time point. Rearing water from Tank_2 and Tank_3 was additionally sampled before fish were transferred into the new tanks (Ctrl).

for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (EST 123).

Samples Preparation and 16s rRNA Gene Sequencing (rrs)

DNA was extracted from mucus and water filters with the DNeasy tissue kit (Qiagen, Germany), following the protocol for Gram positive bacteria with some modifications. Achromopeptidase was utilized (incubation for 1 h at 37°C) in the first step of the extraction process, ensuring the lysis of Gram positive bacteria (Ezaki and Suzuki, 1982). Proteinase K (40 μl) and ATL buffer (180 μl) were added to the samples, and tubes were incubated at 55°C for 1 h. Successively, 200 μl of AL buffer was used as last lysis step (incubation at 70°C for 10 min). The manufacture's protocol was followed during the remaining steps. The DNA extracted was stored at −20°C. Sample preparation for 16S rRNA sequencing analysis by Miseq was performed according the Illumina guide (16S Metagenomic Sequencing Library Preparation, Part. 15044223 Rev A). A primer set targeting the V3-V4 hypervariable regions, Pro341F (5′-CCTACGGGNBGCASCAG-3′) and Pro805R (5′-GACTACNVGGGTATCTAATCC-3′) (Takahashi et al., 2014) were used to amplify 16S rRNA genes. PCR amplification was performed using the polymerase iProof High-Fidelity (Bio-Rad, Hercules, CA, United States) with the following cycle conditions: initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and concluded by a final extension at 72°C for 5 min. The quality of the amplicon DNA was checked by agarose gel electrophoresis. Sample concentrations after amplicon PCR and cleaning steps were quantified using Qubit dsDNA HS Assay (Invitrogen). Miseq (Illumina) was used to sequence mucus and water samples. PhiX Control library was combined with the amplicon library (expected at 15%).

Bioinformatics and Statistics

The pipeline Usearch v.8.1861 (Edgar, 2010) implemented in QIIME v1.8.0 (Kuczynski et al., 2012) was used to analyze the data (all executed command lines are listed in Supplementary Materials and Methods). First, paired reads were merged, quality filtered (E_max = 1) and trimmed (430 nucleotides) to ensure the presence of sequences of sufficient quality. Chimeric sequences were identified and excluded from the dataset using the command cluster_otus in Usearch. The same command (cluster_otus) performs 97% Operational Taxonomic Units (OTU) clustering using the UPARSE-OTU algorithm, thus it was used to construct the set of representative OTUs. Finally, taxonomy was assigned using the UTAX algorithm (Edgar, 2010)

with the full-length RDP training set (utax_rdp_16s_trainset15), enabling the construction of the OTU table. Filter_otus_from _otu_table.py in QIIME was used to filter out OTUs making up less than 0.005% of the total, by using default parameters and –min_count_fraction set to 0.00005 (Bokulich et al., 2013). The samples were further normalized to the smallest library to remove sample heterogeneity. The generated OTU table was utilized to create the taxonomy plots and to construct the phylogenetic tree, which was subsequently used to generate the unweighted and weighted UniFrac distances by QIIME. The OTU table was also used to calculate the Bray-Curtis distance matrix using the R (version 3.2.3), package Vegan (Oksanen et al., 2013). Principal Coordinate Analysis (PCoA) of unweighted and weighted UniFrac matrix (Lozupone and Knight, 2008) were performed to assess phylogenetic distances between water and mucus microbiome at different sampling times (T0, T3, T24, Ctrl). PCoA analysis was also performed on the Bray-Curtis distance matrix (Beals, 1984). The α-diversity of mucus and water samples at T0, T3, T24 and Ctrl were calculated at OTU level using Shannon index. The Dunnett-Tukey-Kramer Pairwise Multiple Comparison Test Adjusted for Unequal Variances and Unequal Sample Sizes (Lau, 2013) was used to compare the bacterial diversity, measured by Shannon index, among groups of samples. Finally, Permutational Multivariate Analysis of Variance (Adonis) (Anderson, 2001) using the weighted UniFrac distance matrix in R, package Vegan, was utilized for significance testing on the water and mucus samples.

Droplet Digital PCR Reaction and Data Analysis

The QX200 Droplet Digital PCR (ddPCR^{TM}) System (Bio-Rad, Munich, Germany) was used to quantify the number of copies per microliter of the extracted DNA from mucus and water samples. The ddPCR reaction contained 10 μl of Eva Green Super Mix (Bio-Rad, United States), 100 nM of each primer (Pro341F-Pro805R, see above) and 2 μl of template DNA. In addition, sterile nuclease-free water (VWR) was included in the ddPCR reaction to reach a final volume of 22 μl. A volume of 20 μl of the ddPCR reaction was used to generate 40 μl of droplets using the QX100 droplet generator (Bio-Rad, Munich, Germany). Droplets were transferred to a 96-well plate and amplified with the following conditions: initial denaturation at 95°C for 5 min, followed by 45 cycles at 95°C for 30 s, 53°C for 30 s, 72°C for 45 s and stabilization signal at 4°C for 5 min and 90°C for 5 min. Afterward, droplets were analyzed in the QX200 droplet reader (Bio-Rad, Munich, Germany). Mucus and water samples were studied in triplicates and all the data were analyzed by the QuantaSoft software 1.7 (Bio-rad, Munich, Germany), which provides the concentration values of the ddPCR target DNA in copies/μl. Successively, the numbers of bacterial copies/μl present in the starting samples were calculated: The concentration given by the QuantaSoft software multiplied by the reaction volume and divided by the volume of the starting material added in the PCR (Bio-Rad Droplet Digital PCR Applications Guide, Bullettin 6407, Rev A).

Nucleotide Sequence Accession Numbers

Sequence data are available at NCBI Sequence Read Archive under accession number SRP107063.

RESULTS

16S rRNA gene sequencing was used to identify the bacterial composition and diversity of the skin-mucus microbiome of *Salmo salar* before and after fish handling. Samples from a total of 45 fish were originally collected. However, 15 of these samples did not generate PCR products, and were thus omitted from the analysis (Supplementary Table S1). The bacterial community in the tank water was additionally characterized to evaluate its influence on the skin-mucus microflora. After quality filtration, a total number of 2690828 reads were obtained from water (21 samples) and mucus (31 samples). The mean of read counts was 51746.692, with the highest values being 98187 (sample W3-T0-1) and the lowest being 22070 (sample W3-Ctrl-3) (Supplementary Table S1). Normalization of the dataset generated a total of 1147640 quality-filtered reads from the 52 samples analyzed. These sequences were assigned to 616 OTUs, which were utilized to construct the phylogenetic tree and generate unweighted and weighted UniFrac and Bray-Curtis distance matrices. Alpha diversity estimates represented by Shannon index indicated that the bacterial diversity was significantly higher in water samples compared to mucus (**Figure 2**). In order to focus on the most abundant taxa, **Figures 3**, **4** visualize mainly the OTUs represented by greater than 1% of the total reads. The OTUs <1% of the total sequences are grouped together as "Others (OTUs<1%)". An overview of the phylogenetic distribution of all the OTUs presented at class level is provided in Supplementary Figure S1.

Species Variation Observed in Skin Microbiomes Prior Fish Handling

The microbial profile analysis of mucus from a total of eleven fish was performed at T0. Quantitative Droplet Digital PCR revealed variation in concentration of copies of 16S rRNA gene sequences among the individual fish (**Figure 3** and Supplementary Table S2). Three phyla were mainly observed on the salmon skin-mucus before handling, with *Proteobacteria*-affiliated phylotypes the most abundant followed by *Firmicutes* and *Acidobacteria*. The most abundant OTUs (≥1%) obtained at T0 were classified at genus level (**Figure 3**), illustrating intraspecies variation among the biological replicates. For instance, *Lysobacter*-affiliated phylotypes were the most abundantly observed in samples M6-T0-1 (84%), M9-T0-1 (37%) and M10-T0-1 (37.5%), while almost absent in samples M2-T0-1, M3-T0-1, M4-T0-1 and M5-T0-1. On the contrary, Gp4-affiliated phylotypes (uncultured bacteria from the phylum *Acidobacteria*) were mostly observed in samples M1-T0-1 (15%), M2-T0-1 (14%), M3-T0-1 (17%), M4-T0-1 (45%), and as well in M5-T0-1 (41.5%). In addition, *Pseudomonas*, *Noviherbaspirillum* and *Burkholderia* (the latter in low abundance) were detected in some of the samples,

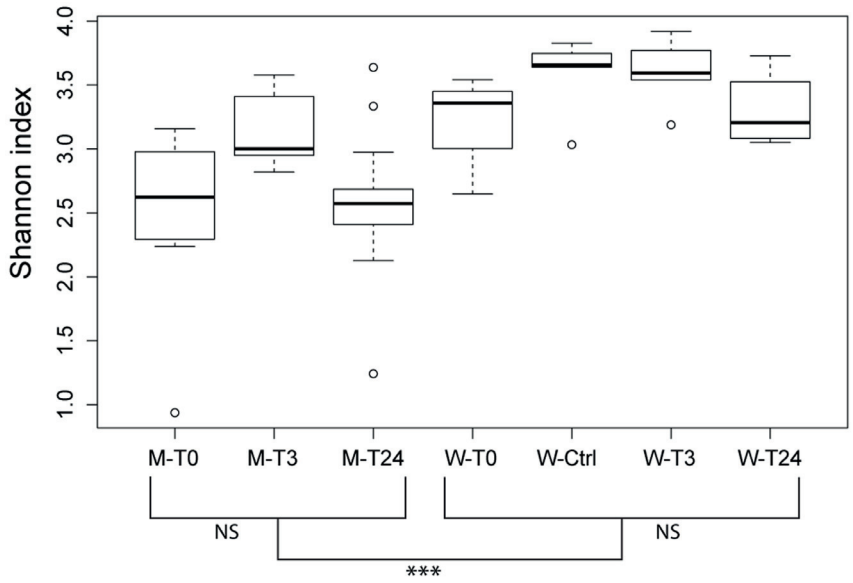

FIGURE 2 | Community diversity estimations by Shannon index at an OTU level. Each bar describe mucus or water samples collected at the different time points (T0, T3, T24 + Ctrl water). Significant differences among the water samples are calculated using the Dunnett-Tukey-Kramer Pairwise Multiple Comparison Test Adjusted for Unequal Variances and Unequal Sample Sizes. Significance degree is represented with stars; $P < 0.05$ with one star (*); $P < 0.01$ with two stars (**); $P < 0.001$ with three stars (***). No significance is indicated as NS.

while OTUs affiliated with the genera *Ralstonia, Lactobacillus* and *Methylobacterium* were observed in almost all biological replicates with fluctuations in relative abundance.

Skin Community Profile and Diversity Post-handling

The same phyla detected in the skin microbiome at T0 were also observed in the samples collected from the handled fish in the two replicate tanks (Tank_2 and Tank_3 at T3 and T24). At T3, the genera-level population of the most abundant phylotypes seemed to be consistent across the assessed biological replicates and the community showed a similar profile as some of the fish sampled at T0 (**Figure 3**). However, intraspecies variation was still observed, with sample M7-T3-3, portraying a different proportion of abundant OTUs compared the other fish sampled at the same time point. Mucus collected at 3 h post-handling (T3) included seven samples, while samples taken at 24 h post-handling included thirteen mucus samples (Supplementary Table S1). Quantitative Droplet Digital PCR (ddPCR) highlighted variation in concentration of copies of 16S rRNA gene sequences within the individual fish at the same experimental time point and among samples collected at the different time points (**Figure 3** and Supplementary Table S2). The skin microbiome of fish collected at T24 showed a shift of the abundant OTUs and had lower intraspecies variation compared to the skin-mucus microbiome detected at T0 and T3 (**Figure 3**). For instance, the average of the relative abundances associated with the genera *Methylobacterium,* Gp4 and *Noviherbaspirillum* showed higher abundance before handling and at 3 h post-handling compared to 24 h post-handling. On the contrary, *Lysobacter* and *Lactobacillus*, which were abundant in some of the samples at T0, were again detected after 24 h post-handling. The most evident shift of the skin-mucus community was associated with the genus *Burkholderia*, which was present in small amount at T0 (1%) and T3 (below 1%), followed by a considerable increase after 24 h (38%). Significant differences existing among T0, T3, and T24 were supported by the beta-diversity distance matrices (UniFrac and Bray-Curtis), which validated the aforementioned intra-animal variation of the total bacterial community and illustrated that the fish mucus microbiomes before and after fish handling were phylogenetically distinct (**Figure 5**). Significant differences existing among T0, T3 and T24 were statistically corroborated by Permutational Multivariate Analysis of Variance (Adonis) (**Figure 3** and Supplementary Table S3).

Water Community Profile and Its Comparison with the Skin-Mucus Microbiome

Water samples were collected from all tanks at the different fish sampling time points, and were subjected to the same *rrs* analysis pipeline to compare its similarity with the fish

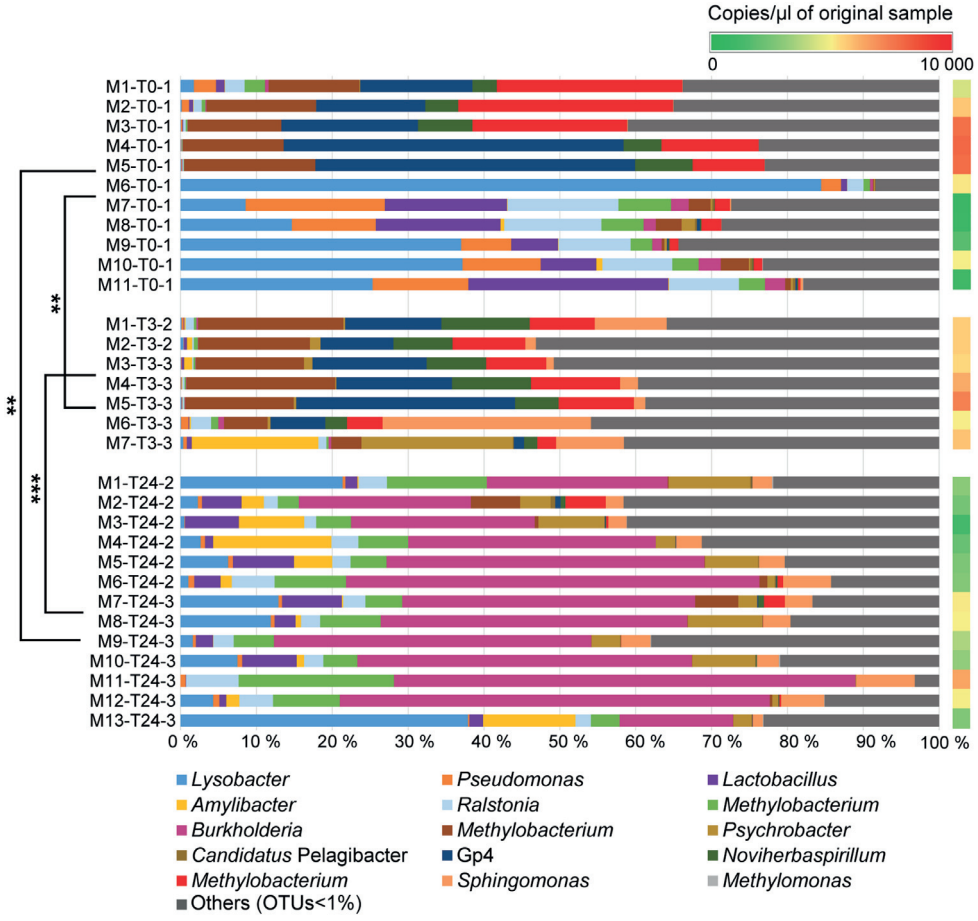

FIGURE 3 | Relative abundances of bacterial genera identified in mucus samples. The bar chart shows relatively abundant genera inherent to the skin mucosal microbiome at the different time points (T0, T3, T24). The OTUs with relative abundance values greater than 1% of the total sequences are mainly considered in the barchart. The OTUs <1% of the total sequences are assembled together as "Others (OTUs<1%)". A comprehensive summary of the taxonomic groups is given in Supplementary Table S4. Permutation Multivariate Analysis of Variance using weighted UniFrac distance matrix calculated from the total OTU dataset are performed among the different experimental time points (shown on the left) (see Supplementary Table S3). Significance degree is represented with stars; $P < 0.05$ with one star (*); $P < 0.01$ with two stars (**); $P < 0.001$ with three stars (***). The color gradient (shown on the right) illustrates the DNA concentration of mucus samples (copies/µl) detected by Droplet Digital PCR (see Supplementary Table S2).

mucus microbiome. Additional water samples were collected from Tank_2 and Tank_3 before transferring the fish (Ctrl) to evaluate any differences with the original water containing the fish in Tank_1 (water T0) and the water to which the fish were transferred and assessed after 3 h and 24 h. Quantitative Droplet Digital PCR (ddPCR) indicated, in overall, a higher number of copies of 16S rRNA gene sequences in the water samples compared to the mucus samples and highlighted variation in concentration among samples, tanks and time points (**Figures 3**, **4** and Supplementary Table S2). The most abundant OTUs (greater than 1%) in each sample were consistently observed in both technical replicates (Tank_2 and Tank_3) and all rearing water samples originating from the different time points (**Figure 4**). *Lysobacter* was the most abundant genus, followed by *Pseudomonas* and *Ralstonia*. Beta-diversity metrics (UniFrac and Bray-Curtis) (**Figure 5**), showed a clear variation between the water and mucus communities, which were visualized by two distinct clusters (outcome supported statistically by Permutational Multivariate Analysis of Variance, Supplementary Table S3). However, the mucus samples M6-T0-1, M7-T0-1,

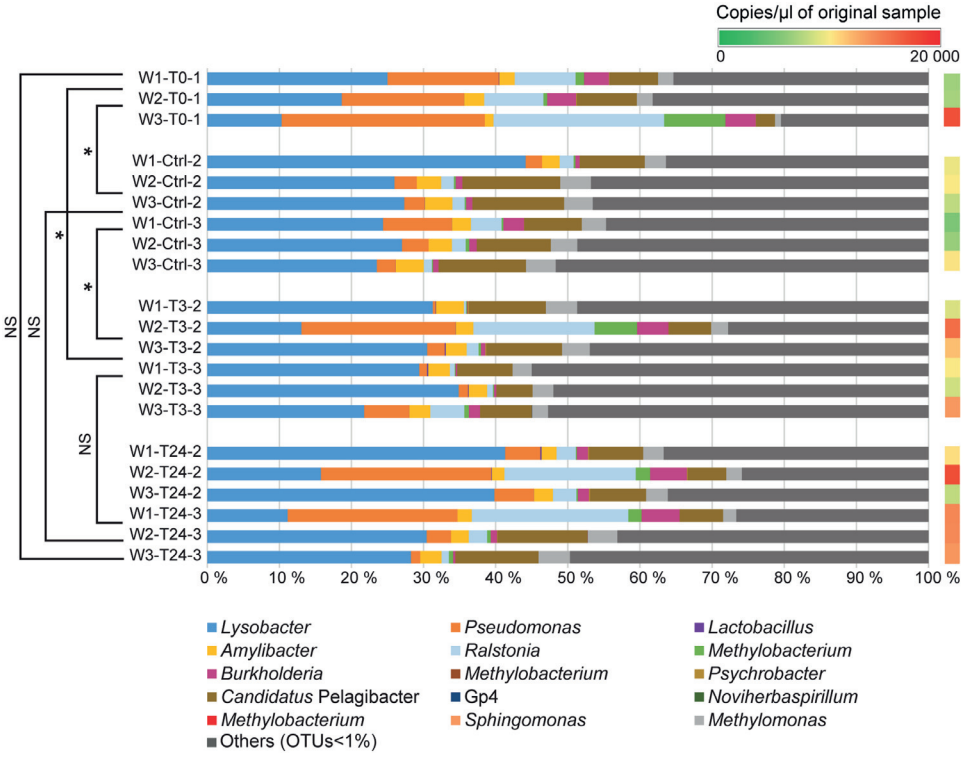

FIGURE 4 | Relative abundance of bacterial genera identified in the rearing water. The bar chart shows the tank water microbiome (3 replicates per tank) at the different time points (T0, T3, T24 + Ctrl water). The OTUs with relative abundance values greater than 1% of the total sequences are mainly considered in the barchart. The OTUs <1% of the total sequences are assembled together as "Others (OTUs<1%). A comprehensive summary of the taxonomic groups is given in Supplementary Table S4. Permutation Multivariate Analysis of Variance using weighted UniFrac distance matrix calculated from the total OTU dataset are performed among the different experimental time points (shown on the left) (see Supplementary Table S3). Significance degree is represented with stars; $P < 0.05$ with one star (*); $P < 0.01$ with two stars (**); $P < 0.001$ with three stars (***). No significance is indicated as NS. The color gradient (shown on the right) illustrates the DNA concentration of water samples (copies/µl) detected by Droplet Digital PCR (see Supplementary Table S2).

M8-T0-1, M9-T0-1, M10-T0-1, M11-T0-1, and M13-T24-1 exhibited a different trend and showed a closer correlation with the water samples (**Figure 5**).

DISCUSSION

In this study, we have used 16S rRNA gene sequencing to investigate the composition of the skin-mucus microbiome of farmed *Salmo salar* and the potential influence of common aquaculture practices on the microbial community. For fish that were sampled and analyzed before netting and transfer, the taxonomic analysis at phylum level exhibited a predominance of *Proteobacteria*, which was in agreement with previous studies conducted on different species of teleost (Wilson et al., 2008; Chiarello et al., 2015). Inspection of the data at genus level indicated differences in the bacterial community among the biological replicates (**Figure 3**). Individual variability has been reported for both wild and captive teleost and cetaceans (Larsen et al., 2013; Apprill et al., 2014). However, the variability observed at T0 cannot be conclusively addressed by this study, due to the limited number of biological replicates and the low cell biomass detected in several samples. The number of 16S rRNA gene copies obtained from the skin-mucus varied between individuals (**Figure 3** and Supplementary Table S2), showing low biomass in some samples, which may be related to low bacterial biomass in the mucus (Austin, 2006), technical challenges in the DNA extraction procedure or a combination of these. This may explain why amplification of the 16S rRNA genes was problematic in several samples (also observed by Lowrey et al., 2015). Notably, some of the mucus samples with the lowest biomasses (M7-T0-1, M8-T0-1, M9-T0-1, M11-T0-1) showed similarity with the microbial profile of the rearing water. This similarity is evident when

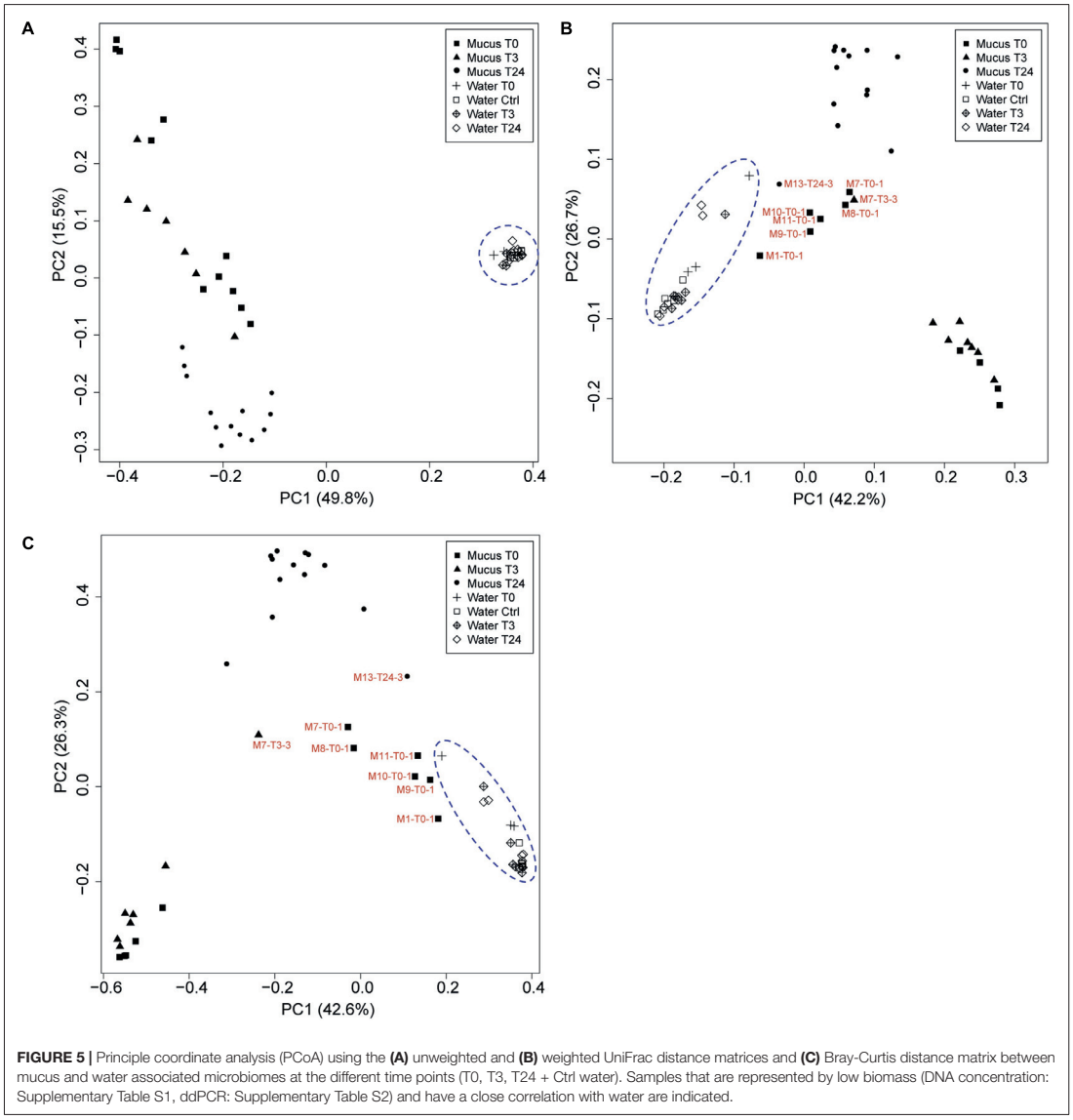

FIGURE 5 | Principle coordinate analysis (PCoA) using the **(A)** unweighted and **(B)** weighted UniFrac distance matrices and **(C)** Bray-Curtis distance matrix between mucus and water associated microbiomes at the different time points (T0, T3, T24 + Ctrl water). Samples that are represented by low biomass (DNA concentration: Supplementary Table S1, ddPCR: Supplementary Table S2) and have a close correlation with water are indicated.

comparing the genera distribution in **Figures 3** and **4** as well as the beta diversity analysis (**Figure 5**), where the abovementioned samples are clustering in close proximity with the water samples. It is therefore tempting to speculate that the low-biomass samples are partly represented by the microbiome present in rearing water, in addition to the mucus microbiome. Unfortunately, there is no appropriate way to sample skin-mucus without collecting some of the water associated with it. Our observations highlight the importance of quantifying the sample biomass using sensitive method like ddPCR to thereby determine the bacterial biomass and to ensure a correct interpretation of the microbiome profile, recognizing the potential bias from co-sampling of the surrounding environment.

The physiological response of the fish skin to stress has barely been scientifically studied, but it is generally accepted that mucus production increases upon stress events and the general immunologic state of the skin is altered (Tort et al.,

2003). Similar responses have been documented in other mucosal systems such as the mouths of mammals, where expression of mucins are increased after stress (Bosch et al., 2000), highlighting the protective role of mucus. The common aquaculture practices such as netting and transfer may cause fish stress and removal of mucus, allowing the growth of potential opportunistic bacteria. For instance, it was observed that a damaged mucus layer caused high mortality in salmonids during challenge experiments with bacteria (Svendsen and Bøgwald, 1997; Madetoja et al., 2000). In the present study, the comparison of the host microbiome before and after handling showed a shift in the composition of the community after 24 h (**Figure 3**). This was supported over both technical replicates (Tank_2 and Tank_3) by the statistic test Adonis (Supplementary Table S3). The most prominent change observed in the microbiome over the 24 h post-handling period, was the rise of the genus *Burkholderia*. The order *Burkholderiales* has also been detected as component of the skin-mucus microbiome of rainbow trout (*Oncochynchus mykiss*) and cow-nose rays (*Rhinoptera bonasus*) (Lowrey et al., 2015; Kearns et al., 2017). In particular, the genus *Burkholderia* is known to either have a beneficial or pathogenic relationship with other host organisms (Compant et al., 2008), but is mostly documented as a pathogen for plants (Jeong et al., 2003), humans (Valvano, 2006) and animals (Whitlock et al., 2007). To the best of our knowledge, this genus has not been linked to pathogenesis in fish. In addition to the increased relative abundance of *Burkholderia*-affiliated phylotyopes at T24, a decrease of *Methylobacterium*-affiliated phylotypes was observed in almost all biological replicates. Since *Methylobacterium* has mostly been detected in healthy fish, the decline of this genus may be associated with an increase of opportunistic bacteria. It is well documented that *Methylobacterium* spp. are able to produce poly-β-hydroxybutyrates, which are recognized to inhibit the growth of pathogens in other host-bacterial communities (Defoirdt et al., 2007; Halet et al., 2007; Boutin et al., 2013). It is also well known from other studies that stressful conditions change the microbial profile of the mucosal surfaces, leading to microbial imbalance (Boutin et al., 2013; Sylvain et al., 2016). The shift observed at T24 in the present study may represent the beginning stage of dysbiosis, but in the absence of a negative control (fish not exposed to netting and transfer at T3 and T24) a firm conclusions cannot be drawn. The inclusion of a true negative control was not possible due to logistic issues in the experimental setup. The fish stocking level in Tank_1 (96 kg/3.3m^3) differed substantially from the stocking level in Tank_2 and Tank_3 used for hosting the fish at T3 and T24 (4.5 kg/0,5m^3). Therefore, given the large discrepancy in fish density among the experimental tanks, the use of fish from Tank_1 as negative control would not be possible. Nevertheless, our data demonstrate for the first time that the Atlantic salmon skin microbiome can change substantially within only 24 h.

Another important aspect of this work was to investigate the similarities and diversities between the bacterial communities found in the rearing water and the skin-mucus microbiome. Analysis at the genus level of the most abundant phylotypes (**Figure 4**) illustrated similarities between water samples collected in the different tanks and at the three experimental time points. Many genera detected in rearing water were also observed in mucus such as *Lysobacter* and *Ralstonia*, in accordance with the hypothesis that fish skin mucosal microbiome is colonized by strains living in the surrounding environment (Horsley, 1973). However, as mentioned above, the low biomass observed in some of the mucus samples may have distorted the results, causing detection of members of the water microbiome in the mucus. Despite the similar genera distribution observed between the two environments, beta-diversity analysis (UniFrac and Bray-Curtis; **Figure 5**) showed distinct phylogenetic distances between water and mucus derived microbiomes. These results were statistically confirmed by Adonis (Supplementary Table S3), suggesting that each microbial community is adapted to its own environment. A similar outcome has been observed in another study on Brook Charr (*Salvelinus fontinalis),* where the role of stress on the skin-mucus microbiome was investigated using 16S rRNA gene sequencing analysis (Boutin et al., 2013). The study by Boutin et al., demonstrated that the water community shared common genera with the fish skin microbiome, although UniFrac analysis indicated significant differences between the two microbiomes. Thus, despite the skin-mucus microbiome is in direct contact with the external environment, it seems to be specifically adapted to the salmon mucus. For instance, a study conducted by McKenzie et al. (2012) showed that the skin bacteria community present on amphibians is species specific, even when different amphibian species co-exist in the same pond.

CONCLUSION

Our data suggest that netting and transfer of fish between tanks represent a potential cause for the rapid rise of Burkholderia (and decline of others). However, the lack of additional controls (fish not exposed to netting and transfer) at T3 and T24 does not allow us to rule out other influences such as the fish's acclimation to the microbial properties of the rearing water, natural temporal changes or other environmental factors (Boutin et al., 2013; Sylvain et al., 2016). The rapid community shift observed in the present study highlights that the fish skin-mucus microbiome is susceptible to significant changes within a time frame of 24 h, underling the need of a controlled bacterial community on the skin-mucus of farmed fish (Hoseinifar et al., 2015; Azimirad et al., 2016). Furthermore, we report that the abundance of bacterial biomass obtained from the skin-mucus samples is highly variable. This is an important point to take into account when analyzing skin mucus of water dwelling animals, as samples with low biomass are vulnerable to contamination by the water microbiome.

AUTHOR CONTRIBUTIONS

GM carried out the experimental work, collected and analyzed data and wrote the paper. GV-K planned the experiment, analyzed data and contributed to writing the paper. SJ planned the experiment and analyzed data. LH analyzed data and contributed to writing the paper. DP analyzed data (performed

statistical analysis). PP analyzed the data and contributed to writing the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02043/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

The skin-mucus microbial community of farmed Atlantic salmon (*Salmo salar*)

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Keywords: skin, mucus, teleost, microbiome, stress, aquaculture, *Salmo salar*

Supplementary Materials and Methods

List of commands and parameters used for the 16S analysis:

Merging:
```
>usearch8 -fastq_mergepairs for_R1.fastq -reverse rev_R2.fastq -
fastqout merged.fastq -fastq_minmergelen 300
```

Quality filtering:
```
>usearch8 -fastq_filter merged.fastq -fastq_maxee 1.0 -fastaout
filtered.fasta
```

The fasta files from all samples were concatenated to one file (also called filtered.fasta) using `cat`.

Identification of unique sequences:
```
>usearch8 -derep_fulllength filtered.fasta -sizeout -fastaout
uniques.fasta
```

Removing singletons:
```
>usearch8 -sortbysize uniques.fasta -fastaout seqs_sorted.fasta -
minsize 2
```

Trimming sequence length:
```
>usearch8 -fastx_truncate seqs_sorted.fasta -trunclen 430 -label_suffix
_430 -fastaout seqs_sorted_430.fasta
```

Removing chimera sequences and clustering:
```
>usearch8 -cluster_otus seqs_sorted_430.fasta -otus otus.fasta -
uparseout out_430.up -relabel OTU
```

Assigning taxonomy and making the OTU table:
```
>usearch8 -utax otus.fasta -db refdbF.ubd -strand both -fastaout
otus_tax_430.fasta

>usearch8 -usearch_global filtered.fasta -db otus_tax_430.fasta -strand
plus -id 0.97 -otutabout otu_table.txt
```

The tab-delimited OTU table was subsequently converted to biom table using biom convert:
```
>biom convert -i otu_table.txt -o otu_table.biom --table-type="OTU
table" --process-obs-metadata taxonomy
```

Filtering out OTUs less than 0.005% of the total sequences in QIIME:
```
>filter_otus_from_otu_table.py –i otu_table.biom -o otu_table_min5.biom
--min_count_fraction 0.00005
```

Performing rarefaction on the OTU table based on the smallest library (22070seqs/sample):
```
>single_rarefaction.py -i otu_table_min5.biom -o
otu_table_min5_even.biom -d 22070
```

QIIME core diversity analyses:
```
>core_diversity_analyses.py -o core_analysis -i
otu_table_min5_even.biom -m mapping_file.txt -c
Category1,Category2,Category3 -e 22070 -t rep_set.tre
```

Supplementary Tables and Figures

Supplementary Table S1. DNA concentrations of mucus and water samples, measured with Qubit fluorometer and the Quant-iT dsDNA HS assay kit. DNA concentrations falling below the minimum quantitative range for the Quant-iT HS assay are noted as not detected (n.d.). The presence (+) or absence (-) of PCR products are specified, in addition to the number of sequences/sample after quality trimming. Samples not included in the analysis are noted as not included (n.i.).

Sample ID	DNA conc. (ng/ml)	PCR	Sequences (n)
M1-0h-1	0.14	+	51521
M2-0h-1	0.33	+	54984
M3-0h-1	0.40	+	41772
M4-0h-1	0.36	+	54460
M5-0h-1	0.40	+	74525
M6-0h-1	0.10	+	50170
M7-0h-1	n.d.	+	47123
M8-0h-1	n.d.	+	29579
M9-0h-1	n.d.	+	45413
M10-0h-1	0.16	+	26697
M11-0h-1	n.d.	+	39172
M12-0h-1	n.d.	-	n.i.
M13-0h-1	n.d.	-	n.i.
M14-0h-1	n.d.	-	n.i.
M15-0h-1	n.d.	-	n.i.
M1-3h-2	0.34	+	43979
M2-3h-2	1.10	+	40594
M3-3h-3	0.23	+	39289
M4-3h-3	0.21	+	56028
M5-3h-3	0.46	+	44608
M6-3h-3	0.12	+	24949
M7-3h-3	0.11	+	57458
M8-3h-3	n.d.	-	n.i.
M9-3h-3	n.d.	-	n.i.
M10-3h-3	n.d.	-	n.i.
M11-3h-3	n.d.	-	n.i.
M12-3h-3	n.d.	-	n.i.
M13-3h-3	n.d.	-	n.i.
M14-3h-3	n.d.	-	n.i.
M15-3h-3	n.d.	-	n.i.
M1-24h-2	0.26	+	30325
M2-24h-2	0.12	+	48690
M3-24h-2	0.22	+	30017
M4-24h-2	0.33	+	69534
M5-24h-2	0.23	+	62707
M6-24h-2	0.21	+	77228
M7-24h-3	0.47	+	54839
M8-24h-3	0.16	+	45539
M9-24h-3	0.20	+	42385

M10-24h-3	0.18	+	68933
M11-24h-3	0.21	+	75218
M12-24h-3	n.d.	+	66863
M13-24h-3	0.31	+	46470
M14-24h-3	n.d.	-	n.i.
M15-24h-3	n.d.	-	n.i.
W1-T0-1	1.23	+	27086
W2-T0-1	2.55	+	34693
W3-T0-1	3.37	+	22070
W1-Ctrl-2	2.05	+	29241
W2-Ctrl-2	3.04	+	57359
W3-Ctrl-2	2.39	+	51639
W1-Ctrl-3	1.13	+	98187
W2-Ctrl-3	2.01	+	62745
W3-Ctrl-3	3.19	+	62536
W1-T3-2	2.97	+	83736
W2-T3-2	4.01	+	85584
W3-T3-2	3.89	+	60564
W1-T3-3	2.10	+	48070
W2-T3-3	2.02	+	48462
W3-T3-3	3.15	+	39664
W1-T24-2	1.99	+	67986
W2-T24-2	4.09	+	63784
W3-T24-2	2.09	+	31459
W1-T24-3	4.00	+	68445
W2-T24-3	3.86	+	32160
W3-T24-3	3.14	+	74109

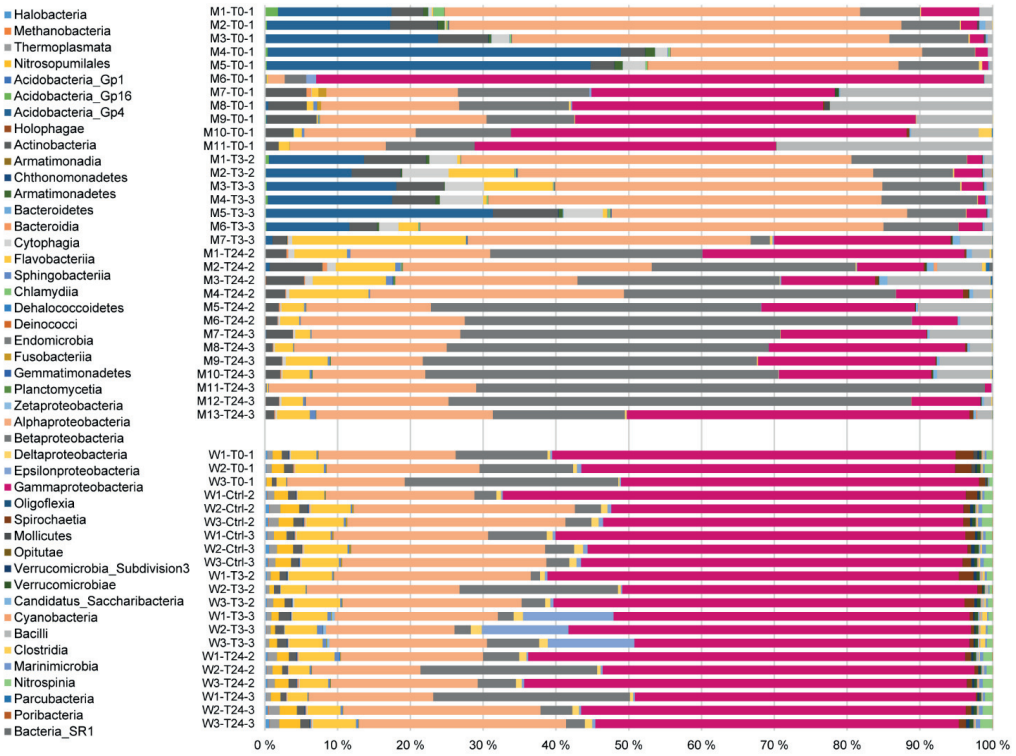
Supplementary Table S2. Droplet Digital PCR concentration values (target DNA copies/µL) of each replicate (R1, R2, R3) obtained from the QuantaSoft software. These values were used to calculate the number of copies/µL in the initial mucus and water samples at different time points (T0, T3, T24 in addition to water control; Ctrl) as explained in Materials and Methods.

Sample ID	R1	R2	R3	Average±StDev	Copies/µL in initial samples
M1-T0-1	19.55	26.35	31.40	25.77 ± 5.95	283
M2-T0-1	162.75	161.35	194.60	172.90 ± 18.81	1902
M3-T0-1	550.75	533.35	633.60	572.57 ± 53.57	6298
M4-T0-1	336.75	715.35	777.60	609.90 ± 238.59	6709
M5-T0-1	610.75	536.35	611.60	586.23 ± 43.20	6449
M6-T0-1	62.25	57.65	58.70	59.53 ± 2.41	655
M7-T0-1	5.65	4.05	3.60	4.43 ± 1.08	49
M8-T0-1	4.85	4.95	4.80	4.87 ± 0.08	54
M9-T0-1	8.45	10.85	10.40	9.90 ± 1.28	109
M10-T0-1	33.55	26.75	29.00	29.77 ± 3.46	327
M11-T0-1	9.35	4.55	3.00	5.63 ± 3.31	62
M1-T3-2	161.75	148.35	132.60	147.57 ± 14.59	1623
M2-T3-2	163.60	122.60	143.60	143.27 ± 20.50	1576
M3-T3-3	115.75	97.35	96.60	103.23 ± 10.85	1136
M4-T3-3	280.75	326.60	204.60	270.65 ± 61.62	2977
M5-T3-3	511.75	457.35	460.60	476.57 ± 30.51	5242
M6-T3-3	39.65	32.95	30.60	34.40 ± 4.70	378
M7-T3-3	183.75	190.35	164.60	179.57 ± 13.38	1975
M1-T24-2	23.15	14.85	14.70	17.57 ± 4.84	193
M2-T24-2	17.75	12.35	13.10	14.40 ± 2.93	158
M3-T24-2	8.35	6.55	5.80	6.90 ± 1.31	76
M4-T24-2	8.45	15.35	12.60	12.13 ± 3.47	133
M5-T24-2	9.55	17.45	19.00	15.33 ± 5.07	169
M6-T24-2	15.75	11.05	22.60	16.47 ± 5.81	181
M7-T24-3	72.65	26.85	33.90	44.47 ± 24.66	489
M8-T24-3	40.05	24.35	27.30	30.57 ± 8.34	336
M9-T24-3	26.10	23.00	15.40	21.50 ± 5.51	237
M10-T24-3	17.05	16.35	22.00	18.47 ± 3.08	203
M11-T24-3	277.75	293.75	315.60	295.70 ± 19.00	3253
M12-T24-3	25.45	31.45	32.50	29.80 ± 3.80	328
M13-T24-3	12.25	17.25	19.10	16.20 ± 3.54	178
W1-T0-1	612.75	535.35	724.60	624.23 ± 95.15	6867
W2-T0-1	589.75	560.35	790.60	646.90 ± 125.31	7116
W3-T0-1	1657.75	1407.35	2084.60	1716.57 ± 342.43	18882
W1-Ctrl-2	793.75	860.35	1008.60	887.57 ± 109.98	9763
W2-Ctrl-2	958.75	650.35	1166.60	925.23 ± 259.75	10178
W3-Ctrl-2	651.75	681.35	878.60	737.23 ± 123.32	8110
W1-Ctrl-3	461.75	424.35	576.60	487.57 ± 79.34	5363
W2-Ctrl-3	598.75	555.35	626.60	593.57 ± 35.91	6529
W3-Ctrl-3	918.75	1003.35	939.60	953.90 ± 44.08	10493
W1-T3-2	882.75	707.35	879.60	823.23 ± 100.37	9056
W2-T3-2	1471.75	1379.35	1758.60	1536.57 ± 197.76	16902

W3-T3-2	1110.75	972.35	1238.60	1107.23	±	133.16	12180
W1-T3-3	845.75	838.35	1093.60	925.90	±	145.28	10185
W2-T3-3	720.75	682.35	954.60	785.90	±	147.35	8645
W3-T3-3	1276.75	1256.35	1333.60	1288.90	±	40.03	14178
W1-T24-2	760.75	1242.90	924.60	976.08	±	245.16	10737
W2-T24-2	1715.75	1490.35	1978.60	1728.23	±	244.36	19011
W3-T24-2	770.75	630.35	824.60	741.90	±	100.29	8161
W1-T24-3	1443.75	1231.35	1487.60	1387.57	±	137.05	15263
W2-T24-3	1357.75	1236.35	1524.60	1372.90	±	144.72	15102
W3-T24-3	929.75	1324.35	1634.60	1296.23	±	353.27	14259

Supplementary Table S3. Permutation Multivariate Analysis of Variance (weighted UniFrac distance matrix) of mucus and water samples at different time points.

Factors	P-value
Mucus: T0 vs. T3	0.012
Mucus: T0 vs. T24	0.001
Mucus: T3 vs. T24	0.001
Mucus at T3: Tank_2 vs. Tank_3	0.650
Mucus at T24: Tank_2 vs. Tank_3	0.082
Mucus time (T0, T3, T24)	0.001
Mucus tanks (Tank_1, Tank_2, Tank_3)	0.011
Mucus vs. water	0.001
T0: water vs. mucus	0.001
T3: water vs. mucus	0.001
T24: water vs. mucus	0.001
Water: T0 vs. T3	0.044
Water: T0 vs. T24	0.214
Water: T3 vs. T24	0.096
Water: T0 vs. Ctrl	0.020
Water: T3 vs. Ctrl	0.016
Water: T24 vs. Ctrl	0.165
Water at T3: Tank_2 vs. Tank_3	0.100
Water at T24: Tank_2 vs. Tank_3	0.500
Water time (T0, T3, T24)	0.186
Water tanks (Tank_1, Tank_2, Tank_3)	0.053



Supplementary Figure S1. Relative abundance of phylotypes at the class level inherent to the mucus and water microbiomes at the different time points (T0, T3, T24 + Ctrl water).

Paper II

The farmed Atlantic salmon (*Salmo salar*) skin-mucus proteome and its nutrient potential for the resident bacterial community

Giusi Minniti, Simen R. Sandve, János T. Padra, Sara Lindén, Phillip B. Pope, Magnus Ø. Arntzen, Gustav Vaaje-Kolstad, 2018, Manuscript submitted to Applied and Environmental Microbiology.

The farmed Atlantic salmon (*Salmo salar*) skin-mucus proteome and its nutrient potential for the resident bacterial community

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Abstract

Norway is the largest producer and exporter of farmed Atlantic salmon (*Salmo salar*) worldwide. Skin disorders correlated with bacterial infections represent an important challenge for fish farmers due to the economic losses caused. Little is known about this topic, thus studying the skin-mucus of *Salmo salar* and its bacterial community depict a step forward in understanding fish welfare in aquaculture. In this study, we used label free quantitative mass spectrometry to investigate the skin-mucus proteins associated with both Atlantic salmon and bacteria. In particular, the microbial temporal proteome dynamics during 9 days of mucus incubation with sterilized seawater was investigated, in order to evaluate their capacity to utilize mucus components for growth in this environment. At the start of the incubation period, the largest proportion of proteins (~99%) belonged to the salmon and many of these proteins were assigned to protecting functions, confirming the defensive role of mucus. On the contrary, after 9 days of incubation, most of the proteins detected were assigned to bacteria, mainly to the genera *Vibrio* and *Pseudoalteromonas*. Most of the predicted secreted proteins were affiliated with transport and metabolic processes. In particular, a large abundance and variety of bacterial proteases were observed, highlighting capacity of bacteria to degrade salmon proteins.

Importance

Fish are in constant contact with the aquatic environment and its reservoir of viruses and microorganisms. The skin represents the critical outer barrier that protects the fish against potential pathogens. Integrity of the skin barrier is vital for fish health. The outermost part of the skin is constituted by a mucus layer, which consists of gel-forming mucins, proteins, peptides and lipids. Despite substantial research on fish-skin mucus, little is known about the presence of bacteria and their ability to utilize the mucus constituents as nutrients. The focus of our research lies in identifying how bacteria interact with the fish skin and especially the mucus. A deeper knowledge of how bacteria persist and proliferate in the skin-mucus is vital both from a fundamental and applied perspective given the importance of the skin for fish health and wellbeing.

INTRODUCTION

Mucus is a complex, viscous and hydrated secretion present at the interface between the epithelial surface and its external environment. The dominant constituent of mucus are mucins, a family of highly glycosylated proteins that form large macromolecular networks through intermolecular disulphide bonds (1). In addition to mucins, mucus contains salts, lipids and a variety of proteins, many related to defense functions like lysozyme, defensins, immunoglobulins, growth factors and trefoil factors (2). In most of the mucosal surfaces, mucus has a protective function, providing a semipermeable, but robust barrier that prevents unwanted chemical compounds, pathogens and parasites from reaching the epithelial cell surface (3). In mammals, mucosal surfaces line various cavities in the body and surround internal organs. In water dwelling animals like amphibians and fish, mucus is also secreted by the skin epidermis, yielding the characteristic slippery property of the skin. In bony fish, the skin-mucus components are secreted by three main cell types: goblet cells, club cells and sacciform cells (4). The properties of skin-mucus have been studied for decades and a variety of functions have been suggested, such as osmo- and ion regulation (5), gas exchange (6), reduction of fluid friction (7) and defense (8). Since the mucus layer represents the main physical barrier separating the skin epithelium from the aqueous surroundings, its role in protecting the fish from infectious agents is of major importance. Indeed, leukocytes (9) and several anti-microbial proteins and peptides (10) have been observed in mucus.

Despite the protective role of mucus against infectious pathogens, skin-mucus surfaces harbor a complex microbial community (symbiotic and/or opportunistic) (11, 12). The capability of fish to maintain a correct balance between symbiotic and opportunistic bacteria seems to be crucial to ensure fish health (13). Indeed, it is well known that skin mucins play a key role in accommodating resident bacterial flora and limiting adhesion of pathogens (1). Specifically, it seems that changes

in mucin glycan composition and possibly release of mucins with altered binding properties enable removal of microbes from the mucosal surface in order to prevent microbial infections (14-17). In a study by Padra and colleagues (18), it was observed that the mucins present on the skin-mucus were characterized by shorter glycans and a lower level of the sialic acid N-Acetylneuraminic acid compared to the mucins of the intestinal regions, which manifested itself in the form of low adhesion of the fish pathogen *Aeromonas salmonicida*. Despite the protective function of mucus, some pathogenic microorganisms have developed efficient strategies to bypass the mucosal barrier (19). Indeed, it should be noted that mammalian intestinal pathogens have been indicated to bypass intestinal mucus layers by using mucin-specific proteases (20, 21). Mucins can also regulate pathogen growth and virulence (22), and the N-acetylglucosamine residue on mucin glycans promote *Aeromonas salmonicida* growth (23). Moreover, a recent study by Sveen *et al.* showed that expression of mucin encoding genes in the skin of Atlantic salmon decreased upon handling stress, but increased in the time period following long term stress (24), highlighting the influence environmental factors have on mucus composition.

The biochemical constituents of fish skin-mucus has been studied by many, using primarily enzyme and immunological assays (25-35) and proteomics (36-43) (see also (4) and (10) for comprehensive reviews). As already noted, many of the free proteins are related to protective roles against infection. Commonly identified antimicrobial proteins are lysozymes (44), histone fragments (45), antibodies (46), lectins (47), complement components, alkaline phosphatase and proteases (48). Most proteomic studies also report findings of proteins related to cellular function such as actin, collagen, ribosome subunits and proteins related to metabolic pathways. It is not known whether such proteins originate from dead or living cells present in the mucus or if they are truly secreted with a specific biochemical function. In addition, as mentioned previously the

presence of a bacterial community in the fish skin-mucus also entails the presence of bacterial proteins secreted for the purpose of protection, adhesion, feeding, proliferation or other life sustaining functions. However, only one study (38) have reported identification of bacterial proteins in mucus.

In the current study, label-free quantitative mass spectrometry was used to investigate the exoproteome of the *Salmo salar* skin mucus and its resident bacteria. In order to identify the bacteria able to utilize mucus components as nutrients, the resulting microbial community was analyzed by 16S rRNA gene amplicon sequencing, accompanied with temporal exoproteome data, yielding an opportunity of not only identifying the bacteria present in the skin-mucus, but also for functional analysis and determination of the microbial metabolic strategies. Since data published earlier had indicated in overall low abundance of bacterial biomass in *Salmo salar* skin-mucus (49), we chose to incubate the mucus samples for 9 days at 10°C before analysis to promote growth of the resident bacteria. Additionally, this ensured sufficient biomass to allow extraction of high quality DNA and identification of the most mucus degrading genera. Finally, the exoproteome associated with the skin-mucus of *Salmo salar* was also analyzed in order to establish its composition.

MATERIALS AND METHODS

Fish and sampling procedure

Eight farmed *Salmo salar* (average weight 300 g) from the Norwegian Institute of Water Research (NIVA), Drøbak, Norway, were randomly sampled, immediately killed by a blow to the head and transferred to an autoclaved plastic bag and stored on ice. Of these fish, three (named F1, F2 and F3) were used for skin-mucus label-free quantitative proteomic analysis, whereas the remaining five (named F4, F5, F6, F7 and F8) were selected to estimate the skin-mucus mucin concentration (see below for method). The net used for sampling the fish was sterilized with 70% EtOH between each round. The tank biomass was approximately 1 kg/m^3 and the tank was supplied with seawater from 60 m depth in the Oslo Fjord with salinity of 34.7 PSU and a temperature of 7.6°C. Fish were fed with Skretting Spirit 4.5 mm pellets at ~0.5% fish weight per day. Mucus was sampled in two steps – firstly by draining the mucus accumulated in the plastic bag into a 1.5 mL plastic test tube, followed by gentle collection of the skin-mucus by a rounded plastic spatula and transfer of this mucus to the same test tube. Sampled mucus from three individuals (F1, F2 and F3) was mixed with sterilized seawater 1:10 (total volume 10 mL), gently vortexed and split into three separate sterile culture tubes, yielding three technical replicates for each mucus sample. All samples were incubated for 9 days at 10°C on a shaker (200 rpm). A total volume of 650 µL was sampled from each technical replicate after 0 (i.e. immediately after dilution of the mucus and the subsequence mixing), 2, 5 and 9 days for proteomic analysis. At day 9, 50 µL of each sample was collected for 16S rRNA gene sequencing analysis. The mucus samples (not diluted) from the remaining four fishes (F4, F5, F6 and F7) were stored at -20°C until mucin quantification. Finally, as mentioned above one additional farmed salmon (F8) was successively collected from the same farming facility for the sake of conducting a supplementing skin-mucus growth experiment. This fish

(~3,000 g) had been exposed to the same conditions as stated above. Skin-mucus collection and cultivation was also performed as stated above, but in this case, the mucus was not diluted in sterile seawater, in order to maintain as high mucin concentration in the sample as possible. Samples were collected at 0, 12, 24 and 48 hours and stored at -20°C until mucin quantification (see below).

Sample preparation for 16S rRNA gene sequencing.

DNA was extracted from bacteria grown in the diluted mucus preparation after incubation for 9 days at 10°C at 200 rpm using the DNeasy tissue kit (Qiagen, Germany), following the protocol for Gram-positive bacteria with some modifications. Achromopeptidase (1h incubation at 37°C) was utilized in the first step of the DNA extraction procedure in order to ensure lysis of Gram-positive bacteria (50). Proteinase K (40 µL) and ATL buffer (180 µL) were added to the samples, and tubes were incubated at 55°C for 1h. Successively, AL buffer (200 µL) was used as last lysis step (incubation at 70°C for 10 minutes). The manufacture's protocol was followed during the remaining steps. The extracted DNA was stored at -20°C. Sample preparation for 16S rRNA sequencing analysis by MiSeq was performed according the Illumina guide (16S Metagenomic Sequencing Library Preparation, Part. 15044223 Rev A). For this analysis, the primer set targeting the V3-V4 hypervariable regions, Pro341F (5'-CCTA CGGGNBGCASCAG-3') and Pro805R (5'-GACTACNVGGGT ATCTAATCC-3') (51) and the polymerase iProof High-Fidelity (Bio-Rad, Hercules, CA, USA) were used to amplify the 16S rRNA genes. The PCR conditions used were as follows: initial denaturation at 95°C for 3 minutes, followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and concluded by a final extension at 72°C for 5 minutes. The quality of the PCR products was validated by agarose gel electrophoresis and quantified by Qubit dsDNA HS Assay (Invitrogen). MiSeq (Illumina) was used to sequence the mucus samples at Day 9.

Bioinformatics analysis of 16s rRNA gene sequencing.

Analysis of the data was accomplished using the Usearch v.8.1861 (52) within the QIIME v1.8.0 (53) pipeline. First, paired reads were merged, quality filtered (E_max = 1) and singletons were removed. Reads were trimmed to 430 nucleotides. Subsequently, the command cluster_otus was used to identify and remove chimeric sequences from the database and to cluster the Operational Taxonomy Units (OTUs), using the UPARSE-OTU algorithm (97%). Finally, taxonomy was assigned employing the UTAX algorithm (52) with the full-length RDP training set (utax_rdp_16s_trainset15), enabling the construction of the OTU table. The data were further normalized to remove sample heterogeneity. In order to focus on the most abundant taxa, the OTUs represented by less than 0.2% of the total reads were filtered out, although they were displayed in the taxonomy plot (Figure 1) as "Others". The most abundant OTUs (>0.2%) were utilized to create a community database used for the proteomic analysis.

Sample preparation and proteomic analysis

The mixture of mucus and seawater was centrifuged at 5,500 × g for 10 minutes and the supernatant was collected and stored at -20°C until use. Protein precipitation was performed by adding 50% (v/v) Trichloroacetic acid to a final concentration of 10% (v/v), followed by incubation for 1h at 4°C. Precipitated proteins were centrifuged for 15 min at 15,000 × g, washed one time with 0.01 M HCl/90% (v/v) acetone and centrifuged again for 15 min at 15,000 × g. Subsequently, pellets were resuspended in SDS sample buffer and separated by SDS-PAGE using the AnykD Mini-PROTEAN gel (Bio-Rad Laboratories, Hercules, CA, USA) at 240 V for 12 minutes and stained using Coomassie Brilliant Blue R250. Each gel lane was cut into five bands and destained two times using 25 mM ammonium bicarbonate in 50% (v/v) acetonitrile. The

proteins were reduced and alkylated at 25°C using 10 mM DTT and 55 mM iodacetamide, respectively, with an incubation time of 30 minutes in each step. Afterwards, proteins were digested overnight with 0.1 µg trypsin (Promega, Mannheim, Germany) in 25 mM ammonium bicarbonate at 37°C as previously described (54). Prior to mass spectrometry, peptides were desalted using C18 ZipTips (Merck Millipore, Darmstadt, Germany), dried under vacuum (45°C) (Concentrator plus, Eppendorf, Denmark) and dissolved in 2% (v/v) acetonitrile and 0.1% (v/v) tri-fluoro acetic acid. Peptides were analyzed using a nanoHPLC-MS/MS system consisting of a Dionex Ultimate 3000 UHPLC (Thermo Scientific, Bremen, Germany) connected to a Q-Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano-electrospray ion source. Samples were loaded onto a trap column (Acclaim PepMap100, C_{18}, 5 µm, 100 Å, 300 µm ID × 5 mm, Thermo Scientific) and back flushed onto a 50 cm analytical column (Acclaim PepMap RSLC C_{18}, 2 µm, 100 Å, 75 µm ID, Thermo Scientific). At the beginning, the columns were in 96% solution A [0.1% (v/v) formic acid], 4% solution B [80% (v/v) acetonitrile, 0.1% (v/v) formic acid]. Peptides were eluted using a 90-min gradient developing from 4% to 13% (v/v) solution B in 2 minutes, 13% to 45% (v/v) B in 70 minutes and finally to 55% B in 5 minutes before the washing phase at 90% B. The flow rate was constant at 300 nL/min. In order to isolate and fragment the 10 most intense peptide precursor ions at any given time throughout the chromatographic elution, the mass spectrometer was operated in data-dependent mode (DDA) to switch automatically between orbitrap-MS and higher-energy collisional dissociation (HCD) orbitrap-MS/MS acquisition. The selected precursor ions were then excluded for repeated fragmentation for 20 seconds. The resolution was set to R=70.000 and R=35.000 for MS and MS/MS, respectively. For optimal acquisition of MS/MS

spectra, automatic gain control (AGC) target values were set to 50.000 charges and a maximum injection time of 128 milliseconds.

Bioinformatic analysis of proteomic data

A community database was generated by extracting and concatenating UniProt or NCBI protein entries for the 18 genera detected in the 16S rRNA analysis (Figure 1). In detail, 16 genera were extracted from UniProt (SwissProt + TrEMBL, release 2017_01) (*Bacteriovorax*, *Colwellia*, *Halomonas*, *Litoreibacter*, *Lysobacter*, *Marinomonas*, *Methylobacterium*, *Neptuniibacter*, *Phaeobacter*, *Polaribacter*, *Pseudoalteromonas*, *Ralstonia*, *Shewanella*, *Sphingomonas*, *Sulfitobacter*, *Vibrio*) and two were extracted from NCBI (*Olleya*, *Rubritalea*). In addition, UniProt entries for *Salmo* were added in the database for the detection of salmon proteins. In total, the database had 2.297.141 protein entries. MS raw files were analyzed using MaxQuant version 1.4.1.2 (55) and proteins were identified and quantified using the MaxLFQ algorithm (56). In details, the data were searched against the abovementioned community database supplemented with common contaminants such as human keratin and bovine serum albumin. In addition, reversed sequences of all protein entries were concatenated to the database for estimation of false discovery rates. The tolerance levels for matching to the database was 6 ppm for MS and 20 ppm for MS/MS. Trypsin was used as digestion enzyme, and two missed cleavages were allowed. Carbamidomethylation of cysteine residues was set as a fixed modification and protein N-terminal acetylation and oxidation of methionines were allowed as variable modifications. The "match between runs" feature of MaxQuant, which enables identification transfer between samples based on accurate mass and retention time (56), was applied with a match time window of one minute and an alignment time window of 20 minutes. All identifications were filtered in order to achieve a protein false discovery rate (FDR) of 1% and further filtering were applied to include at least one

unique peptide and at least two peptides in total. Proteins that could not be unambiguously assigned to one genus (2.5% of total protein identifications), i.e. contained only peptides with shared sequences across different genera, were omitted from further analysis. Regarding protein quantifications, the label-free quantification (LFQ) values reported by MaxQuant were used to calculate the protein abundances at the genus level. The LFQ values were summed for each genus and then log-transformed before developing the heat maps and sample comparisons. The LipoP 1.0 server (57) was used to predict the sub-cellular locations of the detected proteins (http://www.cbs.dtu.dk/services/LipoP/) and dbCAN (58) (http://csbl.bmb.uga.edu/ dbCAN/) was utilized for the prediction of carbohydrate-active enzymes. Finally, Gene Ontology (GO) (59) was used for functional annotation of the detected bacterial proteins and salmon skin-mucus proteins. The proteins were further categorized into groups of GO-terms using high-level GO-SLIMs. The GO-SLIMs, one for bacterial proteins and one for salmon proteins, were constructed using QuickGO (60) and based on the most prevalent GO terms in the dataset and with minimized hierarchical overlap in the GO structure.

Mapping of *Salmo salar* mucus proteins to RNAseq data

The Atlantic salmon RNA-seq expression data samples from 15 tissues were obtained from NCBI SRA (PRJNA72713) (Lien et al., 2016). Fastq files were adapter-trimmed before alignment to the Atlantic salmon genome (RefSeq assembly GCF_000233375.1) using STAR (v2.5.2a) (Dobin et al., 2013). We used HTSeq-count (v0.6.1p1) (Anders et al., 2015) to produce counts of uniquely aligned reads to each gene (RefSeq Annotation Release 100), and then calculated the relative gene expression levels as per kilobase of exon per million reads mapped after normalizing for the samples effective library size (see TMM method from edgeR (Robinson et al., 2010) user manual).

All downstream analyses were carried out in R (version 3.4.1). For the relationship between protein amount and gene expression, a linear model was fitted using the lm function, and spearman correlation test was performed using log2 transformed values. The resampling test for skin-expression bias in the skin protein gene set (derived from mapping salmon proteins detected by proteomics to RefSeq genes), was constructed using the sample function in R without replacement. Number of resampled top expressed genes in skin was computed from 1,000 resampled sets of 3,158 genes from all protein coding genes in *Salmo salar*. Resampling p-value was calculated as followed: p-value = (r+1)/1,000, where r is the number of resampled datasets with equal or more genes having highest expression in skin compared to the genes encoding proteins detected in Atlantic salmon skin-mucus. Heatmap of skin expression levels of the 299 genes with the highest expression in skin was plotted using the pheatmap package. Expression values were scaled by rows and clustered using spearman correlation distance.

Mucin quantification

Atlantic salmon mucin samples were treated with protease inhibitor (Sigma-Aldrich Co.) diluted 1:100, aliquoted and stored at -80°C between experiments. A ten step serial dilution of pig gastric mucin (PGM, Sigma-Aldrich Co.) with known concentration and glycosylation was used as a standard for concentration determination in the range of 500 µg/mL and 1µg/mL. PVDF membranes (Merck Millipore Ltd.) with 0.45 µm pore size and blot papers (Bio-Rad Laboratories) were pretreated with methanol (Sigma-Aldrich Co.) prior to use and 100 µL sample (diluted 1:500; 1:250 and 1:125) and PGM were slot-blotted on to the PVDF membranes. After the samples were sucked through, 100 µl dH_2O were added to the wells to retain all sample material. The membrane was air-dried, dipped in methanol and then dipped in 3% acetic acid. The membrane was stained with 1% Alcian blue 8GX (Sigma-Aldrich Co.)/3% Acetic acid (pH2.5) for 1h and then destained

three times in methanol. The membrane was scanned with a Bio-Rad Gel Doc™ EZ Imager and the intensity of the bands was quantified with the ImageJ software. The concentration of mucin samples was calculated from band intensities using PGM standard curves. The concentrations were corrected for the glycosylation differences between Atlantic salmon and PGM i.e. the glycan chain length and the ratio of charged glycan structures (61). The assay was reproduced three times.

Nucleotide sequence accession numbers

Sequence data are available at NCBI Sequence Read Archive under accession number SRP123429.

Mass spectrometry proteomic data

The mass spectrometry proteomic data have been deposited to the ProteomeXchange Consortium via the PRIDE (62) partner repository with the dataset identifier PXD008838.

RESULTS

16S rRNA gene sequencing analysis of bacteria utilizing salmon skin-mucus as nutrient source

In order to obtain an overview of the bacteria able to proliferate in the *Salmo salar* skin-mucus, 16S rRNA gene analysis was used to investigate the bacterial community established after incubating mucus in sterile seawater for 9 days at 10°C. A total of 258,237 reads were identified from 9 samples (three technical replicates from fish F1, F2 and F3), which could be assigned to 297 OTUs. In order to focus on the most abundant taxa, only the OTUs ≥ 0.2% of the total number of sequences were utilized to create a taxonomy plot (Figure 1) and to construct a community database for proteomics. The resulting data set showed a total number of 249,070 reads distributed among 23 OTUs assigned to 18 genera. The microbial community amongst the three biological replicates showed *Vibrio* as the most abundant genus in samples F1 and F2. *Rubritalea* and *Olleya* were the second most abundant genera in F1 and F2, respectively. Both F1 and F2 exhibited a consistent bacterial profile in the three technical replicates. The bacterial community identified in F3 deviated substantially from F1 and F2, as *Vibrio, Rubritalea* and *Olleya* were present in only low amounts while *Bacteriovorax* and *Ralstonia* were the dominating genera. Furthermore, larger technical variations were observed among the three replicates in F3.

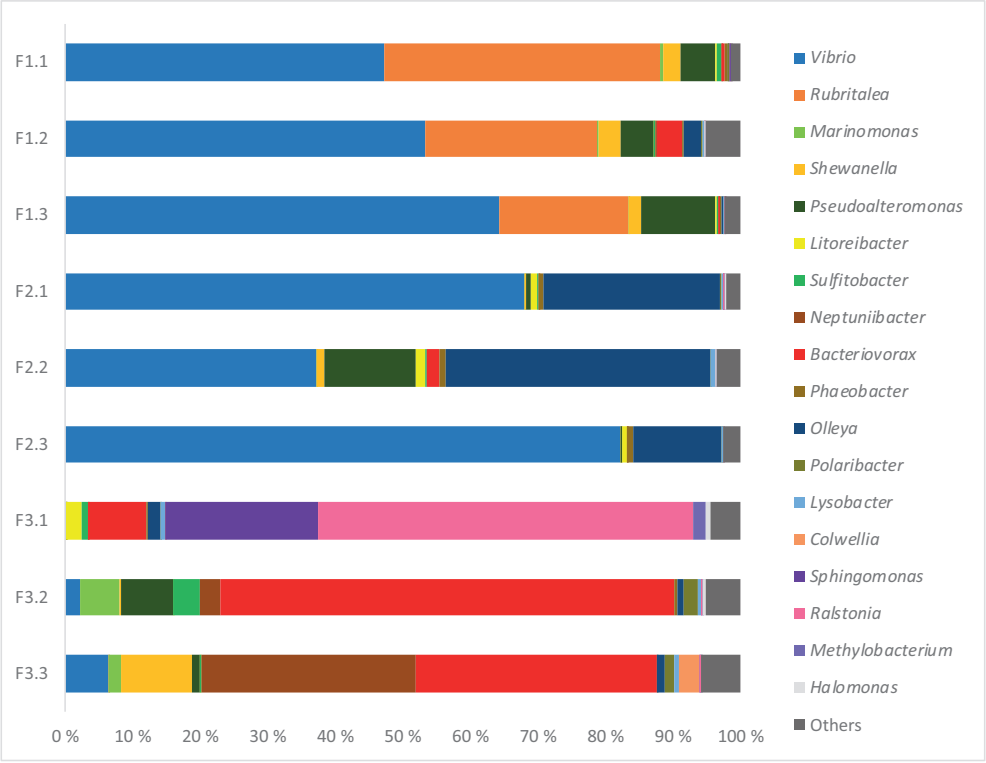

Figure 1. Relative abundances of the bacterial genera identified after 9 days of salmon skin-mucus incubation with sterilized seawater by 16S rRNA gene sequencing analysis. Each technical replicate per sampled fish is visualized separately in this figure. All the OTUs <0.2% are indicated as "Others".

Temporal quantitative proteomic analysis of the salmon-skin microbiome

Label-free quantitative proteomics was utilized to monitor the temporal dynamics of the skin-mucus microbial exoproteome. A total number of 4,563 bacterial proteins were detected in the analysis over the 9 days of incubation (Table 1 and Figure 2). Medium to high reproducibility of quantification was observed between the biological replicates at the different experimental time points (Supplementary figure S1, Pearson correlation R=0.39-0.95). The majority of the proteins detected in the analysis were assigned to *Vibrio* and *Pseudoalteromonas* genera, with 2017 and

256 1621 proteins identified, respectively (Table 1). *Shewanella*, *Neptuniibacter* and *Marinomonas*
257 were also represented with 519, 154 and 146 proteins, respectively. Furthermore, 13 additional
258 bacterial genera were detected with low protein counts. In total, 996 bacterial proteins (22%) were
259 predicted to be actively secreted (Table 1).

Table 1. The total number of proteins, at all the time points, assigned to the different genera and the amount of proteins predicted as secreted.

Genus	Protein Count	Secreted[a]
Salmo	3583	523 (15%)
Vibrio	2017	390 (19%)
Pseudoalteromonas	1621	427 (26%)
Shewanella	519	101 (19%)
Marinomonas	154	27 (18%)
Neptuniibacter	146	24 (16%)
Colwellia	33	12 (36%)
Olleya	22	10 (45%)
Halomonas	18	0
Sphingomonas	11	3 (27%)
Lysobacter	6	0
Methylobacterium	6	2 (33%)
Ralstonia	4	0
Bacteriovorax	2	0
Phaeobacter	1	0
Polaribacter	1	0
Rubritalea	1	0
Sulfitobacter	1	0
Litoreibacter	0	0

[a] Predicted using SignalP

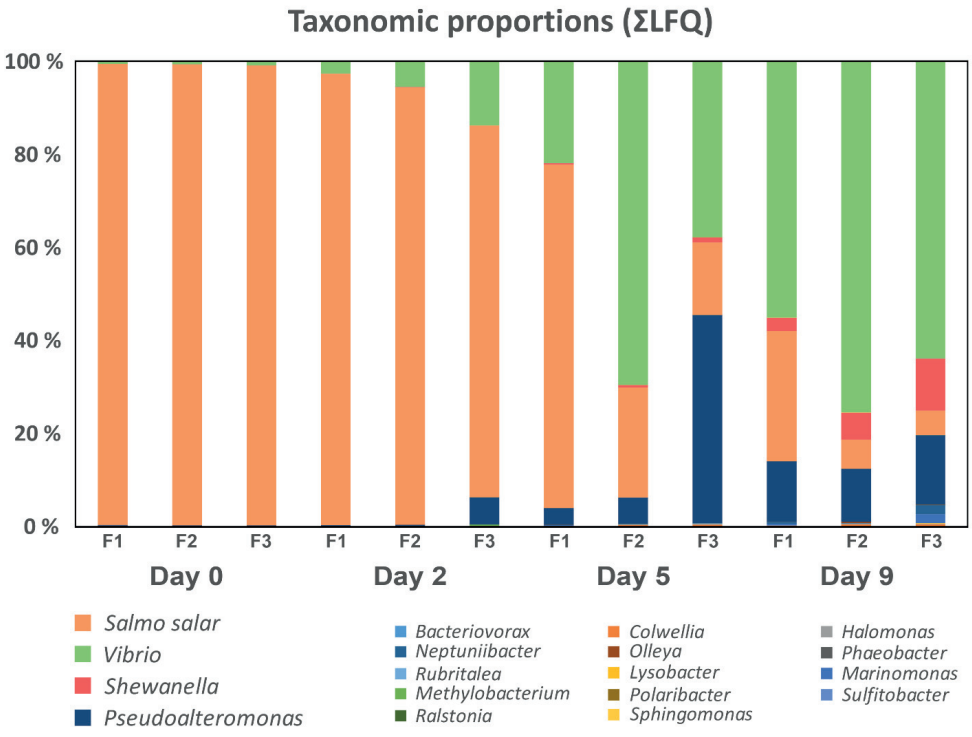

Figure 2. Taxonomic proportions of proteins in mucus over time. The bar chart shows the taxonomic proportions at the genus level, based on expressed proteins, assigned to the bacterial community over 9 days of mucus incubation with sterilized seawater. In addition, the proteins associated with salmon are displayed as "*Salmo salar*".

The taxonomic proportions changed markedly during the 9 days of incubation of salmon skin-mucus in sterilized seawater (Figure 2). At day 0, 99.2% of the proteins belonged to *Salmo salar* while 0.8% of the proteins were from bacterial origin (*Vibrio* 0.6%, *Pseudoalteromonas* 0.14%, *Shewanella* 0.03% and *Olleya* 0.03%). After 5 days, the *Salmo salar* proteins were substantially reduced and after 9 days bacterial proteins dominated the mucus exoproteome. Displaying the protein abundances as a heat map (Figure 3), we observed that *Pseudoalteromonas, Shewanella* and *Vibrio* showed a strong increase of their abundances over time, as did also *Neptuniibacter, Marinomonas, Halomonas and Colwellia*, but in lower amounts. *Ralstonia* and *Sulfitobacter* were only detectable at day 2 and onwards, whereas *Sphingomonas* and *Olleya* showed a consistent abundance during all experimental time points with minor fluctuation in their abundances. *Methylobacterium* showed a decrease in abundance over time. Finally, *Bacteriovorax*, *Polaribacter, Lysobacter*, *Phaeobacter* and *Rubritalea* were identified, but in few replicates and time points. The remaining genus, *Litoreibacter,* could not be identified at all during the analyzed time period.

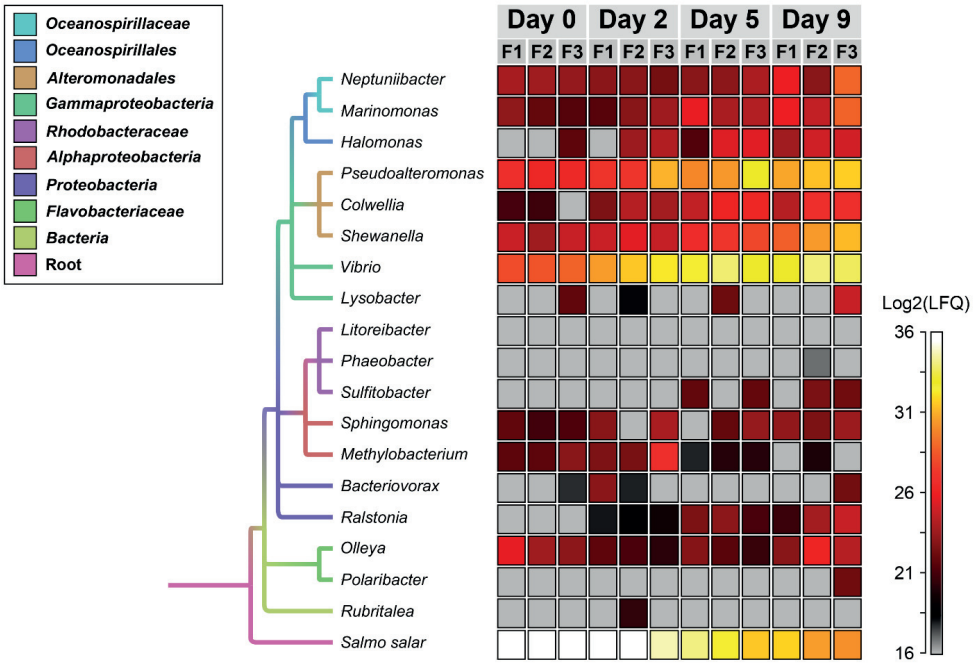

Figure 3. Heat map of the total expressed proteins. The heat map shows the abundances of expressed proteins, summarized at genus level, during 9 days of mucus incubation with sterilized seawater. It ranges from low abundance (grey), to medium abundance (red) and high abundance (white). The genera are sorted based on a phylogenetic tree made with the NCBI Common Tree tool and visualized using the software FigTree version 1.4 (available at: http://tree.bio.ed.ac.uk/).

In order to get an overview of the functional involvement of the expressed bacterial exoproteome, we used Gene Ontology (GO). The resulting table revealed a variety of processes represented in the exoproteome such as regulation, transport, metabolism, pathogenesis, cellular processes and many more (Supplementary table S2 and S3). Since the functionally relevant proteins in an exoproteome are those actively secreted by the bacteria, the identified proteins were also sorted based on the presence of a predicted leader peptide indicating secretion. When focusing on predicted secreted proteins from the dominant *Vibrio* genus, GO clustering showed proteins mainly

associated with transport and metabolic processes, (Figure 4; panel A and B). In particular, proteins assigned to siderophore transport and proteolytic activity were abundant (Figure 4; panel C and D). Of the siderophore transport proteins, nine proteins stand out; the Vitamin B12 transporter BtuB (ADA1C3IP31), ferrichrome iron receptor (ADA1C3IT79), ligand-gated channel protein (ADA1B9QID6), FhuE receptor (ADAA1C3J0C3), ferrichrome-iron receptor (A0A1C3IR55), ligand-gated channel protein (A0A1D2YG10), putative Fe-regulated protein B (A3UTN9), putative ferrichrome–iron receptor (A3UUWW6) and outer membrane receptor protein (A3UTS4). The expression of ion/ siderophore transport proteins seemed to be persistent in all the samples from day 2, although some inter-fish fluctuations occur (Figure 4; panel C). In addition, several types of proteases from *Vibrio* were detected, spanning many of the protease families as defined by the MEROPS database (63) (e.g. peptidase M4, peptidase M16, alkaline serine protease, aminopeptidases). The abundances of the individual proteases also varied within samples and sampling days (Figure 4; panel D). Overall, the most abundant proteases displayed were Peptidase M4 (A0A0P6Z347), Immune Inhibitor A (A0A1C3IQR9 and A0A1C3JD33) and Periplasmic protease (A3UZH0). The second most dominant bacterium detected in the secretome analysis was the genus *Pseudoalteromonas* (Table 1). Similar to *Vibro, Pseudoalteromonas* also showed high expression of proteins associated with metabolic processes (proteolysis) and transport (siderophore transport) (Supplementary figure S2; panel A, B, C and D) indicating the necessity of such enzymes to survive and proliferate in the *Salmo salar* mucus.

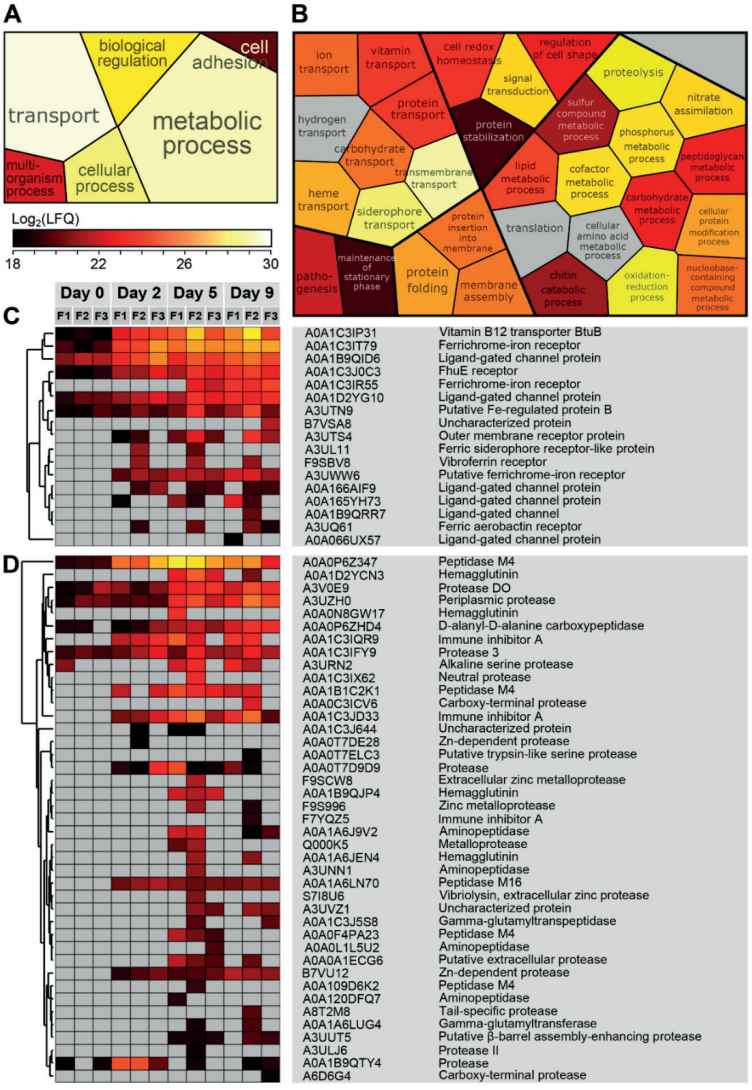

Figure 4. Functional categorization of secreted proteins from the genus *Vibrio*. The secreted proteins from *Vibrio* were grouped according to their biological function using Gene Ontology (GO), and the abundance for each group was calculated as the sum of all the individual proteins. High-level GO terms, i.e. generic biological processes, are shown in Panel A and the color of each block reflects the abundance. Panel B shows a similar representation for medium-level GO terms, i.e. more specific biological processes. Grey blocks indicate that no proteins could be mapped to this GO-term. Panel C shows a heat map of the proteins associated with 'siderophore transport', while Panel Dshows proteins assigned to 'proteolysis'. The heat map ranges from low abundance (black), to medium abundance (red) and high abundance (white).

Host proteins detected in the *Salmo salar* skin-mucus.

Quantitative proteomic analysis of the salmon skin-mucus exoproteome exhibited a total of 3,583 proteins associated with Atlantic salmon (Table 1). As expected, the *Salmo salar* proteins were most abundant at the start of the incubation period (2 days), while the remaining experimental time points were dominated by bacterial proteins (Figure 2 and 3). Similar to the bacterial, the salmon exoproteome was categorized according to biological function using a Gene Ontology (GO) approach. The GO analysis revealed that the majority of the proteins were involved in biological regulation processes, transport processes, metabolic processes, cellular processes and multi-organism processes (Supplementary table S1), typically related to roles in the cytoplasm of cells. Indeed, only 15% of proteins identified were predicted to be actively secreted (Table 1 and Supplementary table S1), indicating cell lysis or secretion by non-classical mechanisms. Some of the hallmarks of mucosal immune responses were identified, such as multiple lysozyme, lectins, calmodulin, galectins, histones, ribosomal proteins and complement related proteins. Structural proteins such as actin, keratin, tubulin, cofilin-2 and filamin-A (Supplementary table S1) were also observed. Interestingly, six of the ten chitinases encoded in the *Salmo salar* genome were detected (accession numbers A0A1S3L8D8, A0A1S3L8T9, A0A1S3LA77, A0A1S3MFN1, A0A1S3N6L1, A0A1S3P6P2 and B5DG80), which may confer protection against chitin containing pathogens (e.g. fungi) or parasites (e.g. sea lice).

In order to investigate if the salmon proteins identified in the day 0 samples are likely formed (and secreted) from active/ living, skin cells, the *Salmo salar* exoproteome was mapped against an existing RNAseq panel from 15 tissues. Of the 3,583 proteins identified in the exoproteome, 3,158 could be uniquely mapped to salmon genes (hereafter referred to as “skin protein genes”) (Supplementary figure S3). There was a significant positive correlation between the protein

abundance and steady state expression level of skin protein genes (Supplementary Figure 3; panel A), Pearson correlation p-value = 4.59^{-75}). A total number of 299 skin protein genes (9%) showed the highest gene expression level in skin in the gene expression tissue panel (Supplementary Figure 3; panel C), which is a significant enrichment of genes with skin-biased expression (Supplementary figure 3; panel B) (resampling test p-value < 0.001).

Quantification of mucins

The concentration of mucin was estimated in four mucus samples, showing concentrations ranging from 9.9 to 22.2 mg/ mL (Table 2). For one sample (F8), the depletion of mucin over time was also monitored in three technical replicates during bacterial growth in undiluted mucus. Over 48 hours the bacteria present had consumed over 50% of the mucin available (Table 2).

Table 2. Mucin concentration estimated for skin-mucus samples.

Sample		Mucin (mg/mL)
F4		9.85
F5		20.73
F6		22.18
F7		13.18
Sample	**Time (h)**	
F8	0	8.9 (3.6)*
F8	12	9.2 (2.9)*
F8	24	6.2 (1.1)*
F8	48	4.1 (0.4)*

*Values are shown as median (n=3) with range in parenthesis.

DISCUSSION

Skin mucus can be used by microorganisms as a source of nutrients

In order to explore the salmon skin-mucus microbiome and its exoproteome, 16S rRNA sequencing analysis was used to determine the bacterial community and to guide construction of the community database. In overall, *Vibrio* was the dominant genus detected (Figure 1). *Vibrio sp.* are gram-negative bacteria which live mainly in freshwater, seawater and sediment (64), although they also have been recovered from the skin of freshwater and seawater fish (65). This genus is often associated with pathogenesis in fish (66-68), highlighting its capacity to proliferate on mucosal surfaces, providing a possible explanation for their dominance in the exoproteome both before and after mucus incubation (Figure 2).

Blast comparison of the sequences assigned to *Vibrio* OTUs against the NCBI nucleotide database showed high percentage of identity (99%) with *Vibrio splendidus* and *Vibrio tapetis* species, which are well documented fish pathogens (69-71). Despite the dominance of *Vibrio sp.*, variations in the bacterial profile was observed among the three sampled fish (Fig.1), which may be correlated with a different bacterial community present in each sample. Variability has been reported among species, individuals and different body parts (12, 72, 73). Moreover, the bacterial diversity identified in the mucus after 9 days of incubation might also be influenced by non-resident bacteria (e. g water microbiome) that adhere to the mucus components (49) and grew after 9 days of incubation.

As mentioned before, the 18 bacterial genera obtained from the 16S rRNA analysis were used to generate a community database, which was utilized for determining the exoproteome associated with the microbiome proliferating in mucus and to investigate its temporal dynamics. Due to the

lack of a sample-specific metagenome database in our proteomic analysis, we used the second best approach, i.e. a sample-specific filtering of protein sequences in public repositories based on the 16S rRNA analysis, a strategy also suggested by other authors (74, 75). When applying this 16S based sample-specific filtering, we could reduce the public repository-derived database from 58M (whole UniProt bacterial section) to 2.2M and by this, increase the sensitivity of the analysis. However, we acknowledge that our approach may fail to include sample-specific strain variations in the microbial community as well as running the risk of being incomplete in terms of community structure; the results must be interpreted in light of this. Using this database, we achieved an overall ID-rate of 42%, i.e. 42% of all MS/MS spectra could be assigned to a protein; this high number indicates that our database showed good quality in terms of completion.

In general, we detected proteins involved in a variety of biological processes (Supplementary table S2 and S3), although representatives of metabolic (proteases) and transport functions (iron transport) dominated. From a proteomic study conducted by Ræder et al on the response of *Vibrio salmonicida* (now known as *Aliivibrio salmonicida*) to mucus exposure, enhanced levels of flagellar proteins, heat shock proteins, chaperonins and a variety of peroxidases were observed and suggested to enable survival of the bacterium in the mucus of salmon (76). Indeed, proteins involved in motility (flagellin and other proteins related to motility), oxidative stress response (peroxidases) and general stress response (heat shock proteins and chaperones) were observed (Supplementary table S2 and S3). The presence of flagellin is documented to be needed by pathogenic bacteria to colonize different environments (77-79). Thus, it may be important for bacterial colonization of viscous environments like mucus. Despite the identification of the important mucus growth factors mentioned above, the dominant group of proteins in the bacterial exoproteome were related to proteolysis and iron transport (see below for further discussion). It

should be noted that as bacteria are growing, cell lysis will ultimately occur giving rise to a substantial amount of cytoplasmic proteins in the exoproteome (80). Thus, the detection of multiple intracellular proteins in the present study may indicate an active defense response of the *Salmo salar* mucus (several antimicrobial proteins from the salmon were identified; see discussion further below), bacterial proteins are secreted by unconventional secretion mechanisms or simply are the result of a natural cell lysis.

Proteases and siderophore transporters dominate the bacterial exoproteome

The majority of proteins identified in the exoproteome were mapped to *Vibrio* and *Pseudoalteromonas*. *Vibrio* was the most dominant genus observed from both 16S rRNA gene sequencing and proteomic analysis (Table 1 and Figure 1 and 2), which was followed by the genus *Pseudoalteromonas* according to our proteomic dataset (Table 1 and Figure 2). For this reason, a greater emphasis will be put on these genera in the discussion below. In general, bacterial proteins were mostly identified after 5 to 9 days of mucus incubation (Figure 2). Specifically, looking at the abundances of intracellular and secreted proteins by *Vibrio* and *Pseudoalteromonas* during the 9 days of mucus incubations, higher protein expression was clearly observed over the last experimental time series (Figure 3). The increase of bacterial proteins was correlated with a decrease of fish proteins (Figure 2 and Figure 3), inferring bacterial utilization of salmon proteins as a nutrient source. Indeed, the GO analysis showed a high number of secreted proteases from both *Vibrio* (Figure 4; panel B and D) and *Pseudoalteromonas* (Supplementary figure S2; panel B and D). In a study on the mollusk pathogens *Vibrio tapetis* and *Vibrio eastuarianus* secretomes, only few predicted extracellular enzymes were detected, these being proteases, lipases and chitinases, all suggested to be virulence factors (81). Especially intriguing are the chitinases, which are also identified in the current exoproteome (Figure 4B and Supplementary Table S3) with no

obvious function, although it has been suggested that *Salmo salar* scales contain chitin (82). Chitinases have been identified as virulence factors in a variety of other studies, but no conclusive evidence has been provided yet regarding their function (83, 84) . However, activity towards other substrates than chitin has been suggested (e.g. the GlcNAc containing glycans of glycoproteins) (85, 86). Moreover, many of the proteases identified in our analysis also represent virulence factors, such as vibriolysine (87), haemmagglutinin (88) and immune inhibitor A (89). In addition, one of the most abundant proteases secreted by the genus *Vibrio* is the peptidase M4 (A0A0P6Z347) (Figure 4; panel D), which is a metallopeptidase. It is well known that different families of metallopeptidases are involved in pathogenesis (90-92).

Beside the proteases, another group of abundant bacterial proteins were related to transport processes, more specifically iron transport via siderophores or heme (Figure 4 and Supplementary figure S2; panel C). Iron acquisition is essential for bacteria, since many enzymes are dependent on this metal as a co-factor (93-95). Siderophores and siderophore receptors have also been associated with bacterial pathogenesis, where their role is to acquire iron within their vertebrate hosts (where the level of free iron is low) in order to survive and proliferate (68, 96). Although siderophores cannot be detected by proteomics, their respective outer membrane receptors (ferrichrome receptor; Figure 4; panel C), and iron complex outer membrane receptor protein; Supplementary Figure S2; panel C) were displayed at low, medium and high abundance from day 0 to day 9. The expression of siderophore transporters and siderophore receptors is associated with virulence in several *Vibrio* species (e.g. the eel pathogen *Vibrio vulnificus*) (97). For other bacteria, such as *Pseudomonas aeruginosa*, siderophores also act as signaling compounds for production of virulence factors such as proteases (98). It is tempting to speculate that the high abundance of both siderophore receptors and proteases observed in the exoproteome may be functionally connected.

An unexpected result was the low amount of bacterial glycoside hydrolases related to mucin degradation, since mucin is thought to represent the most abundant glycoprotein in the mucus (10); only one GH33 bacterial sialidase was identified (Vibrio; A0A178JAP7). In the current study, the average mucin concentration was ~16 mg/mL (Table 2), thus representing a plentiful nutrient resource. The mucus viscosity declined visibly upon incubation and bacterial growth, indicating mucin depolymerization (results not shown). Padra et al showed that the skin mucins of *Salmo salar* are relatively simple, mostly glycosylated by a disaccharide made of N-acetylgalactosamine and sialic acid (18, 61). Since the mucin was so abundant in the mucus samples, one would expect to identify multiple family GH33 sialidases or family GH101 endo-α-N-acetylgalactosaminidase in the exoproteome. However, rather than glycoside hydrolases, proteases were the dominant hydrolase activity identified. Proteases are needed to hydrolyse the peptide backbone of the mucin chains and this family of enzymes also represent important enzymes for mucin depolymerization by bacteria (19). The resulting glycopeptides would contain *O*-glycosylations that could be harvested by the bacteria through hydrolysis of the glyosidic linkages of the glycans. A second possibility could be that mucins are degraded to glycopeptides by outer membrane bound proteases and subsequently transported into the bacterial cell for further processing. Such mucin processing system has been demonstrated for *Capnocytophaga canimorsus,* a bacterium feeding on mucins in dog saliva (99). Indeed, the most abundant bacterial proteins found at day 0 and also in the other time points are proteases and TonB transporters (100) that could transport glycopeptides into the cells.

The *Salmo salar* skin-mucus proteome

In addition to analysis of bacterial proteins in the mucus exoproteome, we also analyzed skin-mucus proteins associated with farmed Atlantic salmon and their putative roles in controlling the

447 bacterial population in the mucus. GO analysis of the *Salmo salar* exoproteome showed the
448 presence of proteins involved in biological regulation processes, transport processes, metabolic
449 processes, cellular processes, multi-organism processes (Supplementary table S1). It is well known
450 that one of the major roles of the skin mucosal surface is to protect fish towards pathogens (10)
451 and the many proteomic studies on fish skin-mucus have identified a variety of proteins considered
452 to be important for the mucosal immune system. In the present proteomic dataset we detected
453 several types of ribosomal proteins, lysozymes and histones (Supplementary table S1), all known
454 for having antimicrobial properties (101, 102). In addition, structural proteins, in agreement with
455 other studies (38, 103, 104), were detected in the salmon mucus. Actin has been identified in
456 mammalian mucus mainly during infection processes (105, 106), thus its presence in fish may be
457 correlated simply with a discharge of damaged cells into mucus. However, some studies (103, 107)
458 suggested the importance of actin and keratin as part of the fish immune response and defense.
459 Surprisingly, hemoglobin was also detected in our analysis. The presence of hemoglobin in mucus
460 may be correlated with blood contamination during the sampling procedure, although it was
461 previously identified in the epidermal mucus of stressed fish (108), suggesting a possible role in
462 the fish mucosal surfaces. For instance, hemoglobin-derived peptides in vertebrates have been
463 shown to have antimicrobial activity (109).

464 Most of the salmon proteins identified in mucus (85%) were predicted to be intracellular (Table
465 1). Proteomic studies of the human intestinal mucus showed similar characteristics, where the high
466 abundance of intracellular proteins was explained by the presence of detached epithelial cells
467 present in the mucus that may leak or rupture upon sample preparation (2). Indeed, epithelial cell
468 shedding is commonly observed in the intestine (110) and is related to the maintenance of tissue
469 homeostasis (111). Although mammalian intestinal mucus and fish skin-mucus are not directly

comparable, it is tempting to speculate the detection of epithelial cells in mucus due to lysis during sample collection, or to bacterial degradation, which may cause the leakage of intracellular proteins. A recent study by (112) showed that intestinal mucus of *Salmo salar* smolts contained a large amount of apoptotic cells resulting in the extraction of host DNA. Other studies of fish skin-mucus also report a dominance of putatively intracellular proteins (38, 103) and it is possible that many of these originate from cells found on the skin surface. Unfortunately, there is no appropriate way to avoid collection of epithelia cells together with mucus. In the present study, we attempted to minimize the inclusion of epithelial cell proteins in our samples during the samples by collecting the mucus from the fish as gently as possible; firstly, by putting the fish in sterile plastic bags so the majority of the mucus could be obtained by draining the plastic bag. Secondly, the remaining mucus on the fish skin was gently removed by using a rounded plastic spatula. Analyses of tissue expression bias of the genes encoding the *Salmo salar* proteins from mucus also supported that that these proteins originated mainly from skin cells (Supplementary figure S3).

Since the concentration of mucins is high in the fish skin-mucus (Table 2), one would expect to find mucins in the proteome despite their high degree of glycosylation and low complexity amino acid sequence. Indeed, proteins annotated as mucin-5B-like (A0A1S3PVV9), intestinal mucin like (A0A1S3QCU3), and mucin-5AC-like (A0A1S3KVA9) were detected in relative large abundances in the proteome. A recent study by *Sveen et al.* characterized seven mucin encoding genes from Atlantic salmon and their tissue specific expression (24). Of these seven, two genes were predominantly expressed in the skin (*muc5ac.1*: gene ID XP_013982550.1, protein ID A0A1S3KVA9 and *muc5b*: gene ID XP_014031349.1, protein ID A0A1S3PVV9). Proteins representing both these genes were identified in the present proteome (see text above and Supplementary Table 1), but none of the other five mucins. This further highlights the tissue

493 specificity of some of the mucins as suggested by Sveen *et al.* In addition, our data suggest that
494 the "intestinal mucin like" mucin (protein ID A0A1S3QCU3) is expressed in the skin.

495 Interestingly, glycoside hydrolases predicted to deglycosylate mucins were also detected in
496 abundance amongst the *Salmo salar* proteins (several putatively secreted GH33 sialidases, GH20
497 N-acetylhexosaminidases and GH89 α-N-acetylglucosaminidases; see Supplementary Table S1).
498 It is not known why such enzymes are present in the mucus, but they may be involved in
499 remodeling of the mucins or degrading/ modifying bacterial lipopolysaccharides; it is established
500 that some bacteria decorate their cell surface with common host glycans like sialic acid in order to
501 evade the immune system (113). In mammals, it has been shown that infection induce mucin
502 glycosylation changes, which in turn affect pathogen adhesion (16, 114). Similarly, infection and
503 microbial products induce changes in mucosal glycosylation in fish (14, 15), indicating that
504 infections can affect interactions with pathogens in the fish too.

505 In conclusion, the present study gives a first glance of the skin-mucus proteins of farmed *Salmo*
506 *salar*, taking into account both proteins associated with the salmon and the bacterial community
507 present on the fish. The Atlantic salmon skin-mucus exoproteome revealed proteins involved in
508 different processes such as biological regulation processes, transport processes, metabolic
509 processes, cellular processes and multi-organism processes. This results indicate the occurrence of
510 cell lysis, which represent a general challenge when dealing with the proteome of mucosal
511 surfaces. Nevertheless, multiple antimicrobial proteins and enzymes were detected, emphasizing
512 the protective role of mucus. Moreover, our study indicates that the antimicrobial function of the
513 skin-mucus did not prevent bacterial proliferation using mucus constituents as the main nutrient
514 source, a property monitored over time by determining the temporal proteome dynamics of the

most abundant genera. At the beginning of the incubation period, the largest proportion of proteins (>99%) belonged to the host. Successively, proteins associated with the genera *Vibrio* and *Pseudoalteromonas* dominated the mucus exoproteome, highlighting a clear decrease of the salmon proteins. Indeed, the GO analysis showed the expression of several types of proteases by these bacteria, which may be used to degrade salmon proteins to grow in mucus. The use of label-free quantification mass spectrometry to characterize the salmon skin-mucus proteome, focusing on both salmon and bacterial proteins, in addition to their temporal proteome dynamics, represent a new approach of study, which may contribute to better understanding the etiology of skin disorders associated with farmed fish.

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SUPPLEMENTARY MATERIAL

The farmed Atlantic salmon (*Salmo salar*) skin-mucus proteome and its nutrient potential for the resident bacterial community

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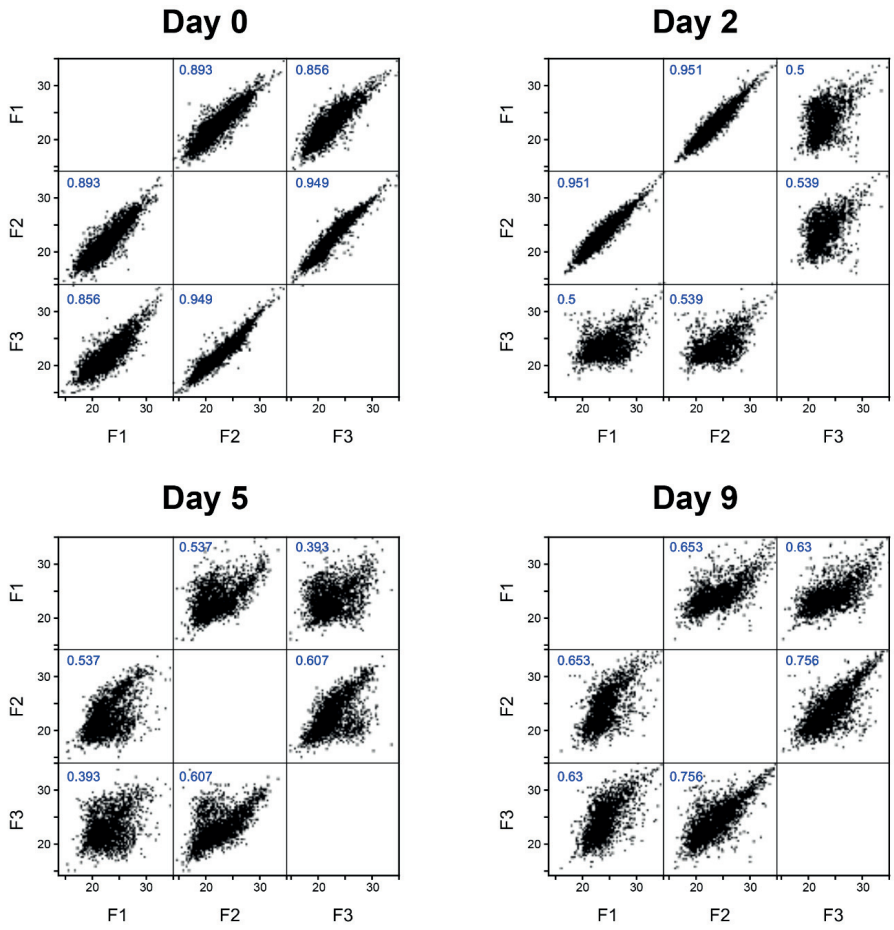
Supplementary tables S1, S2, S3 are available online: arken.nmbu.no/~gustko/Supplementary_tables.xlsx

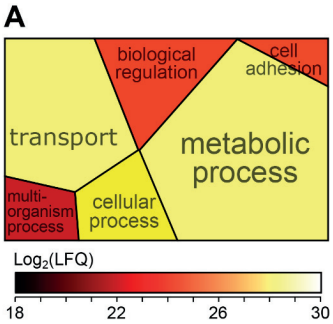
Supplementary table S1 displays all the proteins (intracellular and secreted) assigned to *Salmo salar* and identified by label-free quantitative mass spectrometry. The detected proteins were assigned into different groups according their biological functions. Protein abundances are shown for each experimental time series, together with the posterior error probability, number of identified peptides, number of unique peptides and all protein IDs that were placed in the same protein group, as reported by MaxQuant.

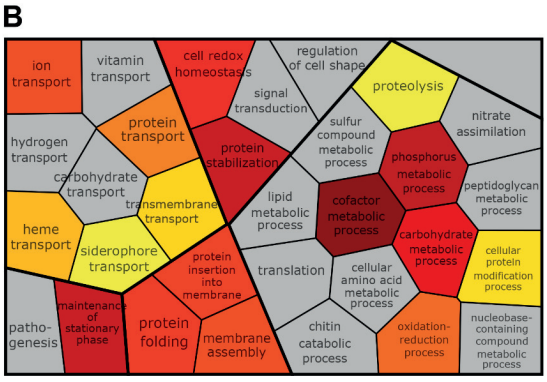
Supplementary table S2 displays all the intracellular proteins assigned to bacteria and identified by label-free quantitative mass spectrometry. The detected proteins were assigned into different groups according their biological functions. Protein abundances are shown for each experimental time series, together with the prosterior error probability, number of identified peptides, number of unique peptides and all protein IDs that were placed into the same protein group, as reported by MaxQuant.

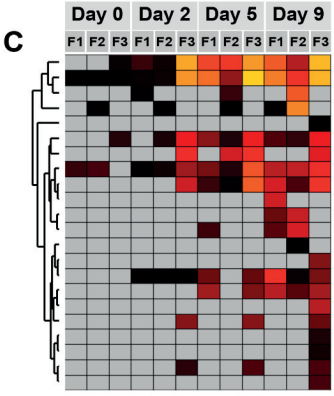
Supplementary table S3 displays all the secreted proteins assigned to bacteria and identified by label-free quantitative mass spectrometry. The detected proteins were assigned into different groups according their biological functions. Protein abundances are shown for each experimental time series, together with the prosterior error probability, number of identified peptides, number of unique peptides and all protein IDs that were placed into the same protein group, as reported by MaxQuant.



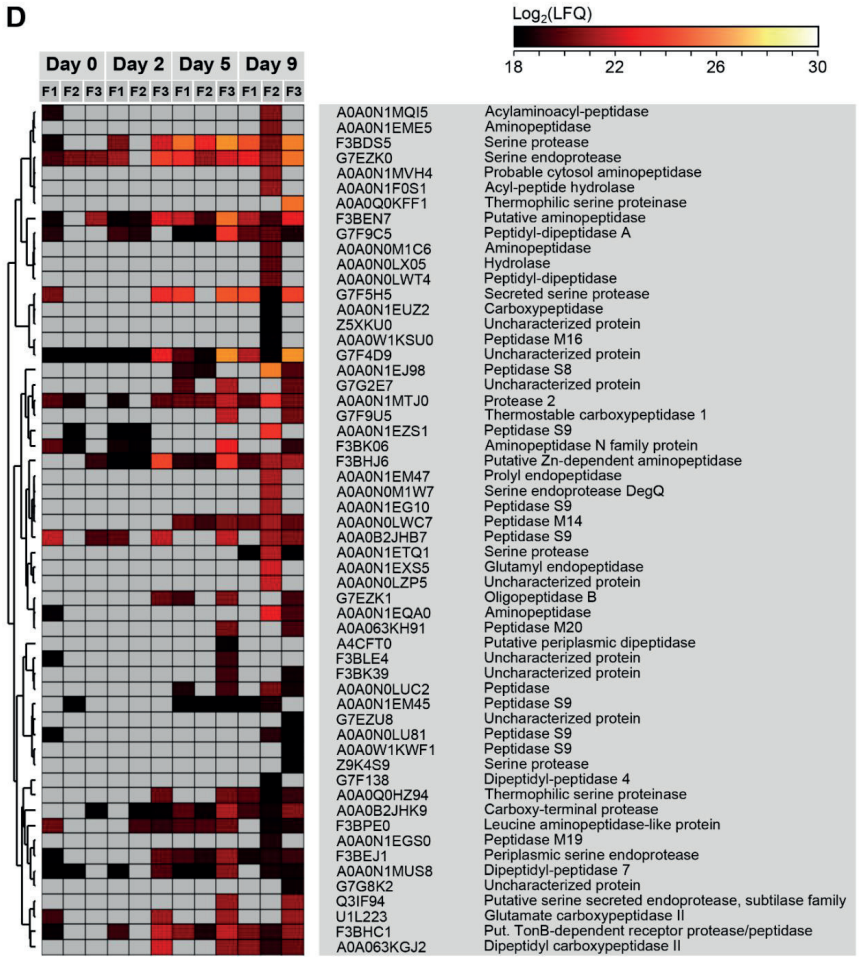
Supplementary figure S1. Scatterplot matrix to visualize replicate consistency of mucus samples (mucus from three fish; F1, F2 and F3) incubated with sterialized seawater and harvested at Day 0, 2, 5 and 9. X- and Y-axis indicate log2-transformed protein abundance and the numbers inside the plots indicate Pearson correlation, ranging between R=0.39-0.95.



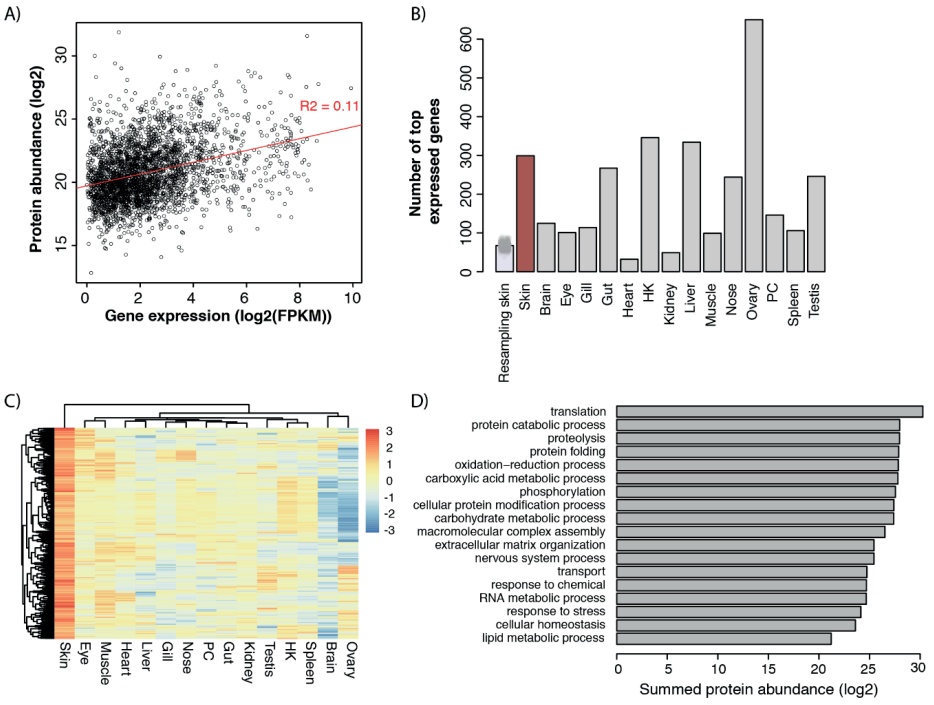




G7F6H9	Iron complex outermembrane recepter protein
A0A0U2WWR8	OM receptor for fe. coprogen and fe. rhodotorulic acid
A0A0N1EJ79	TonB-dependent receptor
A0A0N0M1T1	Membrane receptor protein
Q3IFM9	Putative OM receptor for ferric iron uptake
A0A0W1LBB8	Molybdenum ABC transporter permease
A0A0W1KSF9	TonB-dependent receptor
G7G0U3	Iron complex outermembrane recepter protein
A0A0W1LIS2	TonB-dependent receptor
A0A0Q0NIH6	FhuE receptor
A0A0N1EP68	Uncharacterized protein
A0A0N0M1T4	Ferric siderophore receptor
Q15TJ6	TonB-dependent receptor
F3BL73	Ferrichrome-iron receptor
A0A0Q0NIJ1	FhuE receptor
Q3IDJ5	Putative TonB-dependent receptor
U1L4R3	TonB-dependent receptor
G7EHB3	Iron complex outermembrane recepter protein
G7F518	Iron complex outermembrane recepter protein
A0A0Q0JH34	FhuE receptor
A0A0B2JF29	TonB-dependent receptor
Z9K2P7	TonB-dependent receptor



Supplementary figure S2. *Pseudoalteromonas* secreted proteins assigned into different groups according to their biological functions. The secreted proteins from *Pseudoalteromonas* were grouped according to their biological function using Gene Ontology (GO), and the abundance for each group was calculated as the sum of all the individual proteins. High-level GO terms, i.e. generic biological processes, are shown in Panel A and the color of each block reflects the abundance. Panel B shows a similar representation for medium-level GO terms, i.e. more specific biological processes. Grey blocks indicate that no proteins could be mapped to this GO-term. Panel C shows a heat map of the proteins associated with 'siderophore transport', while Panel D shows proteins assigned to 'proteolysis'. The heat map ranges from low abundance (black), to medium abundance (red) and high abundance (white).



Supplementary figure S3. Tissue gene expression. Panel A shows the correlation between skin surface protein abundance and gene expression of the same proteins in skin tissue. The positive correlation is highly significant in a spearman correlation test ($p=4.59^{-75}$). A linear model fitted to the data and the proportion of the variance explained is indicated with red line and text. Panel B indicates the number of genes with the highest expression across all tissues in the skin protein gene set. Of the 3158 genes in the gene set, 299 had the highest expression in skin. The "resampling skin" bar shows the mean (bar height) and distribution (grey points) of the number of genes with highest expression in skin in 1000 random samples of 3158 protein coding genes. Panel C Heatmap shows the tissue expression distribution for the 299 genes with highest expression in skin. Expression levels are scaled by rows. Red indicates higher expression and blue lower expression. Finally, panel D displays the most prevalent biological processes (GO) and their summed protein abundance of the 299 proteins with highest expression in skin.

Paper III

Characterization of putative virulence factors with chitinolytic activity from *Aliivibrio salmonicida*

Giusi Minniti, Jennifer S. M. Loose, Sophanit Mekasha, Bastien Bissaro, and Gustav Vaaje-Kolstad, 2018, Manuscript.

Characterization of putative virulence factors with chitinolytic activity from *Aliivibrio salmonicida*

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ABSTRACT

Genes encoding putative chitin degrading enzymes are present in the genomes of most bacterial fish pathogens. Logically, one would expect these enzymes to be involved in chitin degradation, but recently both chitinases and chitin targeting lytic polysaccharide monooxygenases (LPMOs) have been identified as virulence factors targeting other substrates. In the current study, enzymes predicted to be active towards chitin (a family GH18 chitinase and two family AA10 lytic polysaccharide monooxygenases (LPMOs) from the causative agent of Cold Water Vibriosis, *Aliivibrio salmonicida*, were cloned and characterized with respect to activity towards chitin and other relevant substrates in order to evaluate their possible role as virulence factors. The characterized enzymes all had operational stability around 30 °C, a common value for cold-adapted enzymes, in accordance with the psychrophilic character of the source bacteria *Aliivibrio salmonicida*. The family GH18 chitinase was able to depolymerize α- and β-chitin, but its activity was up to 50-fold lower compared to other well-studied chitinases from *Serratia marcescens* and *Cellvibrio japonicus*. The two LPMOs showed activity on chitin, cleaving the substrate chains by oxidation. Activity towards more complex, and potentially natural substrates such as *Salmo salar* skin mucus or scales was not detected for any of the enzymes.

INTRODUCTION

Chitin is a linear polysaccharide consisting of β-1,4 linked *N*-acetylglucosamine units. It represents the second most abundant polysaccharide in nature, after cellulose, serving as a structural element in many organisms (Gooday, 1990a; b). Enzymes related to chitin degradation have been studied for more than a century, since their discovery by Noel Bernard in 1911 (Bernard, 1911). Today, the database of carbohydrate active enzymes (CAZy) classifies chitin degrading enzymes in family GH18 and GH19 (chitinases), family GH20 (N-acetylhexosaminidases) and family AA10 and AA13 of the auxiliary activities (lytic polysaccharide monooxygenases) (Cantarel et al., 2008; Levasseur et al., 2013). The activity of these enzymes has been amply described in the soil bacterium *Serratia marcescens* (Vaaje-Kolstad et al., 2013). The processive and non-processive chitinases represent the enzymes that do most of the work in depolymerizing chitin chains to soluble chitooligosaccharides. However, the efficiency of this process is dependent on the action of lytic polysaccharide monooxygenases (LPMOs) that cleave the glycosidic bonds in the crystalline regions of chitin by an oxidative mechanism (Vaaje-Kolstad et al., 2010; Phillips et al., 2011; Quinlan et al., 2011), thereby rendering the substrate more accessible for chitinases. The soluble chitooligosaccharides resulting from the combined action of the chitinases and LPMOs are finally hydrolyzed into monomeric units (*N*-acetylglucosamine, GlcNAc) and chitobionic acid (GlcNAcGlcNAc1A) by *N*-acetylhexosaminidases (Vaaje-Kolstad et al., 2013).

Most chitinolytic enzymes hitherto characterized are related to the depolymerization of chitin for a metabolic purpose. Organic material containing chitin is abundant in the ocean and it is thus not uncommon to find genes encoding enzymes related to chitin degradation in the genomes of marine bacteria, like e.g. *Vibrio anguillarum* and *Aliivibrio salmonicida*. Indeed, *Vibrio anguillarum* grows efficiently on chitin (Takiguchi and Shimahara, 1989). From analysis of the potential chitinolytic machinery of *Aliivibrio salmonicida*, it seems to have as well the necessary genes to depolymerize chitin; a GH18 chitinase, GH20 *N*-acetylhexosaminidases and AA10 LPMOs. However, when attempting to culture the bacteria on chitin, *Aliivibrio salmonicida* is not able to grow despite its theoretical chitinolytic potential (Egidius et al., 1986; Hjerde et al., 2008). Indeed, a deeper analysis of the bacterium genome performed by Hjerde *et al.* revealed that its chitinolytic machinery once had been substantial, but due to gene decay, only half of the genes in the chitin utilization pathway remained intact (Hjerde *et al.* 2008).

In the last years, there is an increasing number of studies that report involvement of both chitinases and LPMOs in bacterial infection mechanisms where chitin is not the obvious substrate target, for example where mammals represent the host of the infection (Joshi et al., 2005; DebRoy et al., 2006; Chaudhuri et al., 2010; Mondal et al., 2014). For instance, it was demonstrated that an extracellular chitinase from *Vibrio cholerae* called ChiA2 is involved in pathogenesis during infections in humans. This enzyme seems to degrade mucins promoting bacterial infection (Mondal et al., 2014). Additionally, another study indicated that a protein called GbpA, later probed to be a multi-domain LPMO (Loose et al., 2014), was able to promote bacterial colonization of the human intestine through mucins binding (Kirn et al., 2005).

Aliivibrio salmonicida is the causative agent of Cold Water Vibriosis (CWV), a disease that caused severe losses of farmed Atlantic salmon in the past (Egidius et al., 1981; Holm et al., 1985; Evensen et al., 1991; Austin et al., 2012). CWV causes hemorrhages on the fish skin surface and internally in the organs (Bruno, 1996). Today, the disease is under control, although its re-occurrence has been observed (Hjeltnes, 2014). The capability of *Aliivibrio salmonicida* to infect Atlantic salmon has been suggested to be correlated with the presence of chitin (Kashulin et al., 2017), which has been detected in fish scales (Wagner et al., 1993; Tang et al., 2015). However, as abovementioned the bacterium is not able to grow on chitin (Egidius et al., 1986; Hjerde et al., 2008), suggesting an alternative substrate target for these enzymes, possibly similar to what has been observed in mammals.

In the present study, the chitinolytic enzymes (*As*Chi18A, *As*LPMO10A and *As*LPMO10B) from *Aliivibrio salmonicida* have been characterized with respect to their ability to degrade different allomorphs of chitin (α- and β-chitin) in addition to other biologically relevant substrates like scales and mucus of Atlantic salmon. Moreover, the activity of *As*Chi18A was compared to the chitinases of *Serratia marcescens* and *Cellvibrio japonicus*, which both are well known for their efficiency in chitin degradation.

MATERIAL AND METHODS

Cloning, expression and purification

Codon-optimized genes encoding the following *As*LPMO10A (residues 1-491, UniProt ID; B6EQB6), *As*LPMO10B (residues 1-395, UniProt ID; B6EQJ6) and *As*Chi18A (residues 1-846, UniProt ID; B6EH15) from *Aliivibrio salmonicida* (LFI1238) were purchased from GenScript (Piscataway, NJ, USA). Gene-specific primers (Table S1), with sequence overhangs corresponding to the pre-linearized pNIC-CH expression vector (AddGene, Cambridge, Massachusetts, USA) were used to amplify the genes in order to insert them into the vector by a ligation independent cloning method (Aslanidis and De Jong, 1990). All the cloned genes contained their native signal peptides. Sequence-verified plasmids were transformed into ArcticExpress (DE3) competent cells (Agilent Technologies, California, USA) for protein expression. Cells harboring the plasmids were inoculated and grown in Terrific Broth (TB) medium supplemented with 50 µg/mL of kanamycin (50 mg/mL stock). Cells producing the full-length *As*LPMO10s were cultivated in flask-media at 37 °C until OD = 0.7, cooled down for 30 min at 4 °C, induced with 0.5 mM IPTG and incubated for 44 hours at 10 °C with shaking at 200 rpm. Cells producing *As*Chi18A were grown in a Harbinger LEX bioreactor system (Epiphyte Three Inc, Toronto, Canada) using the same procedure described above, although the cell were cultured for a shorter time period (12 hours) and air was pumped into the culture by spargers. Successively, cells were harvested using centrifugation and the periplasmic extracts were generated by osmotic shock (Manoil and Beckwith, 1986). The periplasmic fractions, containing the mature proteins (signal peptide-free), were sterilized by filtration (0.2 µm). *As*LMO10A and *As*LPMO10B were purified by anion exchange chromatography using a 5 mL HiTrap DEAE FF column (GE Healthcare) and a running buffer (buffer A) consisting of 50 mM Bis-Tris-HCl pH 6.0. A linear salt gradient of buffer B (Bis-Tris-HCl pH 6.0 and 500 mM NaCl) from 0 to 100 % over 20 column volumes, with a flow rate of 1 mL/min, was applied to the column. Furthermore, hydrophobic interaction chromatography using a 5 ml HiTrap Phenyl FF (HS) column (GE Healthcare) was performed. The running buffer/binding buffer (buffer A) consisted of 50 mM Tris-HCl pH 7.5 and 1 M $(NH_4)_2SO_4$, whereas the elution buffer (buffer B) contained 50 mM Tris-HCl pH 7.5. The latter was applied with a gradient from 0 to 100% over 20 column volumes and with a flow rate of 1.5 mL/min. In addition, *As*LPMOB was further purified by size exclusion chromatography using a HiLoad 16/60 Superdex 75 column operated at 1 mL/min and with a

running buffer containing 1x PBS, pH 7.4. *As*Chi18A was purified by anion exchange chromatography using a running/binding buffer (buffer A) that consisted of 20 mM Tris-HCl pH 8.0 and 5 mM imidazole. A linear gradient of buffer B (20 mM Tris-HCl pH 8 and 500 mM of imidazole) from 0 to 100 % over 20 column volumes, with a flow rate of 3 mL/min, was utilized to elute the bound proteins from the column. All protein purifications were carried out at 8 °C. Protein purity was analyzed by SDS-PAGE. Fractions containing the pure proteins were pooled and concentrated using Amicon Ultra centrifugal filters (Millipore, Cork, Ireland). Protein concentrations were determined by measuring A_{280} and using the proteins' calculated molar extinction coefficients (via ExPASy ProtParam online tool). Before use, *As*LPMO10A and *As*LPMO10B were saturated with Cu(II) by incubation with excess of $CuSO_4$ in a molar ratio of 1:3 for 30 minutes at room temperature. The excess Cu(II) was eliminated by a desalting PD MidiTrap G-25 column (GE Healthcare) equilibrated with 50 mM Tris-HCl pH 8.0 and 150 mM NaCl.

Enzyme reactions

For activity assays, standard *As*LPMO10 reactions contained 10 mg/mL of either squid pen β-chitin (France Chitin, Orange, France), or shrimp shell α-chitin purchased from Chitinor As (Avaldsnes, Norway) as substrate. Chitin was incubated with 20 mM Tris-HCl pH 7.5, in 2 mL Eppendorf tubes at 30 °C and stirred in an Eppendorf Comfort Thermomixer at 700 rpm. The final enzyme concentrations in the reactions were 1.0 μM and reactions were started by the addition of 1.0 mM of ascorbic acid (this activates the LPMOs). Similar reaction conditions were used for *As*Chi18A, although the final enzyme concentration used was 0.5 μM and ascorbic acid was not added in the reactions. At regular intervals, samples were taken from the reactions and the soluble fractions was separated from the insoluble substrate particles using a 96-well filter plate (Millipore) operated with a vacuum manifold. Subsequently, the soluble fraction of *As*LPMO10s-catalyzed reactions were incubated with 1.5 μM of a chitobiase from *Serratia marcescens* (also known as *Sm*CHB or *Sm*GH20A) at 37 °C overnight in order to convert LPMO products to *N*-acetylglucosamine (GlcNAc) and chitobionic acid (GlcNAcGlcNAc1A) (Loose et al., 2014; Forsberg et al., 2016), followed by a sample dilution with 50 mM H_2SO_4 in a ratio of 1:1 prior quantification by HPLC (see below). The soluble fractions of *As*Chi18A reactions, were diluted with H_2SO_4 after the filtration step, which stopped the enzymatic reaction, before quantification

of $(GlcNAc)_2$ by HPLC (see below). Additionally, in order to collect samples for product profiling by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS, see below) of the two *As*LPMO10s-catalyzed reactions, 5 µL of the soluble fraction was sampled after filtration and kept at -20 °C prior to analysis.

Product analysis and quantification

Qualitative analysis of the native and oxidized products of the *As*LPMO10A and -B soluble fractions were performed by MALDI-TOF MS (Vaaje-Kolstad et al., 2010). For this analysis, 1 µL of sample was mixed with 2 µL 2,5-dihydroxybenzoic acid (9 $g.L^{-1}$), prepared in 150:350 H_2O/Acetonitrile), applied to a MTP 384 target plate in ground steel TF (Bruker Daltonics) and dried under a stream of warm air. The samples were analyzed with an Ultraflex MALDI-TOF/TOF instrument (Bruker Daltonics GmbH, Bremen, Germany) equipped with a Nitrogen 337 nm laser beam, using Bruker FlexAnalysis software. For the quantitative analysis of all the enzymes soluble products a Dionex Ultimate 3000UHPLC system (Dionex Corp., Sunnyvale, CA, USA), equipped with a Rezex RFQ-Fast acid H^+ (8%) 7.8% x 100 mm column (Phenomenex, Torrance, CA) was used. The column was pre heated to 85 °C and was operated by running 5 mM H_2SO_4 as a mobile phase at a flow rate of 1 mL/min. The products were separated isocratically and detected by UV absorption at 194 nm. The amount of GlcNAc and $(GlcNAc)_2$ were quantified using standard curves. Pure GlcNAc and $(GlcNAc)_2$ were obtained from Sigma and Megazymes, respectively. In order to quantify chitobionic acid (GlcNAcGlcNAc1A), a standard was produced in-house by treating chitobiose (Megazymes) with a chitooligosaccharide oxidase (ChitO) from *Fusarium graminearum* , which yields 100% conversion of chitobiose to chitobionic acid (Loose et al., 2014). Standards were regularly analysed in each run.

Protein binding assays

The binding capacity of *As*LPMO10s and *AsChi*18A on α-chitin, β-chitin and Atlantic salmon scales was tested, suspending 10 mg/mL of substrate in 20 mM Tris-HCl pH 7.5 to a total volume of 350 µL in 2 mL Eppendorf tubes. The scales of the Atlantic salmon were obtained from a fresh 3.5 kg farmed Atlantic salmon purchased from the local fish store. Scales were scraped off the fish, then washed in MilliQ water by magnetic stirring in a beaker. The water was changed regularly during a period of 6 h until no change in turbidity was observed. Water was then decanted

off and the washed scales were dried overnight in a 65 °C heating cabinet. Scales were stored in a sealed container at room temperature until use. Reactions were started by the addition of *As*LPMO10A or –B (0.75 μM final concentration) or *AsChi*18A (0.50 μM), which were incubated in 2 mL Eppendorf tubes, at 30 °C and shaken in an Eppendorf Comfort Thermomixer at 700 rpm. Samples were taken (100 μL) after 2 hours and immediately filtrated using a 96-well filter plate (Millipore) operated with a vacuum manifold to obtain the unbound protein fraction. In order to assess the percentage of bound proteins to the substrate, control samples with only enzyme and buffer were performed, representing the maximum quantity of protein present in the samples (100%). The protein concentration in each sample was determined using the Bradford assays (Bio-Rad, Munich, Germany).

Table S1. Primers used to amplify the genes encoding *As*LPMO10A, *As*LPMO10B and *AsChi*18A. The overhang sequences are shown as bold letters.

Cloning primers	**Sequence (5'-3')**
pNIC-CH/*As*LPMOA (forward)	**TTAAGAAGGAGATATACTATG**ATGAATAAATGCAGTACCAA
pNIC-CH/*As*LPMOA (reverse)	AATGGCTTGGGACAAAATCTAA**GCGCACCATCATCACCACCATT**
pNIC-CH/*As*LPMOB (forward)	**TTAAGAAGGAGATATACTATG**ACCAACACGATTAAAATCAATTC
pNIC-CH/*As*LPMOB (reverse)	AATGGGGTGTGTGGCGCTAA**GCGCACCATCATCACCACCATT**
pNIC-CH/*As*GH18 (forward)	**TTAAGAAGGAGATATACTATG**AAACGTATCTTTATTAACAGT
pNIC-CH/*As*GH18 (reverse)	TGATGAATGCGCAA**GCGCACCATCATCACCACCATT**

RESULTS AND DISCUSSION

Sequence analysis and homology modelling.

Annotation of putative CAZy domains of the three enzymes using the dbCAN server (Yin et al., 2012) showed that the chitinase sequence (that contains 881 amino acids, which is unusually large for a chitinase) contains a predicted CBM5, CBM73 chitin binding domains and a C-terminal GH18 domain, modest in size (only 324 amino acids) (Figure 1). All proteins sequences also show long regions that are not annotated by the dbCAN server. Attempts to functionally annotate these regions with other sequence analysis servers such as InterPro, Pfam and SMART were inconclusive. The relatively small size of the GH18 catalytic domain indicates an enzyme stripped of most sub-domains that often are in place to form a substrate binding cleft. Indeed, homology modelling using Swiss-Model (Biasini et al., 2014) revealed a model structure with a shallow substrate binding cleft, reminiscent of a non-processive endo-chitinase, which is clearly observed when compared to the exo-chitinase *Sm*Chi18B from *Serratia marcescens* that has a deep substrate binding cleft (Figure 2). The template used for building the model was from *Chromobacterium violaceum* chitinase (PDB ID 4TX8; sequence identity 54%), which has not been biochemically characterized. The (very) accessible active site of *As*Chi18A suggests that it can access more complex substrates than a linear chitin chain. Compared to other virulence related chitinases, *As*Chi18A has a similar size, but different domain topology. For example, ChiA2 from *Vibrio cholerae*, which has been shown to improve survival of the bacterium in the host intestine, also contains around 800 amino acids, but the GH18 domain is close to the N-terminus and contains a CBM44 and a CBM5 chitin-binding domain on the C-terminal side. ChiA2 has been shown to cleave intestinal mucin (releasing GlcNAc), but has a deep substrate binding cleft and resembles an exo-chitinase (85% sequence identity to the structurally resolved exo-chitinase of *Vibrio harveyi*; (Songsiriritthigul et al., 2008)).

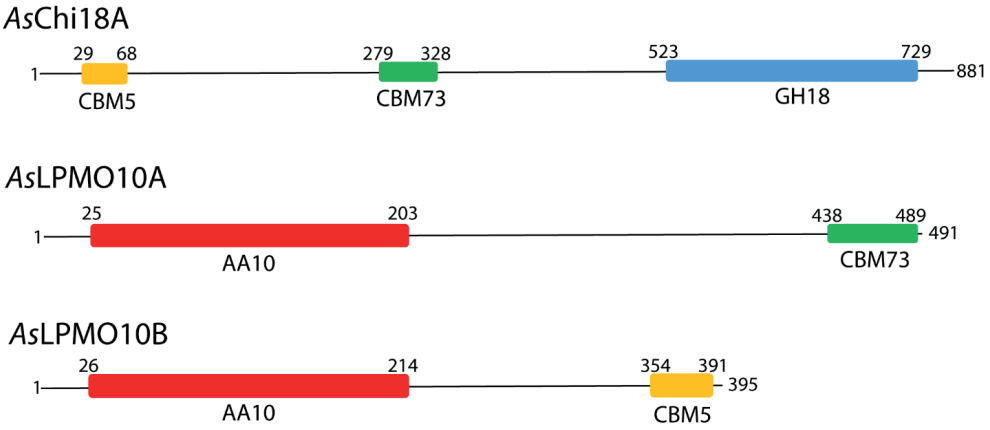

Figure 1. Predicted domains of the *Aliivibrio salmonicida* chitinase and LPMOs. Prediction of CAZy domains of the chitinolytic enzymes was performed using the dbCAN server. Numbers indicate the position in the sequence.

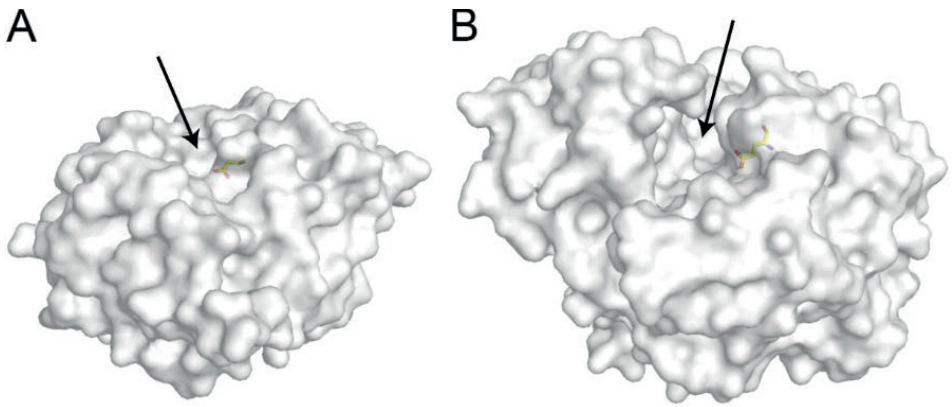

Figure 2. GH18 chitinases structures. The homology model of *As*Chi18A (panel A) and the structure of *Sm*Chi18B deep clefted exochitinase from *S. marcesces* (panel B) are shown in white surface representation with a the catalytic acid shown in yellow colored stick representation and arrows indicating the substrate binding cleft. The template used for modelling the *As*Chi18A GH18 domain was PDB ID 4TX8 and gave a Qmean value of -0.52, which represents a good quality model.

Annotation of the LPMO sequences showed that both proteins contain an N-terminal catalytic AA10 domain and a CBM73 and CBM5 chitin-binding domain in *As*LPMO10A and -B, respectively (Figure 1). Pair-wise sequence alignment of the two LPMOs revealed only a 20% identity between the catalytic domains. Blast search and modelling by homology of the individual catalytic domains showed that the catalytic module of *As*LPMO10A is similar to CBP21 from *Serratia marcescens* and to the catalytic AA10 domain of GbpA, a *Vibrio cholerae* colonization factor (results not shown). In fact, the full-length *As*LPMO10A is very similar to the *Vibrio cholerae* GbpA (61% sequence identity), indicating the possibility of functionally similar roles. The catalytic AA10 domain of *As*LPMO10B is, as already noted, very unlike *As*LPMO10A. From blast searches, the protein seems to be conserved amongst all *Vibrio* genera and also other marine bacteria like *Shewanella* and *Pseudoalteromonas*. None of these related enzymes have hitherto been biochemically characterized. When searching for similar sequences in the PDB database, the most similar structure to the *As*LPMO10B catalytic domain belongs to the viral proteins called "spindolins" (43.5% identity, but the alignment contains many insertions/ deletions; results not shown). There exist no activity data for spindolins, but it is assumed that they are active towards chitin (Chiu et al., 2015). It is therefore not straightforward to assign an activity to *As*LPMO10B based on sequence analysis.

Characterization of catalytic properties.

Mesophilic chitinases such as those from *Serratia marcescens* have their optimal operational stability in the range of 50-60 °C (Brurberg et al., 1996; Suzuki et al., 2002) and similar properties have been shown for plant chitinases (Iseli et al., 1993) and other chitinases from mesophilic bacteria (Frankowski et al., 2001). The progress curves observed for *As*Chi18A indicate an optimal operational stability at approximately 30 °C (Figure 3; panel A), which is lower than observed for the mesophilic enzymes referred to and more similar to what has been shown for other psychrophilic chitinases (e.g. the chitinases of *Moritella marina, Alteromonas* sp. strain O-7 and *Vibrio* sp. strain Fi:7, which all have optima of approximately 30 °C; (Bendt et al., 2001; Orikoshi et al., 2003; Stefanidi and Vorgias, 2008)). Such temperature profile is not surprising since *Allivibrio salmonicida* has an optimal growth temperature of 10 °C (Colquhoun et al., 2002). However, it should be noted that the temperature optimum of enzymes from cold tolerant bacteria

232 can vary substantially (e.g. two metallo-β-lactamases from *Aliivibrio salmonicida* have
233 temperature optimums of 35 and 45 °C; (Kristiansen et al., 2014).

234 Similarly to other GH18 chitinases, the dominant product of chitin hydrolysis by *As*Chi18A was
235 $(GlcNAc)_2$ with small amounts of GlcNAc (< 5%). An interesting property of *As*Chi18A is its
236 double pH optimum, where the enzyme activity is approximately equal at pH 4 and 7 (Figure 3;
237 panel B). Chitinases usually display a single pH optimum, but double pH optima are not
238 uncommon for enzymes. Schwimmer postulated that such phenomenon could arise when the
239 formation of the active enzyme-substrate complex can occur by two different ionic species of the
240 enzyme that differ by at least two protons (Schwimmer, 1962) and several enzymes that display
241 such properties have been characterized, e.g. a phytase from *Aspergillus niger* (Nagashima et al.,
242 1999) and β-galactosidase from *Lactobacillus acidophilus* (Choonia and Lele, 2013). It is not
243 known whether the two pH optima are functionally relevant for *As*Chi18A.

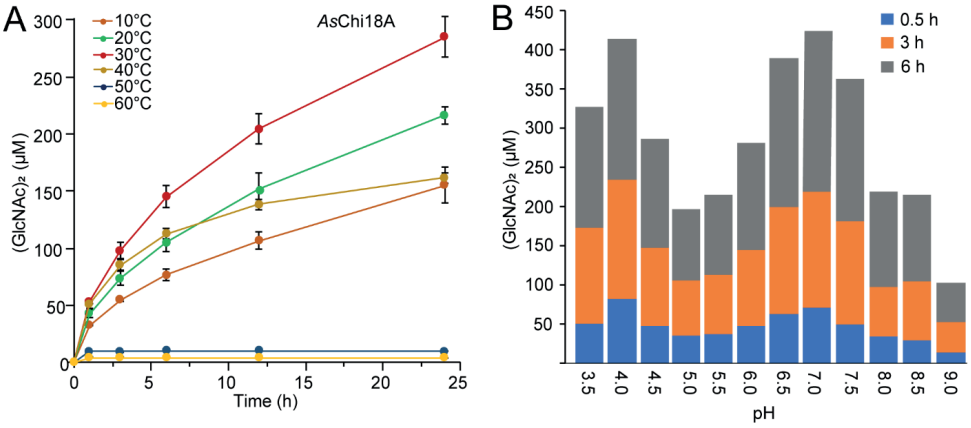

Figure 3. Operational temperature and pH stability of *As*Chi18A. Production of $(GlcNAc)_2$ by *As*Chi18A analysed at various temperatures (A) and pH values (B). All reactions conditions include 10 mg/mL β-chitin and 0.5 µM of enzyme. For data displayed in panel A, reactions were carried out in 10 mM buffer Tris-HCl pH 7.5. For the data displayed in panel B, all reactions were incubated at 30 ºC with the buffers: formic acid pH 3.5, acetic acid pH 4.0 and 4.5, ammonium acetate pH 4.5 and 5.0, MES pH 5.5, 6.0 and 6.5, BisTris-HCl pH 7.0, Tris-HCl pH 7.5 and 8.0 and Bicin pH 8.5 and 9.0. Standard deviations are indicated by error bars (n=3).

The *Aliivibrio salmonicida* LPMOs displayed slightly different operational stabilities when probed at temperatures ranging from 10 to 60°C, (Figure 4). *As*LPMO10A shows activity profiles similar to that of *As*Chi18A, the operational stability (i.e. the temperature where linear progress curves are observed) being approximately 30°C (Figure 4, panels A and C). *As*LPMO10B showed an operational stability lower than 30 °C (Figure 4, panels B and D). Comparison of the *As*LPMO10s activity shows that *As*LPMO10A seems generally more active than *As*LPMO10B, the former enzyme yielding approximately twice as much soluble oxidized products than the latter. This may be related to the chitin binding properties of the enzymes as *As*LPMO10A binds better to both α- and β-chitin than *As*LPMO10B (Figure 5). The chitin binding properties of the enzymes are most likely related to the chitin binding modules attached. Forsberg *et al.* showed that the CBM73 of *Cellvibrio japonicus* LPMO has a stronger affinity to chitin than a CBM5 chitin binding module (Forsberg et al., 2016) and a similar scenario may cause the difference in binding between the two *Aliivibrio salmonicida* LPMOs. It has also been shown that poor binding efficiency, or reaction with "wrong" substrate, leads to oxidative self-inactivation of the LPMOs (Bissaro et al., 2017; Kuusk et al., 2018), which is illustrated by the shorter stable phase displayed by *As*LPMO10B. Altogether, these observations suggest that β-chitin may not be the optimal substrate of *As*LPMO10B. Another difference between this two enzymes is the ratio of oxidized vs native products, which is higher for *As*LPMO10A than for *As*LPMO10B (Figure 4). This indicates that *As*LPMO10B yields higher quantities of products with lower degree of polymerization than *As*LPMO10A. Indeed, from the product profiles obtained by MALDI-TOF MS (Supplementary Figure S1), *As*LPMO10B is observed to yield trimeric products, an oxidized chitooligosaccharide not observed in the *As*LPMO10A profile for β-chitin. This may indicate that *As*LPMO10B and *As*LPMO10A target different regions of the substrate, which is likely given their unlike CBMs.

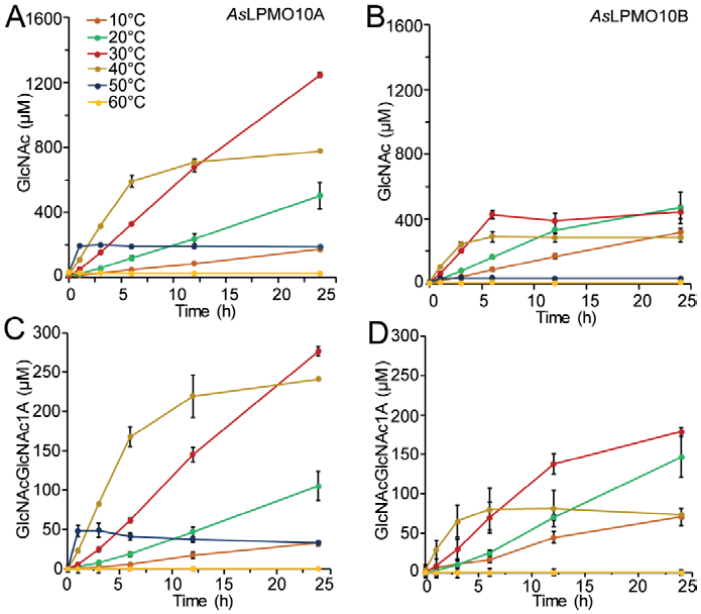

Figure 4. Operational temperature stability of *Aliivibrio salmonicida* LPMOs. The activity of *As*LPMO10A and *As*LPMO10B indicated by the production of GlcNAc is shown in panel A and B, respectively. Formation of oxidized $(GlcNAc)_2$ (i.e. GlcNAcGlcNAc1A), by the same enzymes, is shown in panel C and D, respectively. The amounts presented are based on the average of two independent reactions, which contained 10 mg/mL of β-chitin, 1 µM of enzyme, 1 mM of ascorbic acid and 10 mM of Tris-HCl buffer at pH 7.5, incubated at different temperatures between 10 and 60 °C (colour code provided in panel A). Standard deviations are indicated by error bars.

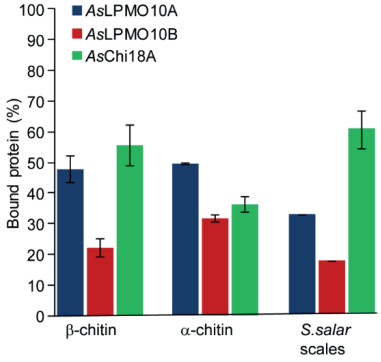

Figure 5. Binding of LPMO10s and Chi18A from *Aliivibrio salmonicida* on different substrates. Each bar shows the percentage of bound proteins after 2 h of incubation at 30°C. Reactions contained 10 mg/mL of substrate, 0.75 µM (LPMOs) or 0.50 µM (Chi18A) of enzymes and 10 mM of Tris-HCl buffer at pH 7.5. All reactions were run in triplicates and the standard deviations are indicated by error bars.

Enzyme synergies

The ability of *As*LPMO10s to cleave chitin chains that are inaccessible to chitinases (i.e. in the crystalline regions of the substrate), often materializes in an increased rate of chitin hydrolysis when both chitinases and LPMOs are present in the same reaction. Several studies have demonstrated this phenomenon (Vaaje-Kolstad et al., 2010; Vaaje-Kolstad et al., 2012; Hamre et al., 2015; Nakagawa et al., 2015; Yang et al., 2017), including a study on the virulence-related LPMO from *Listeria monocytogenes* (Paspaliari et al., 2015). The explanation for this phenomenon lies in the ability of the LPMOs to cleave chitin chains in the crystalline regions of the substrate, which are poorly accessible to the chitinases. The newly formed chitin chain ends formed by LPMO activity, represent new points of attachment for the chitinases, thereby increasing substrate accessibility. Experimentally, this phenomenon can be probed by synergy experiments, where the substrate degradation potential of each enzyme is evaluated and compared to combinations of the same enzymes. For the putative chitinolytic system of *Aliivibrio salmonicida* the situation is different than any other chitinolytic system studied since the chitin degradation potential of the chitinase is substantially lower than that of the LPMOs (Figure 6). Usually the chitinase of a chitinolytic system is significantly more active than the LPMO. Nevertheless, synergies are observed when combining the *As*Chi18A with the individual *As*LPMO10B giving an almost double yield than the sum products calculated by adding the sum of their individual yields. *As*LPMO10A, on the other hand, shows a weaker synergy when combined with *As*Chi18A. Surprisingly, when combining all three enzymes, no synergy is observed for β-chitin degradation and a lower than theoretical yield is obtained for α-chitin. The explanation for this phenomenon is not straight forward, but one could imagine an "impeachment effect" of *As*LPMO10A, potentially occluding binding sites for the other enzymes. It may also be that a total of 2 μM LPMO is too much for these reactions, giving rise to less bound enzyme to the substrate and thereby production of harmful reactive oxygen species (ROS) by the non-bound LPMO (LPMOs are known to form destructive ROS in the absence of a substrate) (Kittl et al., 2012; Loose et al., 2016; Bissaro et al., 2017).

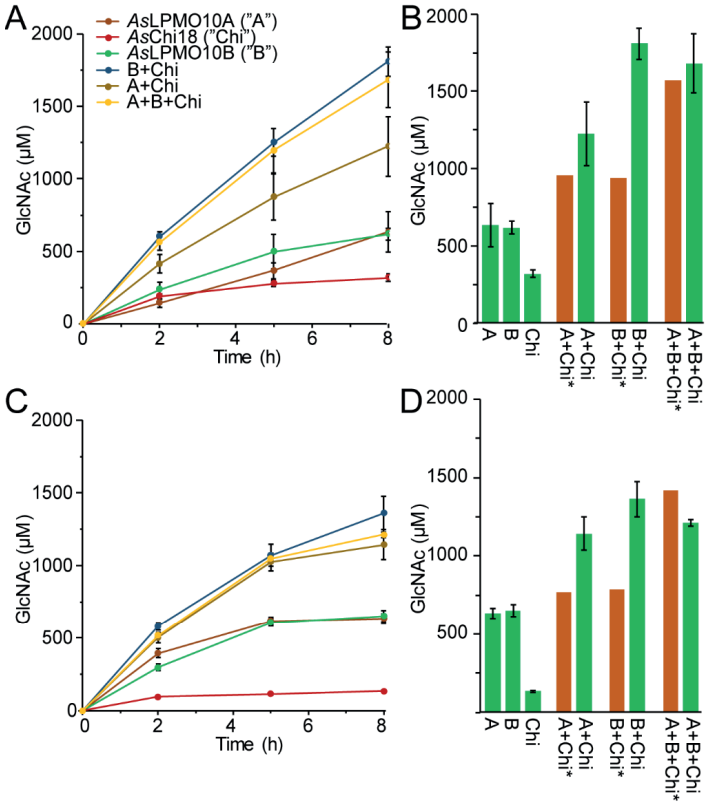

Figure 6. Synergistic activity of *As*LPMO10s and *As*Chi18A on chitin. Panel A and C show the production of GlcNAc by the idividual and combined enzymes on β- and α-chitin, respectively. Panel B and D show the theoretically calculated amounts of GlcNAc based on the sum of its production by the individual enzymes (*) and the detected amounts of GlcNAc by combining the enzymes after 8 h. The amounts presented are based on the average of three independent reactions containing 10 mg/ml of chitin substrate, 1 µM of LPMOs and/or 0.5 µM of GH18, 1 mM of ascorbic acid and 10 mM of Tris-HCl buffer at pH 7.5, incubated at 30 °C for 8 h. Standard deviations are indicated by error bars (n=3).

Comparison of *As*Chi18A with other GH18 chitinases

The apparent low activity of *As*Chi18A prompted comparison of its activity to other well characterized chitinases in order obtain a direct observation of its hydrolytic potential. In order to obtain a relevant comparison, the chitin degradation rate of *As*Chi18A was compared with the four GH18 chitinases of the *Serratia marcescens* (*Sm*Chi18A, -B, -C and -D) (Brurberg et al., 1996;

Suzuki et al., 1999; Tuveng et al., 2017) and, *Cj*Chi18D, which is the most potent chitinase of *Cellvibrio japonicus* (Monge et al., 2018). Activities were monitored at pH 7.5 (Figure 7; panel A), which is the pH of sea water and the near pH-optimum of *As*Chi18A and at pH 6 (Figure 7; panel B), which is the pH where the *Serratia marcescens* and *Cellvibrio japonicus* chitinases have their optimum (Brurberg et al., 1996; Synstad et al., 2004; Synstad et al., 2008). Strikingly, *Sm*Chi18A, -B, -C and *Cj*Chi18D yield more than 50-fold more $(GlcNAc)_2$ than *As*Chi18A after 24 h incubation at pH 6. At pH 7.5, the differences in yields are lower (in the range of 25-40 fold larger yields, except for *Sm*Chi18D; see below for discussion), most likely reflecting the difference in pH optima.

The three GH18 chitinases from the Gram negative soil bacterium *Serratia marcescens* have been studied by many and the bacterium is known for its efficiency in chitin degradation (Monreal and Reese, 1969). The large difference in chitin degrading ability between the *Serratia marcescens* chitinase and the *Aliivibrio salmonicida* chitinase may indicate that the latter has a different role than chitin degradation for a metabolic purpose, especially since *As*Chi18A is the only GH18 chitinase in the bacterium genome. As already mentioned, *Aliivibrio salmonicida* does not seem to be able to utilize insoluble chitin as a carbon and nitrogen source in lab culturing experiments (Egidius et al., 1986; Hjerde et al., 2008), indicating that *As*Chi18A has evolved a different role than chitin degradation. It should be noted that *Aliivibrio salmonicida* can utilize *N*-acetylglucosamine (GlcNAc) as carbon source (unpublished experiments; pers. com. A. Skåne) and that chitin is not the only source of this monosaccharide. GlcNAc is abundant also in vertebrate glycans, like mucins (Bansil and Turner, 2006; Linden et al., 2008).

Although *As*Chi18A is considerably less active than *Sm*Chi18A, -B and -C and *Cj*Chi18D, it showed similar activity to *Sm*Chi18D, the fourth and only recently discovered chitinase from *Serratia marcescens* (Tuveng et al., 2017) (Figure 7; panel inserts). *Sm*Chi18D is not secreted during chitin degradation by the bacterium, and this fact, combined with the poor chitin hydrolyzing properties of the enzyme, made the authors hypothesize that *Sm*Chi18D is not involved in chitin degradation (Tuveng et al., 2017). However, no alternative activities were detected. For virulence related chitinases, alternative substrates have been identified for, e.g. cleavage of mucin glycans by the *Vibrio cholerae* chitinase (Mondal et al., 2014) and hydrolysis of LacdiNAc (GalNAcβ1-4GlcNAc) and LacNAc (Galβ1-4GlcNAc) by the *Listeria*

329 *monocytogenes* and *Salmonella typhimurium* chitinases (Frederiksen et al., 2015). Such substrates
330 were not evaluated by activity assays with *As*Chi18A, but the relatively strong binding of this
331 enzyme to Atlantic salmon scales (Figure 5) may indicate the presence of a target substrate in the
332 scale composite structure. Incubation of *As*Chi18A with mucus collected from Atlantic salmon
333 skin revealed an unidentifiable peak (different from the negative control), but determination of its
334 identity was unsuccessful (data not shown).

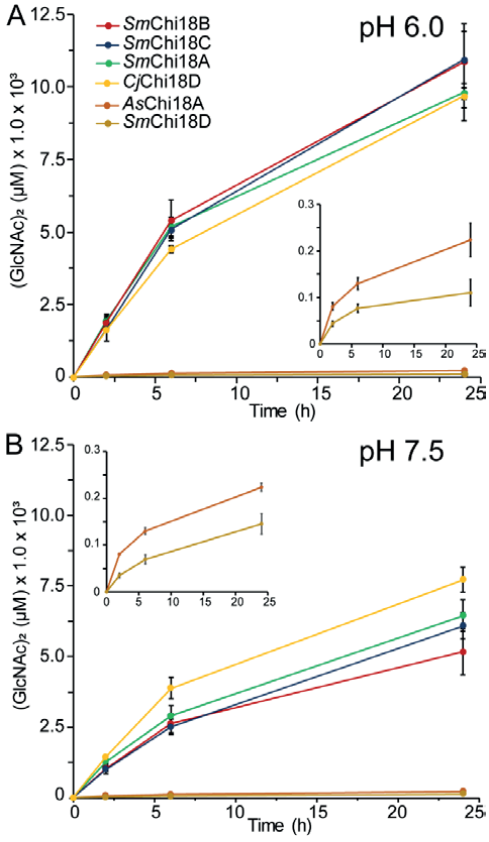

Figure 7. Activity of *As*Chi18A in comparison to different chitinases on β-chitin at different pH. Chitinases from *Serratia marcescens* (*Sm*Chi18A, -B, -C and -D) and *Cellvibrio japonicus* (*Cj*Chi18D) were used. Panel A shows the production of $(GlcNAc)_2$ at pH 6.0, whereas panel B shows the production of $(GlcNAc)_2$ at pH 7.5. The amounts presented are based on the average of three independent reactions containing 10 mg/ml β-chitin, 0.5 µM enzyme and 10 mM buffer at pH 6.0 (MES) and at pH 7.5 (Tris-HCl), incubated for a period of 24 h at 30 °C. The insets in panel A and B shows a magnified view focusing on reactions catalyzed by *As*Chi18A and *Sm*Chi18D. Standard deviations are indicated by error bars (n=3).

Since it seems that the *Aliivibrio salmonicida* chitinase has evolved a different role than chitin degradation, the same question applies for the LPMOs. Both LPMOs are active towards chitin (Figure 4 and Supplementary figure 1), but it is not certain that this is the intended substrate of these enzymes. For instance, GbpA, LPMOs from *Vibrio cholerae*, has activity towards chitin (Loose et al., 2014), but its main function seems to be related to colonization of both transfer vectors (e.g. zoo-plankton), the host epithelium (e.g. human intestine) or both (Kirn et al., 2005; Bhowmick et al., 2008). The LPMO of *Listeria monocytogenes* is also active towards chitin (Paspaliari et al., 2015), but the gene encoding this enzyme is not expressed when the bacterium grows on chitin (on the other hand, the *Listeria monocytogenes* chitinase-encoding genes are expressed when the bacterium is grown on chitin (Leisner et al., 2008; Paspaliari et al., 2015)). Thus, chitin may represent a potential substrate for these LPMOs, but possibly not the biologically relevant substrate.

On the other hand, some LPMOs are designed to only disrupt and disentangle chitin fibers, rather than to contribute to their degradation in a metabolic context, namely the viral family AA10 LPMOs (also called spindolins) (Chiu et al., 2015). These LPMOs are harbored by insect-targeting entomopox- and baculoviruses, and have been shown to disrupt the chitin containing peritrophic matrix that lines the midgut of insect larvae (Mitsuhashi et al., 2007). The main function proposed for the viral LPMOs is to destroy the midgut lining in order to allow the virus particles to access the epithelial cells that are located underneath. Indeed, in pre-LPMO times, by removing from the virus genome what we call today *lpmo* genes, the infectivity of such viruses was shown to drop by more than 1.0 $x10^4$ (Mitsuhashi et al., 1998). Since the scales and gut of fish may contain chitin (Wagner et al., 1993; Tang et al., 2015), it is tempting to speculate that the role of the fish pathogen LPMOs is similar to that of viral LPMOs, namely to disrupt this putatively protective chitin layer in order to provide an entry point to the bacteria for infection. The ability of the *Aliivibrio salmonicida* LPMOs and chitinase to bind Atlantic salmon scales (Figure 5), supports the notion that these enzymes may have their biologically relevant substrate in this host structure. However, no soluble products could be observed in the reactions where the LPMOs were incubated with Atlantic salmon scales (results not shown), but this hypothesis needs to be tested more rigorously before we can make any firm conclusion.

Conclusively, in the present work, we have characterized three chitin depolymerizing enzymes from the fish pathogen *Aliivibrio salmonicida*. The operational stability of the enzymes is optimal at relatively low temperature, possibly reflecting the psychrophilic nature of the bacterium. Compared to chitinases from well-studied chitinolytic bacteria, *As*Chi18A is barely active, suggesting that chitin may not be the biologically relevant substrate for the enzyme. Interestingly, the chitinase also bound strongly to Atlantic salmon scales. The *Aliivibrio salmonicida* LPMOs were both active towards chitin and also bound to Atlantic salmon scales, albeit not as strong as the chitinase. Since such scales have been shown to contain chitin, a role of the three chitin depolymerizing enzymes of *Aliivibrio salmonicida* may be to disrupt the scale chitin in order to promote infection. However, the use of an alternative substrate by these enzymes cannot be excluded.

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SUPPLEMENTARY MATERIAL

Characterization of putative virulence factors with chitinolytic activity from *Aliivibrio salmonicida*

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SUPPLEMENTARY INFORMATION

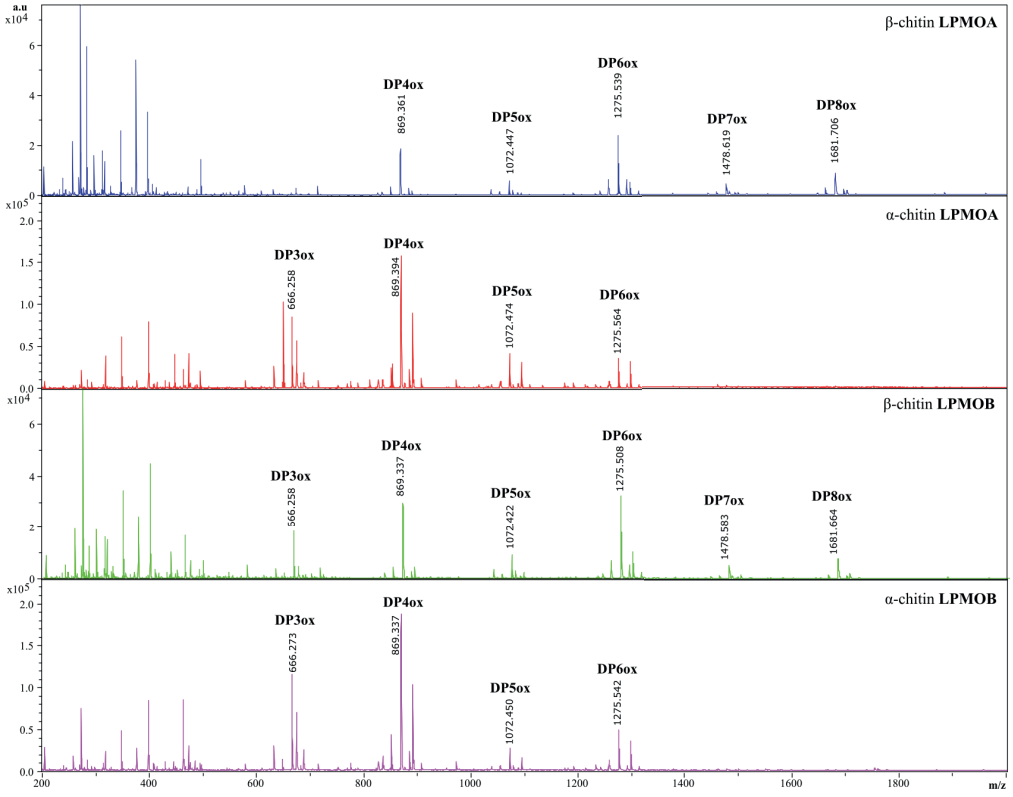

Figure S1. MALDI-TOF MS analysis of oxidized products generated by LPMO10A and -B from *Aliivibrio salmonicida* on chitin (α and β). The MS spectra show soluble C1 oxidized chito-oligosaccarides, i.e. aldonic acids. The degree of polymerization of each product is indicated by "DPn ox", where n equals the number of monosaccharides in the chain. The main peaks are labelled with the respective masses. A complete mass list is provided in table S1.

Table S1. Mass list of potential oxidized chito-oligosaccharides generated by LPMOs from *Aliivibrio salmonicida*. The table shows the degree of polymerization (DP), masses per charge (m/z), the product composition, and the type of adduct and product (Mekasha et al., 2016). GlcNAc: *N*-acetylglucosamine; GlcN: glucosamine; GlcNAc1A: *N*-acetylglucosamine aldonic acid.

DP	Mass per charge (m/z)	Product	Adduct	Product name
3	666.258	$(GlcNAc)_2GlcNAc1A$	$[M+Na]^+$	Aldonic acid
4	827.518	$(GlcNAc)_2GlcNGlcNAc1A$	$[M+Na]^+$	Aldonic acid
	851.416	$(GlcNAc)_3GlcNAc1A$	$[M+Na]^+$	Lactone
	869.419	$(GlcNAc)_3GlcNAc1A$	$[M+Na]^+$	Aldonic acid
	885.500	$(GlcNAc)_3GlcNAc1A$	$[M+K]^+$	Aldonic acid
	891.522	$(GlcNAc)_3GlcNAc1A$	$[M-H+2Na]^+$	Aldonic acid
	907.372	$(GlcNAc)_3GlcNAc1A$	$[M-H+K+Na]^+$	Aldonic acid
5	1054.513	$(GlcNAc)_4GlcNAc1A$	$[M+Na]^+$	Lactone
	1072.523	$(GlcNAc)_4GlcNAc1A$	$[M+Na]^+$	Aldonic acid
	1088.631	$(GlcNAc)_4GlcNAc1A$	$[M+K]^+$	Aldonic acid
	1094.661	$(GlcNAc)_4GlcNAc1A$	$[M+Na]^+$	Aldonic acid
	1110.84	$(GlcNAc)_4GlcNAc1A$	$[M-H+K+Na]^+$	Aldonic acid
6	1191.633	$(GlcNAc)_3(GlcN)_2GlcNAc1A$	$[M+Na]^+$	Aldonic acid
	1207.767	$(GlcNAc)_3(GlcN)_2GlcNAc1A$	$[M+K]^+$	Aldonic acid
	1233.683	$(GlcNAc)_4GlcNGlcNAc1A$	$[M+Na]^+$	Aldonic acid
	1249.811	$(GlcNAc)_4GlcNGlcNAc1A$	$[M+K]^+$	Aldonic acid
	1257.693	$(GlcNAc)_5GlcNAc1A$	$[M+Na]^+$	Aldonic acid
	1275.707	$(GlcNAc)_5GlcNAc1A$	$[M+Na]^+$	Aldonic acid
	1291.847	$(GlcNAc)_5GlcNAc1A$	$[M+K]^+$	Aldonic acid
	1297.865	$(GlcNAc)_5GlcNAc1A$	$[M-H+2Na]^+$	Aldonic acid
	1313.718	$(GlcNAc)_5GlcNAc1A$	$[M-H+K+Na]^+$	Aldonic acid
	1329.852	$(GlcNAc)_5GlcNAc1A$	$[M-H+2K]^+$	Aldonic acid
7	1460.931	$(GlcNAc)_6GlcNAc1A$	$[M+Na]^+$	Lactone
	1478.988	$(GlcNAc)_6GlcNAc1A$	$[M+Na]^+$	Aldonic acid
	1494.991	$(GlcNAc)_6GlcNAc1A$	$[M+K]^+$	Aldonic acid
	1516.97	$(GlcNAc)_6GlcNAc1A$	$[M-H+K+Na]^+$	Aldonic acid
8	1556.145	$(GlcNAc)_4(GlcN)_3GlcNAc1A$	$[M+Na]^+$	Aldonic acid
	1572.256	$(GlcNAc)_5(GlcN)_3GlcNAc1A$	$[M+K]^+$	Aldonic acid
	1598.168	$(GlcNAc)_5(GlcN)_2GlcNAc1A$	$[M+Na]^+$	Aldonic acid
	1614.367	$(GlcNAc)_5(GlcN)_2GlcNAc1A$	$[M+K]^+$	Aldonic acid
	1664.283	$(GlcNAc)_7GlcNAc1A$	$[M+Na]^+$	Lactone
	1682.32	$(GlcNAc)_7GlcNAc1A$	$[M+Na]^+$	Aldonic acid
	1698.339	$(GlcNAc)_7GlcNAc1A$	$[M+K]^+$	Aldonic acid
	1704.617	$(GlcNAc)_7GlcNAc1A$	$[M-H+2Na]^+$	Aldonic acid
	1720.337	$(GlcNAc)_7GlcNAc1A$	$[M-H+K+Na]^+$	Aldonic acid

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