Virulence factors of *Pasteurella atlantica* genomovar *salmonicida* and effect on salmon leucocytes

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Abbreviations

Abbreviation	Full name
Apaf-1	Apoptopic protease activating factor 1
Ata	Acinetobacter trimeric autotransporter adhesin
ATM	Ataxia-Telangiectasia Mutated
ATR	Rad3-related
BAX	Bcl-2 Associated X-protein
BHIB	Brain Heart Infusion Broth
BSA	Bovine Serum Albumin
CdiA	Contact dependent growth inhibition
CDT	Cytolethal Distending Toxin
CFU	Colony Forming Units
colA	Collagenase
DNA	Deoxyribonucleic Acid
E	Extracellular
EsiB	Escherichia coli secretory immunoglobulin A-binding protein
EtOH	Ethyl Alcohol
FAS	Fas Cell Surface Death Receptor
FasL	FasLigand
FCS	Fetal Calf Serum
g	Gram
x *g*	Times Gravitational force
gv	Genomovar
HasA	Haem-scavenging protein

HGT	Horizontal Gene Transfer
HKL	Head Kidney leucocytes
IgA	Immunoglobulin A
IL	Interleukin
kDa	Kilodalton
LPS	Lipopolysaccharides
LptE	Lipoprotein like protein
LPTX	Cell wall anchor domain-containing protein
M-protein	Matrix protein
MAC	Membrane Attack Complex
NaCl	Natrium chloride
NCBI	National Center for Biotechnology Information
nm	Nanometer
NVI	Norwegian Veterinary Institute
OD	Optical density
OM	Outer Membrane
PAMPS	Pathogen Associated Molecular Patterns
PRRS	Pattern Recognition Receptors
Rpm	Revolutions per minute
RTX	Repeat-in-Toxin
RV	Reverse vaccinology
SDS-PAGE	Sodium Dodecyl Sulphate-Polycrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
Ser-Asp	Seryl-aspartic acid

TBS	Tris-Buffered Saline
TLR	Toll-Like Receptor
TNF- α	Tumour Necrosis Factor alpha
TSB	Tropic Soy Broth
VacA	Vacuolating toxin A
VFDB	Virulence Factor Database
YopT	*Yersinia* enterocolitica outer protein T
µg	Micro gram
µl	Micro litre

Abstract

Pasteurellosis has since 2018 caused serious outbreaks of disease in farmed Atlantic salmon in Norway. In 2022 there were 52 reported cases of pasteurellosis in Norwegian salmon farms. Little is known about the bacterium´s virulence factors, immunogenic components, differences between isolates, and differences between the isolates causing disease in salmon and lumpsuckers.

In this thesis, we exposed Atlantic salmon head kidney leucocytes (HKL) to *Pasteurella atlantica* genomovar *salmonicida* and examined the leucocytes and bacteria by Scanning Electron microscopy (SEM) and fluorescence microscopy. We also compared the protein profiles of four isolates of *P. atlantica* gv. *salmonicida* by silver staining SDS-PAGE gels, and their growth requirement of supplemented FCS by cell counting. We studied the biochemical characteristics of the four isolates using the API 20E kit and performed Gram staining. We established a growth curve to ensure that harvest of bacteria was conducted at the same growth stage across all experiments. Based on in silico analyses of the genome of *P. atlantica* gv. *salmonicida* we filtered possible virulence factors present in the outer membrane and as extracellular products.

We found that HKL exposed to *Pasteurella atlantica* gv. *salmonicida* showed more indication of apoptosis compared to non-exposed controls. By in silico analysis a rough list of possible virulence factors and immunogenic components were compiled. All four isolates showed a similar protein profile on the SDS-PAGE gels. We found that *Pasteurella atlantica* gv. *salmonicida* was more biochemically reactive than *Pasteurella atlantica* gv. *cyclopteri*. The gram stain came out gram-negative. Late exponential phase was found to be between 14-16 hours in TSB+ media.

The findings indicate no difference between the included *Pasteurella atlantica* gv. *salmonicida* isolates, and the bacteria require FCS for growth in TSB. As suggested by SEM and in silico analysis the bacteria exhibit possible virulence factors influencing apoptosis of salmon leucocytes.

Sammendrag

Pasteurellose har siden 2018 forårsaket alvorlige utbrudd i oppdrettet Atlantisk laks i Norge. I 2022 var det 52 rapporterte utbrudd av pasteurellose i norsk oppdrettsnæring. Lite er kjent om bakteriens virulens faktorer, immunogene komponenter, forskjeller mellom isolater, og forskjeller mellom isolater som forårsaker sykdom i laks og rognkjeks.

I denne masteroppgaven eksponerte vi hodenyre leukocytter fra Atlantisk laks for *Pasteurella atlantica* genomovar *salmonicida,* og undersøkte dem under SEM. Vi sammenlignet også fire isolater av *P. atlantica gv. salmonicida* protein profiler gjennom silver stained SDS-PAGE gels, og deres vekst krav for FCS ved celle telling. I tillegg ble det gjort et eget forsøk med forskjellige konsentrasjoner av FCS ved å måle celle tall i CASY celle teller. Vi studerte biokjemiske karakteristika av de fire isolatene med API 20E, og gjennomgikk gram farging. Vi etablerte vekstkurver for å finne optimal innhøstingstid til å bli brukt i alle eksperimentene. Basert på in silico analyser etablerte vi en oversikt over mulige virulens faktorer og immunogene komponenter lokalisert ekstracellulær, og i ytter membran.

Vi fant at hodenyre leukocytter eksponert for *P. atlantica* gv. *salmonicida* hadde mer indikasjon på apoptose enn i kontroll. Ved in silico analyser ble en grov liste av mulige virulens faktorer og immunogene komponenter dannet. Alle fire isolater hadde lignende protein profil i SDS-PAGE gels. I API 20E eksperimentet ble det vist at *P. atlantica* gv. *salmonicida* var mer biokjemisk reaktivt enn *P. atlantica* gv. *cyclopteri.* Gram fargingen ble gram negative. I vekstkurve forsøket ble det vist at bakterien når sen eksponensiell fase innen 14-16 timer i TSB+ media.

Funnene i oppgaven indikerer ingen forskjell mellom *P. atlantica* gv. *salmonicida* isolatene, og at bakterien er avhengig av FCS i dyrkningsmediumet for vekst i TSB. Foreslått av SEM og in silico undersøkelser har bakterien virulens faktorer.

Table of contents

List of Figures

List of Tables

1. Introduction

In 2022, 52 Norwegian Atlantic salmon (*Salmo salar L.*) aquaculture farms were diagnosed with pasteurellosis, and the disease was recognized as the 9th largest fish health problem in the industry according to a survey performed by the Norwegian Veterinary Institute (NVI) (Sommerset et al., 2023).

Knowledge is needed on the pathogenesis and pathology of fish suffering from pasteurellosis. To understand this, knowledge is also needed on virulence factors, possible virulence differences across isolates and on immunogenic components of the causative agent *Pasteurella atlantica* genomovar *salmonicida*. There is also a lack of knowledge concerning the growth requirements for the bacteria, and on how to bulk produce it for high yield (high cell counts) for use in vaccines.

1.1 Pasteurellosis

The *Pasteurella* genus is named after the scientist Louis Pasteur (Aktories *et al.*, 2012). Pasteurellosis is the name of the disease caused by bacterial infections by bacteria belonging to the genus *Pasteurella.* The term pasteurellosis in fish was first used for sea bass in Chesapeake bay, USA (Snieszko, 1964). Based on morphology, physiological and serological characteristics the bacteria were named *Pasteurella piscicida* (Janssen and Surgalla, 1968). However, it was later demonstrated by 16S rRNA sequencing that *P. piscicida* is more related to *Photobacterium damselae*, and it was thus renamed *Photobacterium damselae subsp. piscicida* (Gauthier et al., 1995).

The disease outbreak that got the name varraccalbmi in 1989 was the first detection of pasteurellosis in retrospect in Norwegian farmed salmon (Valheim et al., 2000). The disease caused panophthalmitis, resulting in haemorrhagic eyes and necrotizing inflammation (Valheim et al., 2000). After this outbreak, cases of pasteurellosis in Atlantic salmon occurred sporadically until the spring of 2018, when the frequency of outbreaks increased and a new member of the *Pasteurella* species was discovered in diseased salmon (Sommerset et al., 2023). The NVI proposed a working nomenclature for this new species "*Pasteurella atlantica* genomovar *salmonicida*".

Pasteurellosis in lumpsucker (*Cyclopterus lumpus*), commonly used as biological treatment against salmon sea lice, is associated with another genetic variant *P. atlantica* gv. *cyclopteri* (Alarcón et al., 2016). In 2012, the first confirmed outbreak of pasteurellosis caused by *P. atlantica* gv. *cyclopteri* in lumpsucker was reported by the NVI from a farm in southern Norway

(Johansen, 2013; Ellul et al., 2019). Later, more cases have been reported at the west coast of Norway, in both production facilities for juveniles and after they are deployed to salmon cages (Alarcón et al., 2016).

Pasteurellosis caused by *Pasteurella skyensis* pose problems for the farming industry in Scotland and has been detected in one salmon farm in Norway but is not considered a problem (Sommerset et al., 2023). It was proposed in 2002 that *P. skyensis* should be classified as a new species (Birkbeck et al., 2002). *P. skyensis* and *P. atlantica* are genetically similar, yet distinct from each other (Alarcón et al., 2016; Ellul et al., 2021).

Clinical signs of disease caused by *P. atlantica* gv. *salmonicida* infection are purulent inflammation of the pericardium, abdominal wall, and pseudobranchia, and presence of abscesses filled with pus in skeletal muscle and at the base of the pectoral fin (Legård & Strøm, 2020; Sommerset et al., 2023). Pathological changes to the eyes are common, but not present in all affected fish. The histopathological changes are consistent with macroscopic observations. Acute and chronic inflammation, abundant inflammatory cells, tissue fluid and the presence of short rod-shaped bacteria in affected organs (Legård & Strøm 2020; Sommerset et al., 2023).

Interestingly, *Phocoenobacter uteri* associated with harbor porpoise (*Phocoena phocoena)* is closely related to *P. skyensis* and *P. atlantica* and it is thus suggested that they should be moved to the genus *Phocoenobacter* (Gulla et al., 2023). It is also suggested that marine mammals may be reservoirs for *P. skyensis* and *P. atlantica* (Gulla et al., 2023).

1.2 Virulence factors and immunogenic components

There are several effects of pathogen virulence factors, all of which contribute to their ability to infect and cause disease in a host organism. Virulence factors are specific molecules that enhance a pathogen´s ability to colonize, replicate, and evade the host immune system. Virulence factors can be divided into secretory, or membrane associated. Membrane associated virulence factors are important in adhesion and evasion of the host cells. Secreted (extracellular) virulence factors may act to kill the host cell, evade the immune system, and contribute to nutrient acquisition (Sharma et al., 2017).

Virulence factors can help bacteria breach the physical barriers of the immune defense and help with adherence to the host tissues (Sharma et al., 2017). Immune evasion is where pathogens use virulence factors to evade the host immune responses, for instance inhibition of the

formation of the membrane-attack complex (result of activation of the complement system) by having long side chains in cell-wall lipopolysaccharides (LPS) (Sharma et al., 2017). Nutrient acquisition can be acquired by for example secreted enzymes that break down host tissues (Sharma et al., 2017). Toxins can function to disrupt host cell function by punching holes in the cell membrane (Sharma et al., 2017). Pathogens also secrete small quorum sensing molecules that are important for the pathogen to establish infection (Sharma et al., 2017). Knowledge of the virulence factors that may be expressed by the bacterium, and when they are expressed, is important because it can help in the development of strategies to manage and control fish diseases.

Repeat-in-Toxin (RTX) toxins are virulence factors that are synthesized by a diverse group of Gram-negative bacteria (Benz, 2020). RTX toxins need cell-surface receptors to adhere to host cells (Benz, 2020). To adhere to host cells. Numerous RTX toxins attach exclusively to β2 integrins (cell surface proteins) that are only found on leukocytes, including neutrophils and monocytes, the first responders to inflammation following bacterial infection, leukotoxins are an example of this (Benz, 2020). Thus, some RTX toxins can inhibit the first stage of phagocytosis by binding to pathogen recognition receptors (PRRs) and rapidly penetrating the immune cells plasma membrane to form a pore (Benz, 2020).

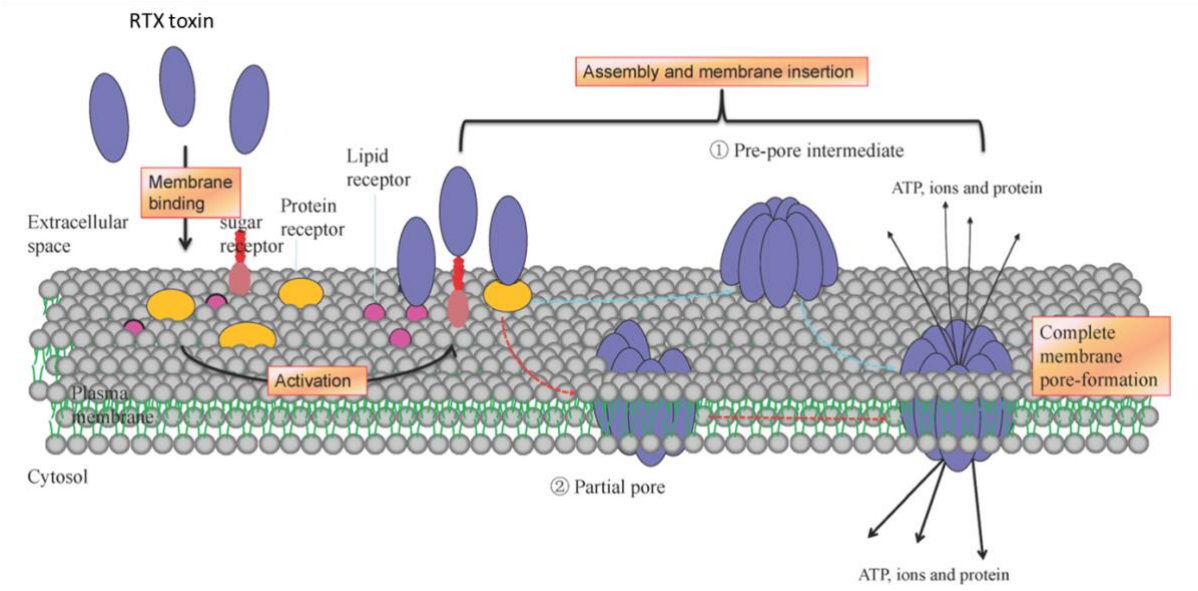

Figure 1: *Illustration of pore formation strategy of RTX toxins. Figure modified from Li Y. et al 2021.*

RTX toxins are one of the important virulence factors described in *Pasteurellaceae* (Sasaki et al., 2009). Multiple pathogens belonging to the family *Pasteurellaceae* possess one of the pore-

forming protein toxins that are seen to give hemolysis caused by the formation of pores in red blood cells, a toxin determined to be a RTX toxin (Sasaki et al., 2009). RTX toxins have been described as eligible antigens for use in vaccines against bacterial diseases (Benz, 2020), attributing them with both virulence and immunogenetic properties.

Immunogenic components are molecules or structures on the pathogen that trigger an immune response in the host. Immunogenic components can be surface proteins such as LPS, flaggelin (the building components of the bacterial flagella) and several other pathogen associated molecular patterns (PAMPs). Immunogenic components can also be in the form of secreted proteins. Immunogenic components are recognized by the host´s immune system, leading to the activation of immune cells, secretion of cytokines, inflammation, activation of the complement system, and the production of antibodies.

Secretion of bacterial proteins may be triggered by the recognition of host receptors by specific adhesion molecules called adhesins (Costa et al., 2015). Secreted products by pathogenic Gram-negative bacteria are often associated with nutrient acquisition (for example HasA, an iron scavenger protein) and virulence (for example pore-forming protein haemolysin A) (Costa et al., 2015). Worth mentioning, siderophores are used by bacteria to accumulate iron molecules (Kramer et al., 2020). A possible alternative to antibiotics is to design antivirulence drugs, targeting virulence factors using small molecules, making pathogens harmless (Costa et al., 2015).

Bacteria can release proteins to the extracellular environment, expose them on their surface or inject them into a neighboring cell using different bacterial secretion systems (Maffei et al., 2017). Some gram-negative pathogenic bacteria use a type 3 secretion system (molecular syringe, sometimes called an injectisome) to inject toxins directly into host cell (Hotinger, 2020). Toxins can hijack essential host functions such as cytoskeleton assembly and apoptosis control (Oswald et al., 2005). These cytolytic polypeptides can disturb host cell membranes, resulting in disruptions of ion balance, ultimately leading to the entry of water, cellular swelling and cell lysis (Johnson, 2018). CDT toxin for example induces apoptosis in human T-cells, and VacA toxin can induce cell death via activation of the mitochondrial apoptosis pathway (Oswald et al., 2005).

Endotoxin, which is synthesized by Gram-negative pathogens, consists of the outer membrane LPS, which is released upon the lysis of Gram-negative bacteria (Johnson, 2018). In contrast to exotoxins, the impact of endotoxin is indirect, leading to the improper activation or excessive

stimulation of inflammatory responses of pro-inflammatory cytokines such as TNF−α, IL-1, IL-6, and IL-8 that may harm host cells (Johnson, 2018).

1.3 Apoptosis

Apoptosis is an important cellular process in growth and development. The term apoptosis was proposed by Kerr and colleagues in 1972 and apoptosis from Greek means “falling off” (Fink & Cookson, 2005). Apoptosis is defined as a programmed cell death without inducing an inflammatory response (Weinrauch & Zychlinsky, 1999), and regarded as a regulated and controlled process (Fink & Cookson, 2005). Apoptosis can be naturally activated in the crucial role of keeping homeostasis of organs or turnover of cells (Weinrauch & Zychlinsky, 1999).

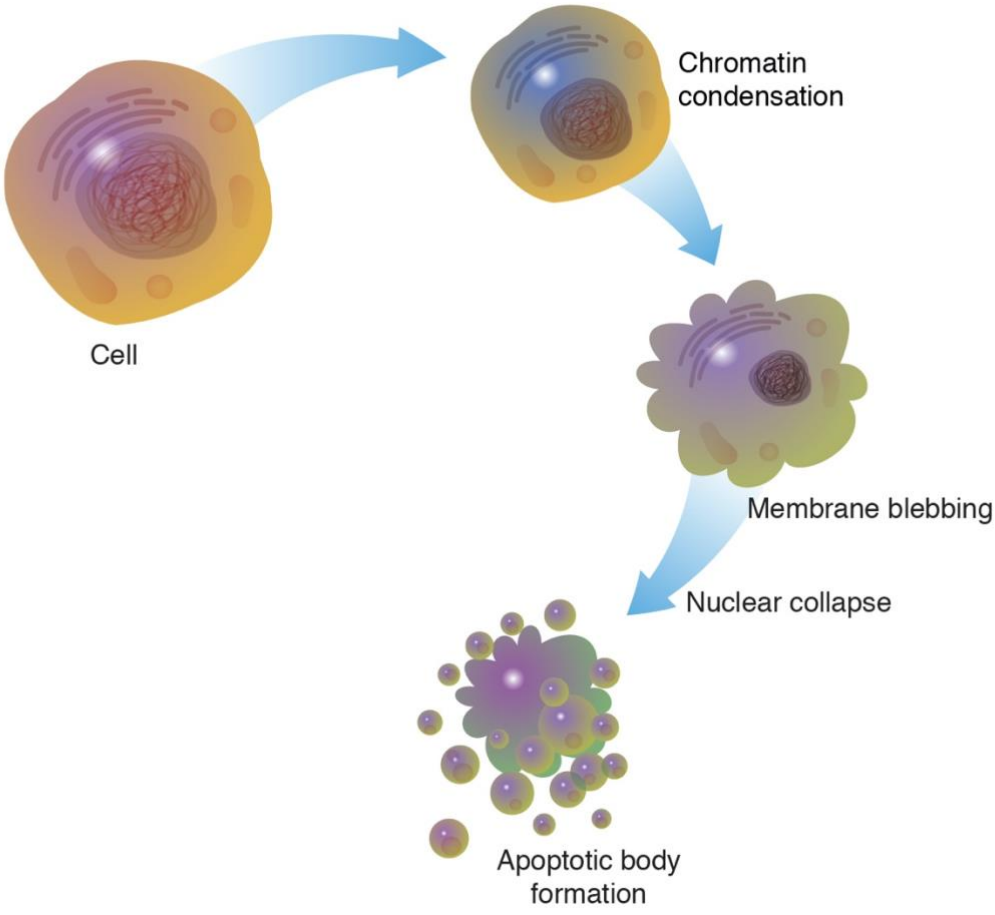

Figure 2: *Apoptosis. Figure taken from National Human Genome Research Institute*

Caspaces, a group of endoproteases functioning in cleaving proteins, are centrally involved in apoptosis (Fink & Cookson, 2005). Caspases are activated via extrinsic (initial signal coming from outside the cell) or intrinsic pathway (signal coming from inside the cell).

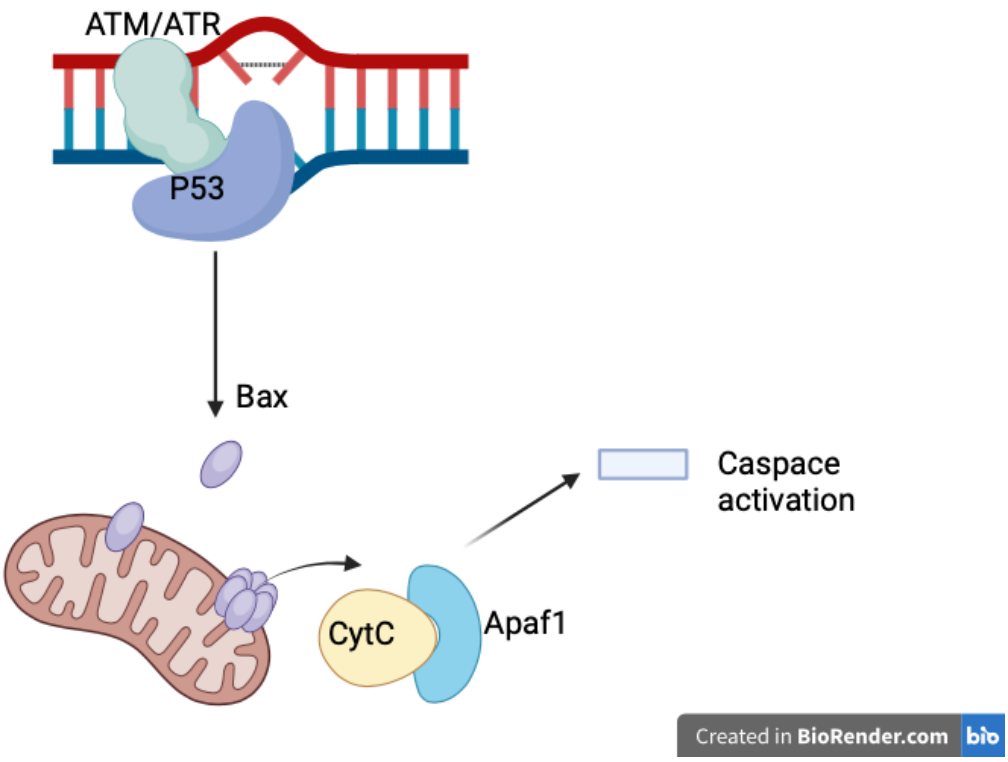

Figure 3: *Intrinsic apoptosis pathway. The intrinsic pathway starts with an irreparable DNA damage detected by ATM/ATR protein kinases and p53 protein tumor suppressor gene. The p53 increase the transcription of pro-apoptotic proteins (Bax) which punches a series of pores in the mitochondria, allowing molecules such as cytochrome-C to leak into the cytoplasm. The leaked cytochrome-C molecules bind to Apaf-1 proteins to activate the caspase cascade. Made with BioRender.*

The extrinsic pathway may be activated by T-lymphocytes with the surface molecule FasLigand (FasL), FasL binds to Fas receptors on the target cell. This sets off a chain of intracellular events that will result in apoptosis by a caspase protein cascade.

Pathogenic microorganisms have been demonstrated to cause eukaryotic cell death, either by infecting host cells or by producing toxic products (Fink & Cookson, 2005). Pathogen-induced modulation of the host cell-death pathways may function to eliminate key immune cells or evade host defenses that can limit the infection (Weinrauch & Zychlinsky, 1999). Bacteria can secrete pore-forming toxins or protein synthesis inhibitors which have been associated with host cell apoptosis (Fink & Cookson, 2005). Plasma membrane blebbing can results from the caspase-mediated activation of gelsolin, an actin depolymerizing enzyme (Fink & Cookson, 2005).

Pathogens have different strategies to activate programmed cell death to undermine normal host defense responses either by virulence determinants which interact with key components of the

cell death pathway or interfere with the regulation of transcription factors monitoring cell survival (Weinrauch & Zychlinsky, 1999). Numerous extracellular pathogens trigger apoptotic pathways indirectly through the process of translocating bacterial toxins onto the host cell's plasma membrane or introducing them into the host cell's cytoplasm. This mechanism underscores the sophisticated interplay between pathogens and the host's cellular machinery, illuminating the intricate strategies employed by these pathogens to manipulate and subvert host defenses (Gao & Kwaik, 2000).

There are five ways virulence factors may induce apoptosis. Secretion of pore-forming toxins that interact with the host cell membrane will result in leakage of cellular components (Weinrauch & Zychlinsky, 1999). Some toxins express their enzymatic activity in the host cytosol (Weinrauch & Zychlinsky, 1999). Effector proteins may be delivered directly into host cells by a highly specialized type-3 secretory system (Weinrauch & Zychlinsky, 1999). Some superantigens target immune cells and others are modulators of host cell death (Weinrauch & Zychlinsky, 1999).

There are different types of apoptotic pore formers. Amphiphilic toxins that are inserted directly into the cell membrane and thereby creating an ion-permeable pore (Weinrauch & Zychlinsky, 1999). *Pasteurella haemolytica* are known to produce RTX toxins that has been shown to cause cell death in bovine leukocytes (Weinrauch & Zychlinsky, 1999).

Pyroptosis is a type of apoptosis involving a high degree of inflammation as the process is lytic and immunostimulatory molecules are released by the cell (Yu et al., 2021). “Pyro” means fire from Greek indicating the inflammation properties of pyroptosis (Yu et al., 2021). Pyrotopic cells are swelling and have membrane blebbing (Yu et al., 2021). Pyroptosis causes flattening of the cytoplasm due to plasma membrane leakage (Yu et al., 2021). Caspase activation is present in both apoptosis and pyroptosis (Yu et al., 2021).

1.4 Immunology and host-pathogen interactions

Fish, like all vertebrates, possess immune systems to defend against pathogens. While the immune system of fish shares some similarities with that of mammals, there are also significant differences, reflecting the unique challenges and environmental conditions faced by aquatic organisms (Gudding 2010). The immune system is divided into innate and adaptive immunity. In contrast to mammals, fish are exposed directly for microorganisms from embryonic stages, this makes the innate part of the immune system more important than the adaptive in early

stages (Uribe et al., 2011). The innate immune system is the first line of defense and acts in the activation of the adaptive immune system (Magnadóttir, 2006).

A minority of microbes are true pathogens that can infect those with normal intact immune defenses, most of the microbes are opportunistic pathogens that strike immunocompromised individuals (Murphy & Weaver, 2017). Pathogens are divided into those who can establish an acute infection, replicate quickly, and re-infect another host, and with pathogens who can establish chronic infections, and live over a longer term in the host while evading elimination by immune defenses (Murphy & Weaver, 2017). After millions of years of coevolution with the host they have evolved a great diversity of strategies to evade the host immune responses (Murphy & Weaver, 2017).

The cellular arm of the innate immune system consists of immune cells that recognize foreign substances and pathogen PAMPS, by binding to PRRs, one strategy of immune evasions used by pathogens is shielding of surface PAMPS for example by modifying LPS so that TLR-4 binding is inhibited (in mammals) (Murphy & Weaver, 2017). Bacterial adhesion to surfaces is the first step in colonization, invasion, and biofilm formation (Klemm, 2010). It is worth mentioning that adherence can have both positive and negative consequences for the pathogen. Binding to phagocytic cells like neutrophils, macrophages, and dendritic cells can result in phagocytosis (Johnson, 2018).

Humoral and cell mediated immunity are needed to fight bacterial infections, humoral immune response is the main protective response against extracellular bacteria. Antibodies secreted by plasma cells can act as an opsonin to increase phagocytosis of the bacterium by neutrophils, macrophages, and dendritic cells (Punt et al., 2019). Antibody-mediated activation of the complement system can lead to recruitment of lymphocytes and neutrophils from the blood into tissue spaces (Punt et al., 2019). Consequently, a bacterial pathogen's capacity to impede complement activation is considered a significant virulence factor (Johnson, 2018).

Bacteria have developed a variety of evasion strategies to host immune responses throughout the infection process. This includes attachment to host cells, inhibiting complement activation, changing antigenic structures, and inhibiting phagocytosis (Punt et al., 2019). The immune system can block attachment of bacteria by binding of secreted antibodies, and the bacteria can evade this by secreting proteases that cleave secretory immunoglobulins or by antigenic variation in attachment structures pili or fimbrae (Punt et al., 2019). Bacterial proliferation can be stopped by the immune system by phagocytosis helped by complement opsonization and

can be evaded by bacteria with production of surface structures (such as polysaccharide capsule, M protein, fibrin coat) that inhibit phagocytic cells, or by some bacteria phagocytosis can be avoided with induction of apoptosis in macrophages (Punt et al., 2019). When bacteria invade host tissues, the immune system can agglutinate bacteria, and some bacteria are able to secrete elastase that inactivates the complement proteins C3a and C5a (Punt et al., 2019). When bacteria are secreting toxins to host cells, the immune system normally neutralize these toxins with antibodies, but some bacteria can secrete hyaluronidase, which enhances bacterial invasiveness in this case (Punt et al., 2019).

Intracellular pathogens can evade immune defenses by invading and growing within phagocytic and non-phagocytic cells. Cytokine responses and macrophages are the main effectors for intracellular bacteria (Punt et al., 2019).

Extracellular pathogens can block phagocytosis with the expression of a polysaccharide capsule or biofilm (Johnson, 2018). Bacterial capsules are typically made up of proteins and sugars that resemble those found in host cells. Consequently, bacterial capsules are not identified as foreign substances by macrophages and neutrophils (Johnson, 2018). An additional obstacle to phagocytosis is the proliferation of pathogens within biofilms. Biofilms are collections of microbial cells adhered to either non-living or living surfaces, held together by a matrix of extracellular polymeric substances that comprises of sugars, proteins, and DNA (Johnson, 2018).

1.5 Aims of the study

In the present study we wanted to study the growth characteristics of *P. atlantica* gv. *salmonicida*, identify virulence factors and immunogenic components by in silico analyses and observe if *P. atlantica.* gv. *salmonicida* and its metabolites caused apoptosis of salmon head kidney leucocytes.

The aims of the study were:

- To investigate *P. atlantica* gv. *salmonicida* growth requirements of FCS when culturing in TSB.
- To examine the biochemical characteristics of *P. atlantica* gv. *salmonicida* and compare it to *P. atlantica* gv. *cyclopteri.*
- To perform in silico analyses for identification of possible virulence factors and immunogenic components.
- To observe leucocytes exposed to *P. atlantica* gv. *salmonicida* by immunostaining and fluorescence microscopy, by confocal microscopy and by SEM.
- To observe differences or similarities between isolates of *P. salmonicida*.

2. Materials and methods

2.1 Bacteria

The bacterial isolates included in this thesis had previously been isolated from clinically sick Atlantic salmon during outbreaks of pasteurellosis in Vestland County. The four isolates of *P. atlantica gv. salmonicida* will be referred to as PaL-1, PaL-2, PaL-3, and Pal-4 in this thesis.

Table 1. *Overview of bacterial isolates included in the thesis, fish species they were collected from, year of isolation and location.*

Isolate abbreviation	Isolated from	Year	Origin
PaL-1	Atlantic salmon (*Salmo salar L.*)	2019	Vestland county (Norway)
PaL-2	Atlantic salmon (*Salmo salar L.*)	2020	Vestland county (Norway)
PaL-3	Atlantic salmon (*Salmo salar L.*)	2020	Vestland county (Norway)
PaL-4	Atlantic salmon (*Salmo salar L.*)	2021	Vestland county (Norway)
V. anguillarum	Lumpfish (*Cyclopterus lumpus L.)*	2021	Vestland county (Norway)

2.2 Cultivation of bacteria

P. atlantica gv. salmonicida were cultured in a liquid growth media termed TSB+ containing Tropic Soy Broth (TSB) supplemented with 1.5 % NaCl (Honeywell International) and 10 % Foetal calf serum (FCS)(Gibco).

Glycerol stock bacteria (1 ml) containing 10 % glycerol stored at -80 ℃ (Thermo Scientific™, Revco™) were suspended in 40 ml TSB+ (Appendix). The bacteria were cultured in 50 ml tubes (Sarstedt AG & CO. AS) and incubated in a shaking incubator (INFORS AG CH-4103 BOTTMINGEN) at 200 rpm and 20 ℃ for 24 hours.

Vibrio anguillarum was cultured by suspending glycerol stock bacteria (1 ml) containing 10 % glycerol stored at -80 ℃ (Thermo Scientific™, Revco™) in TSB supplied with 1.5 % NaCl (40ml). The bacteria were cultured in 50 ml tubes (Sarstedt AG & CO. AS) and incubated in a shaking incubator (INFORS AG CH-4103 BOTTMINGEN) at 200 rpm and 20 ℃ for 5 hours. The main culture was then made by suspending 1 ml of the pre-culture in 40 ml TSB

supplemented with 1.5 % NaCl and further cultured at 20 ℃ with shaking at 200 rpm for 9 hours.

***Table 2**. Overview of growth conditions and media used for cultivation of P. atlantica gv. salmonicida isolates and V. anguillarum.*

Bacteria	Liquid medium	Growth conditions
PaL1	TSB 1.5 % NaCl + 10 % FCS	20 ℃ and 200 rpm for 14h
PaL2	TSB 1.5 % NaCl + 10 % FCS	20 ℃ and 200 rpm for 14h
PaL3	TSB 1.5 % NaCl + 10 % FCS	20 ℃ and 200 rpm for 14h
PaL4	TSB 1.5 % NaCl + 10 % FCS	20 ℃ and 200 rpm for 14h
V. ang	TSB 1.5 % NaCl	20 ℃ and 200 rpm, 5h for pre-culture and 9h for main culture

2.3 Preparation of bacterial glycerol stocks

Freeze stock cultures were prepared to ensure sufficient and identical bacterial stocks for all procedures. After incubation, 16 ml bacterial cultures were added to new 50 ml tubes (SARSTEDT AG & Co) containing 4 ml glycerol (> 99 %) giving a 4:1 ratio of bacterial suspension: glycerol. The solution was gently mixed before the suspension was transferred to cryo tubes (VWR), 1 ml/tube, and frozen at -80 ℃ (Thermo Scientific™, Revco™).

2.4 Growth rate of *P. atlantica* gv. *salmonicida*

To identify when the cultures reached the late exponential growth phase, bacterial growth curves were compiled. This was performed by measuring optical density at 600 nm (OD 600 nm) in the cultures over time using a spectrophotometer (Spectroquant® Pharo 300, Merck). Light spread through the suspension and how much of the light spread is dependent on the density of bacteria in the suspension. A cuvette (VWR Cuvettes PS macro, VWR) with sterile TSB+ was used as a blank. The bacterial cultures were incubated as described in section 2.1.1. and OD was measured at 600 nm wavelength from the time of inoculation of bacteria in TSB+ until the stationary growth phase was reached.

2.5 Analysis of growth with different concentrations of supplemented FCS

In this experiment, the four isolates of *P. atlantica* gv. *salmonicida* were incubated as described in section 2.1.1, but with different concentrations of supplemented FCS (0 %, 5 % and 10 %).

OD (Spectroquant® Pharo 300, Merck) and cell count (Roche Innovatis AG CASY Model TT cell counter) was measured after 16 hours. The cultures were then sub cultured into new 50 ml tubes containing the corresponding concentration of FCS (0 %, 5 %, and 10 %) and incubated for 16 hours before repeating measurements of OD and cell count.

For measuring cell numbers, filtered (2 µm) CASYton buffer was supplied to 10 ml tubes. From the 50 ml tubes containing bacterial cultures 10 µl were extracted and mixed with the 10 ml of CASYton. The bacterial cultures were then analyzed for cell number, viability, and aggregation factor according to preset protocols using a Roche Innovatis AG CASY Model TT cell counter.

2.6 SDS-PAGE gels and silver staining

Sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining were conducted to visualize and compare the protein profiles of the *P. atlantica* gv. *salmonicida* isolates (PaL-1, PaL-2, PaL-3, and PaL-4). Bacterial isolates cultured with different concentrations of supplemented FCS as described in section 2.5 were harvested after 16h in the late exponential growth phase, 1 ml of each of the cultured bacteria suspensions were pipetted into 2.0 ml Eppendorf tubes and centrifuged at 1300 x g for 3 minutes to obtain a pellet of bacteria. The supernatant was discarded, and excess fluids were removed using a pipette. The pellets were stored at –20 ℃.

The pelleted bacteria were taken out of the –20 ℃ freezer and then 100 µl MilliQ were added to each pellet, then 10 µl of the suspension were extracted to new Eppendorf tubes and mixed 1:1 with SDS Reducing Buffer supplemented with β-mercaptoethanol (1:20) (Appendix) and heated in a heating block at 98 ℃ for 5 minutes. After this 10 µl of the samples were supplied to the wells. 5 µl of unstained low-range SDS-PAGE standard (Bio-Rad) diluted 1:20 in the SDS Reducing Buffer, treated equally as the bacterial samples, were used as a ladder. The protein gels (12 % acrylamide Resolving gel and 4 % Stacking gel) were prepared according to the method of Laemmli (Laemmli, 1970). The stacking gel at the top will concentrate proteins in one band and the resolving gel at the below allows for protein separating according to their molecular weight. The gels were electrophoresed at 190V for 50 minutes in a Mini Protean Tetra cell electrophoresis chamber (Bio-Rad, Mini-PROTEAN Tetra Cell).

Post electrophoresis the gels were fixed in a fixative solution (Appendix) for 20 minutes and washed twice for 10 minutes using Milli-Q. The gels were stained using the Silver Stain Plus

kit (Bio-Rad). Once protein bands were visible the reaction was stopped by adding a 5 % acetic acid solution. The gels were photographed in a Bio-Rad, Universal Hood II.

2.7 Gram staining

Bacteria were cultured as described in section 2.1.1. A small sample of bacterial culture was placed on a glass slide and allowed to air dry. When dry, the slide was flooded with crystal violet for 1 minute, this dye enters all bacterial cells, staining them purple. Then the glass slide was rinsed in tap water for 1 minute. Then an iodine potassium solution was applied to the slide, iodine forms a complex with crystal violet inside the bacterial cells, trapping the dye within the cells. Then the slide was rinsed in tap water for 1 minute. Alcohol, 96 % EtOH was applied dropwise. Then the glass slide was rinsed in tap water for 1 minute. The preparation was then counter stained with safranin for 45 seconds. Then the glass slide was rinsed in tap water for 1 minute. Then the slide was observed under a Leica microscope.

2.8 Analytical Profile Index 20E test (API 20E)

Bacteria cultures (44 ml) of PaL-1, PaL-2, PaL-3, and PaL-4 was centrifuged at 2500 x g for 10 minutes at 4 ℃ (Beckman Coulter Inc., Allegra X-15R Centrifuge). Then the supernatant was discarded, and the pellet re-suspended in 4 ml NaCl 0.85 %, then applied on the API 20E strip as described in the API 20E-kit manual, the strips were incubated at 22 ℃ for 48 hours. *V. anguillarum* was used as a control for API 20E and a positive control for the oxidase test. The oxidase test was performed by using filter paper and following the procedure described in the kit.

***Table 3**. Showing OD of bacterial cultures and dilution used for API 20E strip inoculation to test bacterial biochemical characteristics.*

	PAL-1	**PAL-2**	**PAL-3**	**PAL-4**	***V. ang* (control)**
OD	3.006	2.884	2.911	2.875	3.036
0.85 % NaCl	4 ml	4 ml	4 ml	4 ml	4 ml

2.9 Leucocyte isolation

Leucocytes were collected from the head kidney of salmon parr. The head kidney (HK) was aseptically dissected and transferred into 500 µl of L-15+ cell culture medium (Appendix). The tissue was then gently forced through a cell strainer (Falcon, 100 µm. BD Biosciences Discovery Labware, Bedford, USA) using bent glass pipettes and supplementing 1,5 ml of L-15+ media.

4 ml of 1.075 g/ml Percoll solution was pipetted to a 10 ml Eppendorf tube, then 3 ml of 1.060 g/ml Percoll solution were gently pipetted upon it. Lastly 500 µl sample of HK solution was gently pipetted on top of the Percoll gradient. Then the gradients were centrifuged at 400 x *g* for 45 minutes at 4 °C (Beckman Coulter Inc., Allegra X-15R Centrifuge). The top layer was discarded, and the middle and bottom layers were collected containing the leucocytes. The collected leucocytes were thoroughly but gently washed with L-15 medium and centrifuged at 200 x g for 10 min at 4 °C (Beckman Coulter Inc., Allegra X-15R Centrifuge). The cells were resuspended in a small amount of L-15 containing 5 % FCS and counted in a Roche Innovatis AG CASY Model TT cell counter.

2.10 Exposure of leucocytes to PaL-1 and immunostaining

The leucocytes were isolated as described in section 2.9. For all leucocyte exposure experiments, the bacteria were harvested in the late exponential growth phase. The leucocyte counts were adjusted to $3*10^6$ cells/ml. From this leucocyte suspension 200 µl were transferred to sterile 1,5 ml Eppendorf tubes**,** giving a cell count of 6*10^5 cells/tube. The samples were divided into control and exposure samples. For the infected samples the procedure was as follows. An overnight culture of bacteria PaL-1 was centrifuged and re-suspended into L-15 + 5 % FCS. Bacteria counts were then adjusted to $3*10^6$ bacteria/ml. To Eppendorf tubes for leucocyte exposure 200 µl were added giving a ratio of 1:1 between leucocytes and bacterial cells. For the control samples 200 µl of sterile L-15 + 5 % FCS was used instead of bacteria in each tube. The tubes were incubated for 5 hours at 15 °C in a Panasonic Cooled Incubator. The samples were then cleaned by centrifugation through a glucose cushion (Appendix). The sample was gently added on top of a to a 3 ml glucose solution and centrifuged at 100 x g for 10 minutes at 4 °C (Beckman Coulter Inc., Allegra X-15R Centrifuge). The pellet was collected and resuspended in 500 µl PBS-E (Appendix).

Cytospin preparations were made by loading 100 µl of the samples on a prepped slide and centrifuged (Thermo Shandon., Cytospin 3 Cytocentrifuge) at 1000 x g for 3 minutes. The slides were dried in the dark overnight prior to staining**.**

Firstly, the area around the cells was encircled using a hydrophobic PAP-pen. The cells were then fixed using a 1:1 solution of acetone and methanol for 90 seconds and washed for 5 min using PBS in a Coplin jar. To avoid background staining blocking solution (0.5 % BSA in PBS) was supplied and the slides incubated for 30 min. Then slides were incubated for 1h, in darkness, with 500 µl rabbit anti-Pasteurella polyclonal antibodies (1:1000 in 0.5 % BSA in PBS). The slides were then washed three times with 500 µl PBS for 5 min each. The slides were then incubated for 45 min in darkness with 200 µl goat anti-rabbit Ig Alexa Fluor 555 (1:400 in PBS-E) and with 100 µl Alexa Fluor 488 Phalloidin (1:400 in PBS-E). Washed with PBS for 5 min and then incubated for 10 min in darkness with DAPI (1:1000 in PBS). The sample was washed again before mounting a coverslip with ProLong Gold anti-fade mounting media. The immunostained cells were observed with the use of a fluorescence microscope (Zeiss Axioskop 2 plus).

2.11 Confocal microscopy of leucocytes exposed to PaL-1

Head kidney leucocytes were isolated as described in section 2.9. The number leucocytes were adjusted $5*10^5$ cells/well in a total volume of 250 µl. The samples were divided into control and exposure samples using 4-chambered chamber slides (Lab-Tek ® Chamber slide (tm) System 177445 4 Well Permanox ® Slide). For the exposure samples the procedure was as follows. An overnight culture of bacteria PaL-1 was centrifuged and re-suspended into L-15 media. Bacteria were then adjusted to $5*10^6$ bacteria per well and a volume of 250 µl were added to each well over a 4-chambered chamber slide. For the control samples 250 µl of L-15 + 5 % FCS was used instead of bacteria. The slides were incubated for 4 hours at 15 °C on an Panasonic Cooled Incubator. The supernatants, containing free bacteria from exposed samples or medium from control wells were removed and replaced with 500 µl of clean L-15 supplemented with 5 % FCS. And further incubated for 6 h and 24 h respectively.

After exposure period ended the L-15 + 5 % FCS were discarded. The cells were washed 3 times with PBS. From now on between every step the cells were washed with PBS 3x5 minutes. The cells were then fixed using 3.7 % paraformaldehyde for 10 minutes. 0.1 % Triton-X in PBS was applied for 5 minutes to permeabilize cells. To avoid background staining the samples was then incubated with 2 % BSA in PBS for 1 hour. The wells were incubated for 1 hour in

darkness with 500 µl of rabbit anti-Pasteurella polyclonal antibodies (1:10000 in 0.5 % BSA in PBS). Then incubated for 1h in darkness with 200 µl goat anti-rabbit lg Alexa Fluor 555 (1:400 in 0.5 % BSA in PBS). Then incubated for 20 minutes in darkness with 100 µl Alexa Fluor 488 Phalloidin (1:400 in 1 % BSA in PBS), then incubated for 10 minutes in darkness with 100 µl DAPI (1:10000 in PBS). Mounted in 25-30 µl Vectashield (Vector Laboratories) and coverslip. Edges were sealed using nail polish. Slides were stored at 4 ℃ until microscopy and imaging with Leica TCS SP8 STED 3x confocal at MIC (Molecular Imaging Center UIB).

2.12 SEM Scanning electron microscopy

Head kidney leucocytes were isolated as described in section 2.9. The number leucocytes were adjusted $6{,}6*10^5$ cells/well in a volume 125 µl of L-15 + 5 % FCS. The samples were divided into control and exposure samples. For the infected samples the procedure was as follows. An overnight culture of PaL-1 was centrifuged and re-suspended into L-15. Bacterial counts were then adjusted to $6{,}6*10^6$ bacteria per well and a volume of 250 µl were added to each well in an 8-chambered chamber slide (Lab-Tek ® Chamber slide (tm) System 177445 8 Well Permanox ® Slide). For the control samples 250 µl of L-15 + 5 % FCS was used instead of bacteria. The slides were incubated for 4 hours at 15 ℃ in an Panasonic Cooled Incubator. The bacteria or medium from control wells were removed and replaced with 250 µl of clean L-15 supplemented with 5 % FCS. And further incubated for 6 h and 24 h respectively**.** The 6 h samples were fixed according to protocol below and stored at 4 ℃ over night before continuing with the protocol.

Leucocytes were sampled at 6 and 24 h post-exposure. The medium was discarded, and cells were washed three times using PBS. The cells were then fixed in 2,5 % glutaraldehyde diluted in 2x PBS for 45 min at 4 ℃. These were then washed five times with PBS for 10 min at 4 ℃. Post-fixations were then done in 1 % osmium tetroxide diluted in 2x PBS for 20 min at 4 ℃. Cells were then washed twice in dH2O and dehydrated alcohol series as follows, 30 % for 5 min at 4 ℃, then allow the sample to reach room temperature. Followed by 50 %, 70 %, and 96 % EtOH each for 5 min, respectively, followed by 100 % EtOH, three times for 10 min each.

Samples were then submitted to critical point drying (Balzers CPD 030 Critical Point Dryer), mounted on aluminum stubs and carbon tape, and coated (Gatan etching coating System Model 662) with 10-20 nm Iridium and imaged with a Zeiss Supra 55 VP using 5 KV and InLens detector at the Electron Microscopy Laboratory (ELMILAB), University of Bergen.

2.13 In silico analysis of PaL-1 genome

The genome of PaL-1, which was previously sequenced was annotated by Professor Håkon Dahle (UIB). The proteome of PaL-1 was screened against several databases to identify potential virulence factors. Firstly, the proteome was uploaded to PSORTb c3.0.3 to identify subcellular localization of the proteins.

Proteins that came out as extracellular and proteins present on the outer membrane were filtered out into respective lists. Any hypothetical proteins in these lists were identified through the BLASTp database (Blastp BLAST National Center for Biotechnology Information (NCBI)). Proteins identified as localized extracellularly or on the outer membrane were screened against VFDB (Virulence Factor Database) to identify those having potential virulence characteristics.

3. Results

3.1 Growth curves

The growth curves presented in Figure 4 show OD at 600nm measured over time (hours) for isolates PaL-1, PaL-2, PaL-3, and PaL-4 grown in 40ml TBS+. The measurements were done every two hours until the cultures reached stationary phase. The isolates grew in a similar fashion to each other. After 10 h the curves show an early exponential growth phase. The cultures reached late exponential growth after 14-16 h. After 18 h the culture reached maximum optical density at about 0,8-0,9 OD.

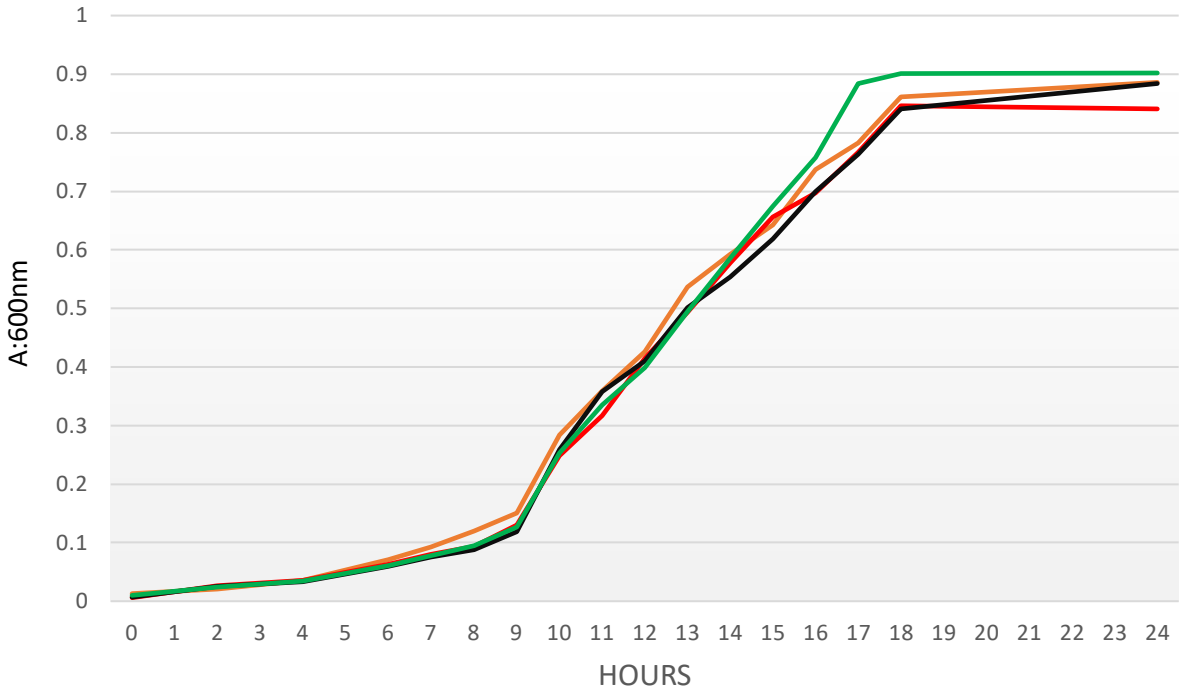

***Figure 4**: Growth curves showing optical density over time of broth cultures of PaL-1, PAL-2, PaL-3 and PaL-4. Orange: PaL-1, Red: PaL-2, Black: PaL-3, Green: PaL-4.*

3.2 Analysis of growth with different concentrations of supplemented FCS

Cell counts (bacteria/mL) measured in the CASY cell counter were compiled as graphs showing number of cells and cell sizes (µm) (Figure 5).

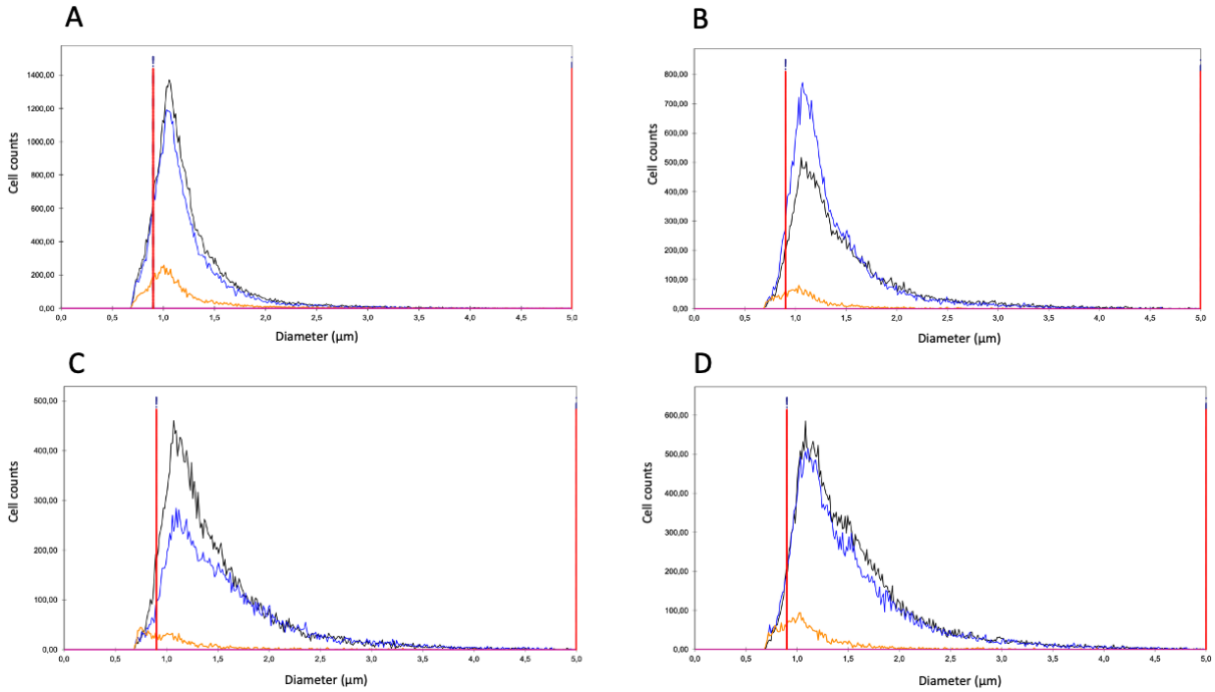

Figure 5: *Showing cell counts and size of cells in the bacteria samples. Black line show bacteria1 grown in medium containing 10 % FCS, blue line bacteria grown in medium containing 5 % FCS and orange bacteria grown in media containing 0 % FCS. A: PaL-1, B: PaL-2, C: Pal-3 and D: PaL-4*

The graphs (Figure 5) show cell count and size of cells in the bacteria cultures after 16 hours of incubation in TSB with three different concentrations of FCS. The colors of the lines indicate the concentrations of FCS supplemented to TSB. Black lines have 10 % supplemented FCS, blue line has 5 % supplemented FCS and orange line has 0 % supplemented FCS. Figure 5A, B, C and D show results for isolates PaL-1, PaL-2, PaL-3 and PaL-4 in that order. The figure indicates a dependency of FCS for the cultures to brow in TSB. The cultures supplemented with 5 % FCS (blue line) and 10 % FCS (black line) are quite similar regarding cell count. The cultures without FCS supplement 0 % (orange line) differs by having a lot lower cell count.

The cell counts presented in Table 4 show the importance of FCS supplemented in the growth media. The cell count is higher for the 5 % and 10 % FCS concentrations after both the initial incubation with bacteria from glycerol stock cultures (1ml) described in section 2.3 and after the subcultivation with bacteria from the initial incubation (1ml).

Table 4. *Cell counts and aggregation factor for Pasteurella isolates cultivated with different concentrations of FCS.*

Isolate	Initial incubation (16h)			Subcultivation (16h)		
	FCS (%)	**Cell Count (cells/mL)**	**Aggregation Factor**	**FCS (%)**	**Cell Count (cells/mL)**	**Aggregation Factor**
PaL-1	10 %	1,9E+09	2,448	10 %	1,943E+09	3,646
	5 %	1,6E+09	2,394	5 %	1,742E+09	4,139
	0 %	4,6E+08	3,656	0 %	8,371E+07	9.578
PaL-2	10 %	1,806E+09	3,962	10 %	1,763E+09	3.882
	5 %	1,659E+09	3,027	5 %	1,727E+09	4,160
	0 %	1,383E+08	3,601	0 %	8,457E+07	4,762
PaL-3	10 %	1,498E+09	3,727	10 %	1,755E+09	4,008
	5 %	1,403E+09	4,638	5 %	5,819E+08	4,168
	0 %	8,726E+07	4,634	0 %	5.808E+07	3,967
PaL-4	10 %	2,451E+09	4,219	10 %	2.024E+09	3.924
	5 %	2,189E+09	4,245	5 %	1,334E+09	4,360
	0 %	1,716E+08	3,611	0 %	7,737E+07	4,770

3.3 SDS-PAGE and silver staining

The FCS dependency was supported by the results from silver stained SDS-PAGE gels (Figure 6). The growth is much lower in the 0 % FCS groups and the number of bacterial cells was not adjusted to the same amount, therefore there were some differences in the intensity of the bands between the isolates. Figure 6A-D shows dark colored protein bands when cultured with 5 % and 10 % supplemented FCS (lane 2 and 3 respectively), and weak protein bands when cultured without FCS supplement lane 1 in Figure 6A-D. On the other hand, location or presence of protein bands seems to be similar between the four isolates.

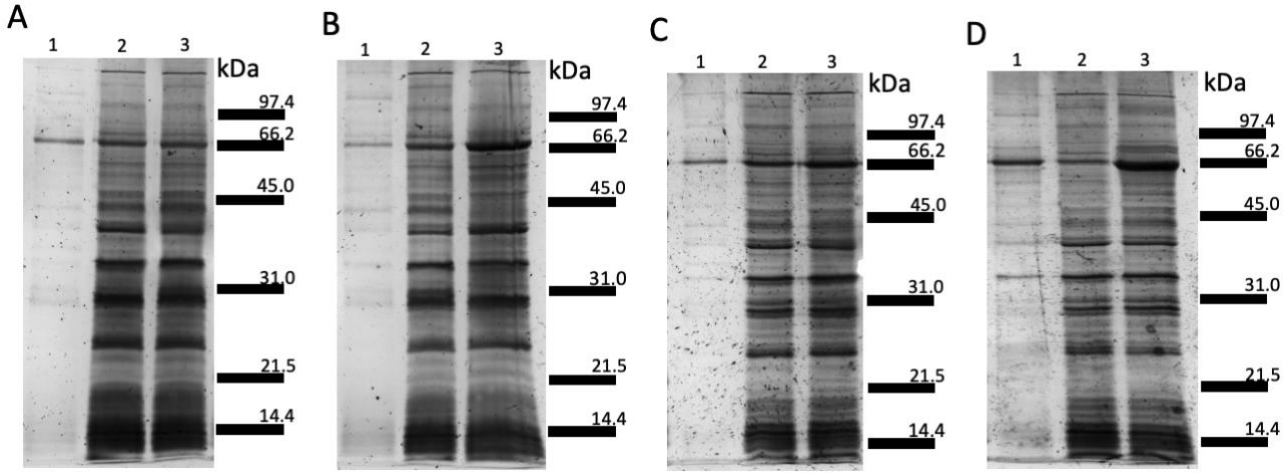

***Figure 6**: Silver stained SDS-PAGE gels showing protein expression of PaL-1(A), PaL-2 (B), PaL-3 (C) and PaL-4 (D) with different concentrations of FCS in cultivation. Lane 1: 0 % FCS, lane 2: 5 % FCS and lane 3: 10 % FCS.*

3.4 Gram staining

The pictures (Figure 7) show gram stains of samples collected from all four isolates. They look morphologically similar and with a blue/purple color showing that this is gram negative bacteria. Figure 4A, B, C and D are the isolates PaL-1, PaL-2, PaL-3 and PaL-4 in that order.

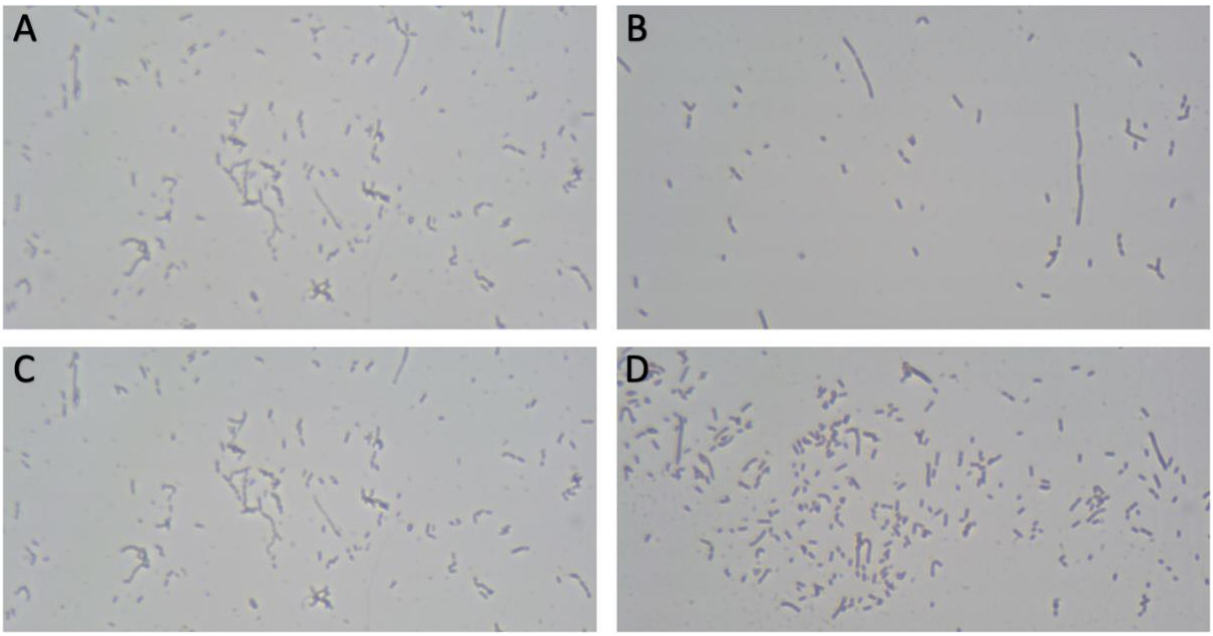

***Figure 7**: Gram stains photographed using a Leica microscopy, showing P. atlantica gv. salmonicida, rod shaped bacteria connected in chains. PAL-1 (A), PAL-2 (B), PAL-3 (C) and PAL-4 (D).*

3.5 API 20E

The table (Table 5) shows biochemical characteristics of *Pasteurella atlantica* gv. *salmonicida* compared to *Pasteurella atlantica* gv. *cyclopteri* and *V. anguillarum*. There are differences in fermentation of sugars as presented in the table below.

***Table 5**. The table shows biochemical characteristics of P. atlantica gv. salmonicida, P. atlantica gv. cyclopteri and V. anguillarum when tested on API 20E strips incubated for 24 hours at 15 ℃.*

	V. anguillarum	*P. atlantica gv. cyclopteri*	*P. atlantica gv. salmonicida*
ONPG	+	+	+
ADH	+	-	-
LDC	-	-	-
ODC	-	-	-
H2S	-		
URE	-	-	-
GLU	+	+	+
MAN	+	-	+
SOR	+	-	+
SAC	+		
MEL	+	-	+
RHA	+	-	+
ARA	+	-	+

3.6 Immunofluorescence

The pictures (Figure 8) show head kidney leucocytes stained with Dapi (blue, nuclei) and Phalloidin (green, actin). Figure 8A showing leucocytes photographed by light microscopy (20x). Figure 8B shows leucocytes stained with Dapi (blue) and phalloidin (green) (20x) and figure 8C the same but at 63x magnification. Figure 9 shows the same order as Figure 8, but the head kidney leucocytes are exposed to bacteria. In figure 9C bacteria can be observed both inside and outside the leucocytes.

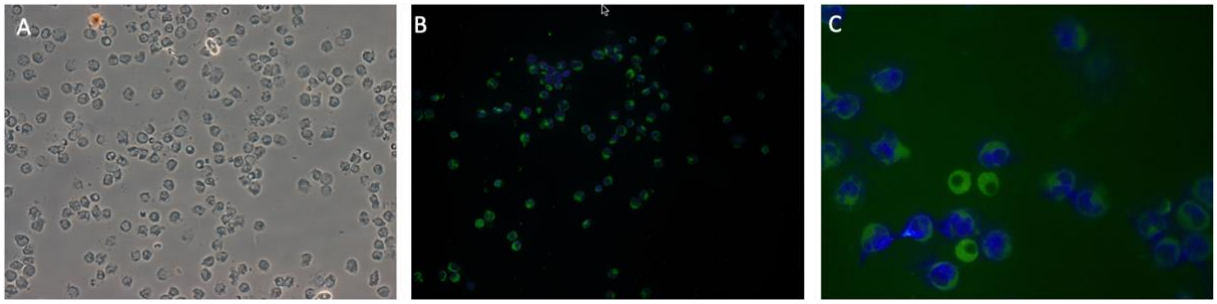

***Figure 8**: A: showing leucocytes light microscopy (magnification 20x). B: leucocytes (magnification 20x) stained with dapi (blue) and phalloidin (green). C: leucocytes (magnification 63x) stained with dapi (blue) and phalloidin (green).*

The pictures (Figure 9) show head kidney leucocytes with the same staining exposed to PaL-1.

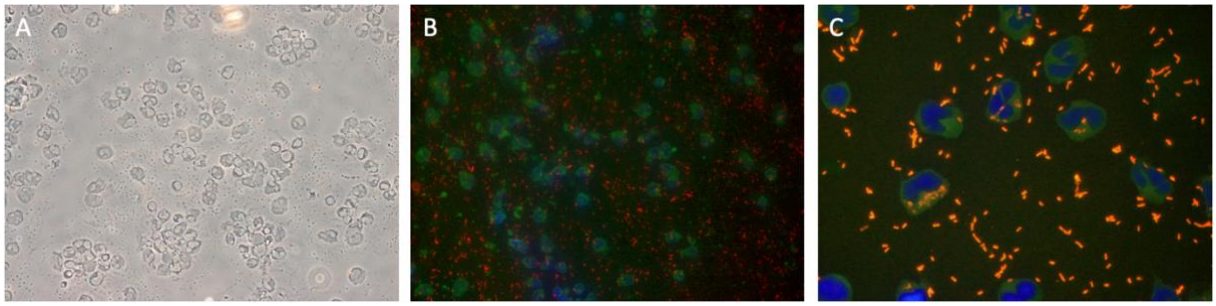

***Figure 9**: A: showing infected leucocytes light microscopy (magnification 20x). B: Infected leucocytes (magnification 20x) showing bacteria, leucocytes stained with dapi (blue) and phalloidin (green). C: Infected leucocytes (magnification 63x) stained with dapi (blue), phalloidin (green) and bacteria (red).*

Unfortunately, there were no successful pictures from the confocal microscopy. Upon confocal microscopy all samples, both challenged and controls, appeared with a green blur and it was not possible to identify leucocytes.

3.7 SEM

The head kidney leucocytes have a round shape after isolation. When applied to wells in L-15 and incubated over time (6h and 24h) they attach at the base of the wells and some change morphologically increasing in diameter and spreading over the bottom of the wells shown by a variety of projections. Some appear rounded with rough surfaces. The ones that do not attach are washed off with L-15 and discarded. At the right concentration of leucocytes per area, the majority of cells are observed alone. Figure 10 shows leucocytes within 6 h groups (A, B) and 24h groups (C, D).

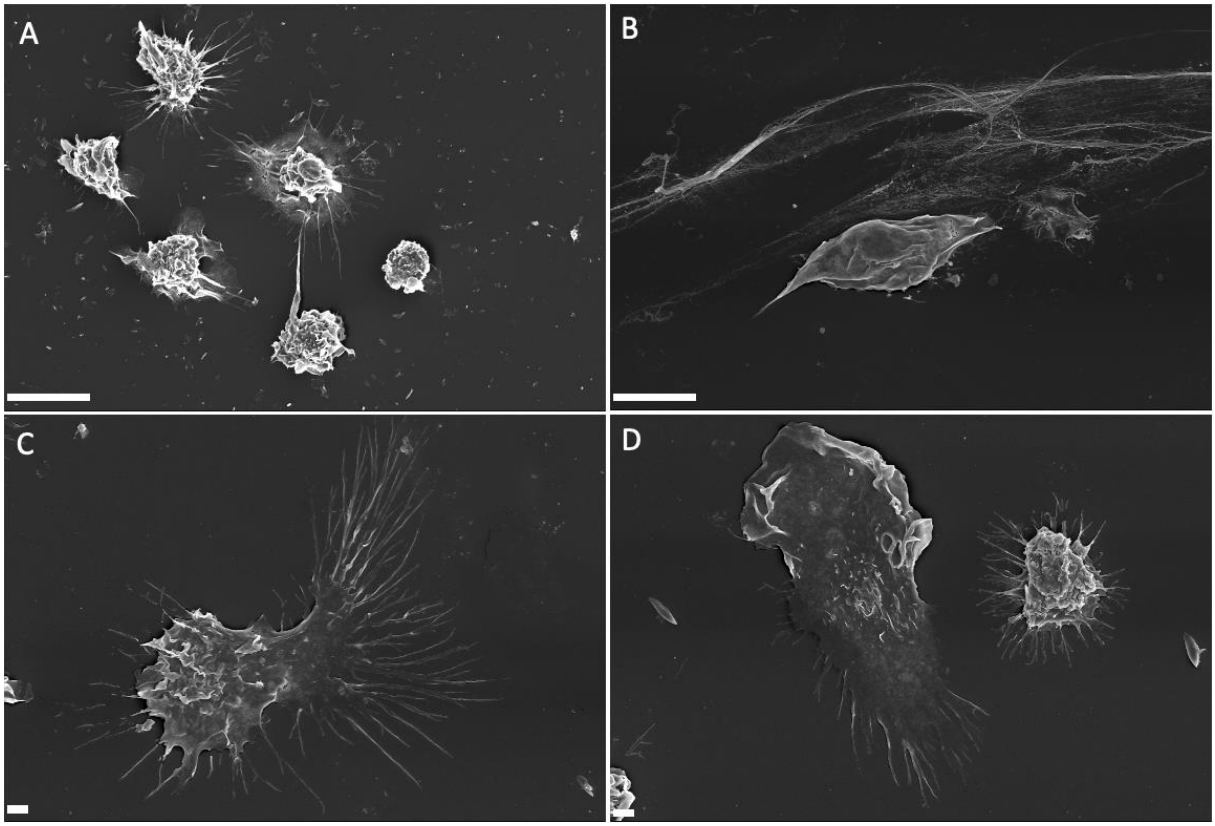

***Figure 10**: Non-infected leucocytes harvested after 6 h and 24 h showing varying cell shapes. **A** SEM picture from 6 h control group. Scalebar = 10µm. Displays intact cells, like round coconut ball. **B** picture from 6h control group. Scalebar = 10µm. Displays intact cells that has attached to the surface. **C** picture from 24 h control group. Scalebar = 2µm. Displaying intact cells. **D** picture from 24h control group. Scalebar = 2µm. Displaying intact cells.*

Figure 11 shows leucocytes exposed to PaL-1 within the 6 h groups (A, B) and 24 h groups (C, D). Figure 12 A shows leucocytes in the 24 h control group, 12B, C and D shows stages of apoptosis in leucocytes exposed to PaL-1.

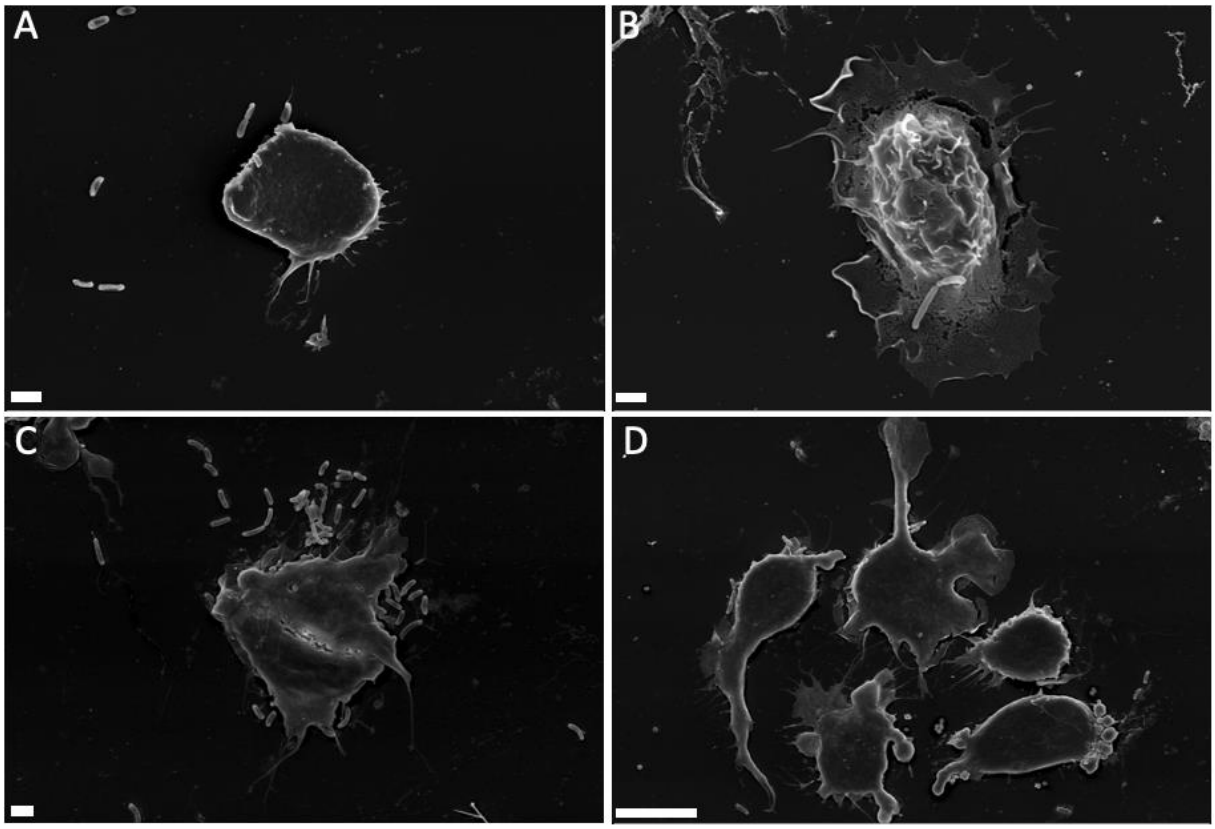

***Figure 11**: **A** picture from 6h infected group. Scalebar = 2μm. Displaying intact leucocytes with bacteria close, no impact. **B** picture from 6 h infected group. Scalebar = 2μm. Displaying bacteria in contact with leucocyte. **C** picture from 24 h infected group. Scalebar = 2μm. Displaying bacteria in contact with leucocyte. **D** picture from 24 h infected group. Scalebar = 10μm. Displaying many apoptotic leucocytes and membrane blebbing in contact with bacteria.*

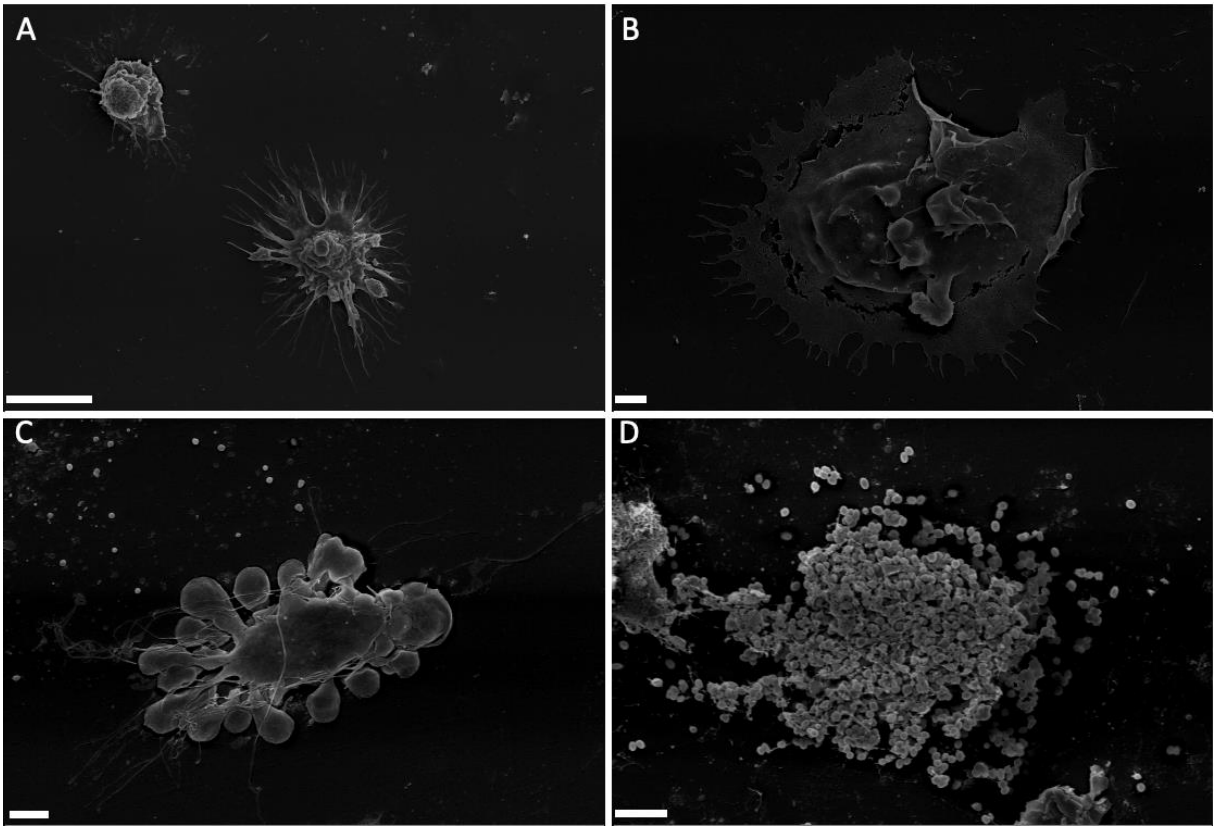

***Figure 12**: Pictures of infected (B-D) and non-infected (A) leucocytes harvested after 6h and 24h. **A**. 24h control group showing what the cells look like without being exposed to bacteria. Scalebar = 10μm. **B**. picture from 6h infected group. Scalebar = 2μm. Displaying beginning of apoptosis. **C**. picture from 24h infected group. Scalebar = 3μm. Displaying stages of apoptosis and clearly visible membrane blebbing. **D**. head kidney leucocyte 24 h post infection. Scalebar = 3μm. Displaying late state of apoptosis.*

After exposure with PaL-1 the cells looked more swelled than in the non-infected controls. Also, membrane blebbing is seen in figure 11D and 12C. By comparing the leucocyte shown in Figure 12C to the leucocyte shown in Figure 12A it is observed that the first is both swelled and have membrane blebbing, and the second does not have membrane blebbing and is not swelled. Bacteria is seen in Figure 11 both in contact with leucocytes and close to them, the bacteria appear rod shaped, and they form strings together. In Figure 11 C many bacteria can be observed, but all pictures in Figure 11 have bacteria close to the cells, and in Figure 12 bacteria is not seen in close contact with the cells.

3.8 In silico analysis of the PaL-1 genome

The table (Table 6) shows name of protein, what function it has, E-value obtained from the Virulence Factor Database, Blast score from Blastp and a localization score for the protein. The proteins listed are potential virulence factors and are located on the outer membrane (OM).

***Table 6**. The table shows possible virulence factors and immunogenic proteins on the outer membrane. Their function, E-value (VFDB), blast score (identities) and localization score (Loc. score).*

Name	**Function**	**VFDB E-value**	**Blast Score**	**Loc. score**
Outer membrane protein P2-like protein (hypotethical protein)	Forms pores that allow passive diffusion of small molecules across the outer membrane.	4E-24	100 %	10
NspA - Immune modulation	Immune modulation	3E-30	100 %	10
porin family protein	Forms a channel through the mitochondrial outer membrane	7E-21	100 %	9
TonB-dependent receptor	Transport of siderophores into the periplasm	E-170	100 %	10
porin family protein	Forms a channel through the mitochondrial outer membrane	1E-54	100 %	9
Type IV pilus biogenesis	Possible involved in attachment	4E-59	100 %	10
Possible EspP (hypothetical protein)	Serine protease with cytotoxic effect. Contribute to biofilm formation.	6E-41	100 %	10
YadA C-terminal domain-containing protein (hypothetical protein)	Promote pathogeneicity and virulence in host cell	7E-07	100 %	10
filamentous hemagglutinin/adhesin	Metabolic process	1E-46	100 %	10
type IV pilus biogenesis/stability protein, pilW-like		2E-20	100 %	10
TonB-dependent hemoglobin/transfer	Transmembranetransporter activity	0,0	100 %	10

rin/lactoferrin family receptor				
haemoglobin-haptoglobin binding protein HhuA	Involved in oxygen transport from the lung to the various peripheral tissues	0,0	100 %	10
outer membrane protein P5 (ompA), human factor H binding protein	Acts as a fimbriae subunit, allowing adhesion to host cells	3E-12	100 %	10
outer membrane protein P5 (ompA), human factor H binding protein	Acts as a fimbriae subunit, allowing adhesion to host cells	3E-87	100 %	10
autotransporter outer membrane beta-barrel domain-containing protein	Potent bacterial adhesin that mediates bacterial attachment to a broad variety of human and other mammalian cells.	0,006	100 %	10
hemolysin activator protein	Interacts with the cell-bound hemolysin. Necessary for the extracellular secretion and activation of hemolysin.	1E-30	100 %	10
Dot/Icm type IV secretion system effector LepB, Rab1 GTPase activating protein (GAP)		1E-08	100 %	10
Putative protein		E-137	100 %	10
filamentous hemagglutinin/adhesin		2E-29	99,95 %	10
multidrug efflux pump channel protein MtrE	Toxin and drug export protein A	3E-20	100 %	10
outer membrane protein A		1E-21	100 %	10
polysaccharide lyase, family 8	Polysaccharide lyase cleaving glycosidic bond between two adjacent sugar residues	6E-07	46,94 %	10

filamentous hemagglutinin/adhesin	Metabolic process	2E-35	100 %	10
hemolysin activator protein	Interacts with the cell-bound hemolysin. Necessary for the extracellular secretion and activation of hemolysin	3E-31	100 %	10
(colA) collagenase [Kappa-toxin - Exoenzyme] (hypothetical protein)	Toxin. Break down host tissue.	6E-04	99,74 %	10

The proteome was blasted against VFDB using an e-value of $1*10^{-3}$. A high e-value was chosen because this is a broad search. Subcellular localizations for all proteins were examined by PSORTb 3.0 with localization score threshold > 6.0.

The table (Table 7) shows the name of protein, what function it has, E-value obtained from the Virulence Factor Database, Blast score from Blastp and a localization score for the protein. The proteins listed beneath are possible virulence factors and are located extracellular (E).

Table 7*. The table shows possible extracellular virulence factors and immunogenic proteins. Their function, E-value (VFDB), blast score (identities) and localization score (Loc. score).*

Name	**Function**	**VFDB E-value**	**Blast Score**	**Loc score**
Secretory immunoglobulin A-binding protein EsiB	Possible inhibits neutrophil activation	1E-46	100%	9,72
Hypothetical protein	functions as a cell surface receptor in chitin elicitor	2E-18	100%	9,45
Hypothetical protein - YopT-type cysteine protease domain-containing protein	inhibiting actin assembly in infected cells	3E-36	99,95%	8,44

Hypothetical protein -prepilin-type N-terminal cleavage	cleavage/methylation	4E-11	100%	9,64
Hypothetical protein	autotransporter protein [BoaB - Adherence]	1e-24	100%	6,26
Secretory immunoglobulin EsiB	Possible inhibits neutrophil activation	5E-57	100%	8,44
Hypothetical protein - trimeric autotransporter adhesin Ata	Important for biofilm formation, binding to various extracellular matrix	5E-57	100%	6,04
Ser-Asp rich fibrinogen-binding	Cell adhesion	8E-05	99,8%	9,52
Hypothetical protein - Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein	Cell adhesion	1E-13	51,68%	9,64

The table (Table 8) show name of protein, what function it has, E-value obtained from the Virulence Factor Database, Blast score from Blastp and a localization score for the protein. The proteins listed beneath are possible immunogenic proteins. The table holds both outer membrane and extracellular proteins.

Table 8*. The table shows possible immunogenic proteins that were not identified as virulence factors (No hits in VFDB). Showing Localization (Loc) where OM = outer membrane and E = extracellular.*

Name	**Function**	**Blast Score**	**Loc score**	**Loc**
Maltoporin	Involved in the transport of maltose and maltodextrins	100 %	10	OM
copper resistance protein NlpE		100 %	10	OM

glycine zipper 2TM domain-containing protein	Structural constituent of ribosome. Biological process: translation	100 %	10	OM
patatin-like phospholipase family protein	Hydrolase activity	100 %	9	OM
ORF6N domain-containing protein	ORF6N domain-containing protein	100 %	9	OM
rod shape-determining protein MreC	Involved in formation and maintenance of cell shape	100 %	10	OM
autotransporter assembly complex family protein	Part of the translocation and assembly module (TAM)	100 %	10	OM
translocation/assembly module TamB domain-containing protein	Protein secretion	100 %	10	OM
47 kDa outer membrane protein	porin	100 %	10	OM
murein transglycosylase A	Murein-degrading enzyme.	100 %	10	OM
LPS assembly lipoprotein LptE		100 %	10	OM
outer membrane protein assembly factor BamE		100 %	10	OM
outer membrane protein assembly factor BamA		100 %	10	OM
OmpH family outer membrane protein		100 %	10	OM
LPXTG anchor domain surface protein	Cell wall-anchored surface receptor	25,42 %	10	OM
TonB-dependent receptor plug domain-containing protein	Signaling receptor activity	65,15 %	10	OM
peptidoglycan DD-metalloendopeptidase family protein		100 %	10	OM

Hypothetical protein	Transferrin-binding protein-like solute binding protein	47,39 %	9,64	E
tRNA N6-adenosine threonylcarbamoyltransferase	Catalytic activity	99,71 %	10	E
Penicillin-binding protein 1A	Possible resistance against host AMP	100 %	9,71	E
Hypothetical protein	LysM-like peptidoglycan-binding domain-containing protein	100 %	9,45	E
Nucleoside diphosphate kinase	Major role in the synthesis of nucleoside triphosphates other than ATP	100 %	9,45	E
tRNA threonylcarbamoyladenosine biosynthesis protein TsaB	Required for the formation of a threonylcarbamoyl group	100 %	9,71	E
Toxin CdiA	Contact dependent growth inhibition to suppress growth of neighboring target cells.	100 %	9,64	E

4. Discussion

The isolates of *P. atlantica* gv. *salmonicida* used in this work had been isolated and reported as the causative agent of confirmed outbreaks of pasteurellosis in farmed Atlantic salmon in Norway. Even though pasteurellosis has been a major problem in Norwegian salmon farming since 2018 (Legård & Strøm, 2020) we still have little knowledge when it comes to the bacterium's growth characteristics, immunogenic components, and virulence factors. Knowledge on these factors is important to enable the development of prophylactic measures such as efficient vaccines against the disease.

In this study, the growth dynamics of *P. atlantica* gv. *salmonicida* was analyzed including growth with different concentrations of supplemented FCS. It was established that all isolates are dependent on supplemented FCS when cultured in TSB. Biochemical characteristics were evaluated by growing bacteria and inoculating them on API 20E strips, and it was found that *P. atlantica* gv. *salmonicida* were more biochemically reactive than *P. atlantica* gv. *cyclopteri*. The low biochemical reactivity of *P. atlantica* gv. *cyclopteri* is consistent with results in previous work described by Alarcón et al (Alarcón et al., 2016). Importantly, in silico analysis of the PaL-1 genome was done to provide an overview of possible virulence factors and immunogenic components. Further, to study the effect of possible virulence factors, leucocytes were harvested from the head kidney of Atlantic salmon and exposed to PaL-1 before analysis by immunocytochemistry and SEM.

4.1 Growth curves

The isolates PaL-1 – PaL-4 show similarity in growth rate, profiles of expressed proteins, and requirements for FCS in the growth medium. The compiled growth curves help to create a standardized protocol for culturing and harvesting the bacteria and ensures reproducibility across experiments. This ensures good control of experimental conditions so that observed differences are due to experimental variables instead of variables in growth conditions. The isolates PaL-1-PaL-4 reach late exponential growth phase after 16 hours. This stage is where bacteria are most active, and thus the time point selected for sampling in every experiment. After 18 h the cultures reached a maximum optical density, at the growth conditions provided in our experiments, an OD of about 0,8-0,9. After 14-16h the cultures reached an OD of 0,6-0,7 that was used as a harvest point in all subsequent experiments.

Bacteria replicate by binary fission with two identical daughter cells as a result. Therefore, we obtain exponential growth only limited to a lack of nutrients. A spectrometer was used to measure the optical density (OD) over time for making the growth curves. Light travels through the suspension and depending on the density in the solution it gives us an indication of biomass. On the other hand, it does not separate between living and dead cells. The CASY cell counter gives us number of cells/mL in the solution and does not separate well between living and dead cells, but information on cell sizes is provided, making it possible to exclude small dead cells and debris from the analysis. Using agar growth medium and counting colony-forming units (CFU) will provide an estimate of the number of live bacterial cells.

4.2 FCS dependency experiment

To enable thorough study of *P. atlantica* gv. *salmonicida* and pasteurellosis it is important to understand the growth requirements of the bacterium. Cultivation of *P. atlantica* in broth has been known to be challenging. In this master thesis the bacteria were grown in TSB supplemented with 10 % FCS and it took 16h to reach late exponential growth phase. It was also found that the bacterium will grow with 5 % supplement of FCS, and that the maximum bacterial concentration was reached after 18h.

In previous studies of *P. atlantica* gv. *cyclopteri* (Ellul et al., 2019) it has been reported to take 18-19h to reach late exponential growth phase, and it was also reported that a 10 % FCS supplement was demanded by the bacteria, and the maximum bacterial concentration were obtained after 20 h.

In previous work Brain Heart Infusion Broth (BHIB), also supplemented with 10 % FCS, has been used to culture *P. atlantica* gv. *cyclopteri*, resulting in a slightly higher number of bacteria obtained, but a slower growth rate compared to culture in TSB+. For growth in BHIB *P. atlantica* gv. *cyclopteri* was reported to require 6-10 % of supplemented FCS (Ellul et al., 2019). For this master thesis TSB+ is chosen because the medium is more appropriate for vaccine production compared to BHIB as it has less unwanted biological components (Ellul et al., 2019). Additionally, other studies including broth growth of *P. atlantica* have used the TSB+ media, including growth of bacteria for challenge studies of salmon and lumpfish (Ellul et al 2019; Sandlund et al., 2021).

A common and shared goal in science is to use as little animal products as possible. The usage of Fetal Calf serum (FCS) is not wanted, because of ethics and because it serves as a potential

risk of transmission of infection and it is not suited for vaccine development (Gudding and Reitan 2010), but some bacteria strictly demand it for growth, and unfortunately there is no good alternative. What components in the FCS the bacteria demand remains uncertain.

The protein profiles of proteins expressed by the isolates (PaL-1 – PaL-4) were examined over different concentrations of supplemented FCS by silver staining SDS-PAGE gels. For bacteria cultured without FCS there are barely visible bands, supporting that bacterial growth are dependent on FCS supplemented in the growth medium. For the silver stained SDS-page gels the bacterium growing on 10 % supplemented FCS provided the darkest and thickest protein bands and bacteria cultured with 5 % supplemented FCS gave similar results. There was no apparent difference in proteins present at different sized (kDa) when comparing bacteria cultured with 10 % and 5 % supplemented FCS. The bacterium growing on 0 % FCS had provided almost invisible protein bands. Exactly what component(s) in the FCS that the bacteria demands is currently not known.

In this experiment, it is found that 5 % or 10 % of supplemented FCS are needed for growing *P. atlantica* gv. *salmonicida*. There was little difference between 5 % and 10 % FCS supplemented in the TBS. For instance, PaL-2 had the highest growth counts when supplemented with 5 % FCS. PaL-3 had the highest growth with 10 % FCS supplemented in TBS. For all isolates growth medium without FCS shows low growth, with an increasing amount of aggregation and debris in the CASY cell count experiments. Also, the Scottish variant *P. skyensis* did not grow in the absence of blood, growth is reported to be faster on salmon-blood agar than in sheep blood agar (Birkbeck et al., 2002).

4.3 Biochemical characteristics

P. atlantica gv. *salmonicida* was in this experiment shown to be more biochemically reactive compared to *P. atlantica* gv. *cyclopteri* when tested using the API 20E kit, this is consistent with previous findings (Valheim *et al.,* 2000; Alarcón 2016). For example, in the fermentation of sugars without oxygen. *P. atlantica* gv. *salmonicida* was found to be mannose (a hexose sugar), sorbitol (sugar alcohol), melibiose (disaccharide “double sugar” of monosaccharide), rhamnose (methyl pentose sugar), and arabinose (pentose sugar) positive where *P. atlantica* gv. *cyclopteri* were negative on all of them. Fermentation is a catabolic process where sugars are partially degraded without the use of oxygen. The isolates, PaL-1, PaL-2, PaL-3, and PaL-4 showed no difference in biochemical properties, demonstrated by API 20E strips.

PaL-1, Pal-2, PaL-3, and PaL-4 were tested for oxidase properties. After multiple oxidase test the results from the experiment were unfortunately inconclusive. This could be because of interference where certain substances in the bacterial culture can interfere with the test and lead to incorrect results, and old or degraded reagents can lead to false-negative results. *P. atlantica* gv. *cyclopteri* have in previous studies been shown to be weakly oxidase positive (Alarcón *et al.*, 2016) and another Pasteurella isolate, the causative agent of "varracalbmi" in salmon, has also been shown to be oxidase-positive (Valheim *et al.*, 2000). For the *P. skyensis* the oxidase activity was very weakly positive (Birkbeck et al., 2002).

For the API 20E analysis *V. anguillarum* was included as control. Broth cultures of *V. anguillarum* grows a lot faster than *P. atlantica* gv. *salmonicida*, and therefore, when used as a control group, to be timed for harvest simultaneously as *P. atlantica* gv. *salmonicida* subcultivating a pre-bacteria culture before harvesting were conducted. Unfortunately, after multiple attempts, the *V. anguillarum* was not identified by the API 20E strips, possible errors could be wrong interpretations of results by comparing color changes in the wells, tipping point colors did not make this easy, or it could also be because of contaminated reagents.

4.6 In silico analysis of the PaL-1 genome

Extracellular or outer membrane proteins have good chances to have a role in bacterial virulence. In silico analysis of a *P. atlantica* gv. *cyclopteri* genome (Ellul et al., 2021) showed the presence of 11 genomic islands and 5 prophages, underlining the potential of mobile gene elements in the development of this species (Ellul et al., 2021). The present in silico analysis of the PaL-1 genome shows that there is one extracellular protein in particular that are a possible virulence factor, the extracellularly located YopT type cysteine protease domain-containing protein as listed in Table 4. YopT is one of the virulence factors produced by *Yersinia pseudotuberculosis* and *Y. enterocolitica*, and disrupt the actin cytoskeleton of the host cell, and in this way, it can inhibit phagocytosis (Schmidt, 2011; Iriarte et al., 1998).

Also, two secretory immunoglobulin EsiB are identified, and are reported to function to inhibit neutrophil activation. Another two identified extracellular located proteins, Ser-Asp rich fibrinogen-binding proteins are associated with cell adhesion. On the outer membrane there are identified four proteins that are involved with adhesion. Two toxins are identified as extracellular and in the outer membrane, the first one is a colA collagenase kappa-toxin exoenzyme like toxin located on the outer membrane. The colA collagenase kappa-toxin

exoenzyme is associated with *Clostridium spp.* and has function in breaking down host tissues during infection. The second is an extracellular CdiA toxin associated with contact dependent growth inhibition to suppress growth of neighboring target cells. A challenge in this experiment is the lack of data in sequenced aquatic *Pasteurellaceae*, making the results a little harder to rely on. Even so, with this analysis a rough list of possible virulence factors and immunogenic components were established.

Immunogenic components refer to the substances that can trigger an immune response in an organism. Outer membrane and extracellular proteins have a good chance of being immunogenic. Two of the outer membrane proteins identified and rated as possible immunogenic components are a LPTX anchor domain and a lipoprotein LptE like protein. Outer membrane LPXTG anchor domain surface protein is implicated in contributing to infections and has a role in directing interactions with the host during infection and is described in *Enteroccus faecalis* (Dramsi S et al., 2017). The outer membrane lipoprotein LptE, found in *Escherichia coli*, contributes to form a complex that assembles LPS into the outer membrane (Malojčić G et al., 2014).

Reverse vaccinology is a data driven approach used in vaccine development. Instead of the traditional method of identifying vaccine candidates by growing and studying pathogens in the laboratory, reverse vaccinology starts with the pathogen's genetic information. The pathogen's genome is analyzed to identify potential vaccine targets, such as surface proteins or antigens. This approach allows for a more targeted and efficient development of vaccines, especially for emerging or rapidly evolving pathogens, as it doesn't require the actual pathogen to be cultured in the lab. This method of vaccinology may reduce the vaccine production time from 5-10 to 1-2 years (Mondal et al., 2022) In the research findings presented by Ellul and colleagues in 2021, the report underscores the promising prospects of employing reverse vaccinology in the context of lumpfish, *Cyclopterus lumpus*, as a preventive strategy against *Pasteurella atlantica*-induced pasteurellosis in aquaculture. Their investigation included in silico analyses and functional assessments, leading to the identification of prime gene targets with a high priority for inclusion in vaccine development, aimed at preventing potential disease outbreaks in this aquatic environment (Mondal et al., 2022; Ellul et al. 2021).

4.4 Confocal microscopy

Confocal microscopy provides optical sectioning capabilities that enables to view thin slices of layers of the sample. To see if exposure to bacteria caused apoptosis of salmon leucocytes and if the bacteria was localized intracellularly confocal microscopy was conducted. Unfortunately, no clear picture was obtained during microscopy of the samples. A strong green background noise was observed, and it was not possible to identify leucocytes or bacteria with the microscopy. One reason could be because the vectashield were 6 months over expire date. Another reason could be a mistake during the staining procedure of the samples.

4.5 SEM

With SEM a high-resolution image of the surface topography of our samples were obtained. The pictures from SEM show no sign of intracellular behavior of the bacteria. Flow cytometry could be used in further studies to confirm the assessment of possible intracellular behavior or replication in this experiment. The result from SEM shows a higher grade of apoptosis in PaL-1 exposed leucocytes compared to non-bacteria exposed control samples. The control samples had also added TSB+ like the exposed ones, showing that it is not the growth medium that is causing apoptosis, but the bacteria. On the other hand, it could be pyroptosis that is observed since swelling and membrane blebbing of host cells are also seen here (Fink & Cookson, 2005). This method is not able to distinguish between the two of them. For the purpose of SEM, freshly sampled Atlantic salmon head kidney leucocytes were exposed to live PaL-1 bacteria and its potential toxins for 6 h and 24 h and observed under a Scanning Electron microscope. No difference was observed between the 6 h and 24 h groups.

In Figure 9, showing PaL-1 exposed leucocytes, no bacteria are seen, and the leucocytes are both swelled, have membrane blebbing and in Figure 9D dead leucocytes are observed, this can indicate that the virulence factors are extracellular secreted rather than outer membrane associated.

Extracellular metabolites are known to be potent virulent factors in multiple bacterial pathogens that cause disease in fish for example *V. anguillarum* and *Aeromonas salmonicida* (Frans et al., 2011, Magorinos et al.m 1992b, Jutfelt et al., 2008). Little is known about the virulence of *P. atlantica* gv. *salmonicida* and how it affects the fish cells and if toxins have an effect *in vitro*. Genes encoding RTX-toxins are found in the lumpfish isolate, but so far not in salmon isolate (unpublished work, fish immunology group).

In SEM pictures, not included in the results but presented in appendix, possible Horizontal Gene Transfer (HGT) by conjugation pilus and possible biofilm formation is shown. One protein identified to be located extracellular, listed in Table 4, known from *Acinetobacter baumannii*, are the trimeric autotransporter adhesin Ata that is reported to be important for biofilm formation (Bentancor LV et al., 2012). Possible biofilm and autoaggregation capabilities were observed by SEM and indicate that hygiene is important and thorough cleaning and changing treatment water in mechanical delousing.

4.7 Immunofluorescence

To study the effect of possible virulence factors identified by the in silico analysis, leucocytes were harvested from the head kidney of Atlantic salmon and exposed to PaL-1 before analysis by immunocytochemistry and SEM. By SEM microscopy apoptosis or pyroptosis were observed. Using immunofluorescence no apoptosis or pyroptosis was observed, but a hypothesis was that the bacteria attacked the actin of the leucocytes, possibly making the phalloidin (actin staining) less green compared to the non-exposed control leucocytes, this was suspected because of the extracellular located YopT- type cysteine protease domain-containing protein, found in the in silico analysis, that is reported to have effect by inhibiting actin assembly in infected cells (Shao et al., 2002). Also, the confocal microscopy showed a green blur on the cell preparations, and it was not possible to identify leucocytes, possible supporting the hypothesis of the bacteria attacking the actin in leucocytes. But after further investigating the control samples by immunofluorescence microscopy, this could not be concluded as the control samples also had weak green color of phalloidin. In figure 6C it could look like bacteria is both inside and outside the cells, unfortunately there were no successful confocal pictures to further investigate the localization of the bacteria.

Immunofluorescence microscopy revealed that there were many bacteria present on the cytospin slides of exposed leucocytes. In fact, there were more bacteria than expected in the samples. In that regard, the glucose cushion used to filter out some of the bacteria and the debris, did not function as expected. There were also some red background noises from the samples, but it was still easy to distinguish the background from the bacteria and leucocytes.

4.8 Directions for future research

The increase in *Pasteurella* infections in Norwegian aquaculture calls for a need of increased knowledge about these bacteria. In future research, it may be beneficial to investigate further the proteins identified by the in silico analysis. For example, one can produce the proteins recombinantly and use them to expose leukocytes, thereby verifying their role in virulence. Immunogenic proteins can be further identified; it is essential that isolates included in the vaccine express these proteins.

5. Conclusions

- *P. atlantica* gv. *salmonicida* strictly demands supplements of FCS for growth in TSB (Table 4).
- *P. atlantica* gv. *salmonicida* are more biochemically reactive than *P. atlantica* gv. *cyclopteri* when tested using the API kit (Table 5)
- Several possible virulence factors and immunogenic proteins are identified (Table 6, 7 and 8)
- Immunofluorescence microscopy showed no difference between control and exposed leucocytes regarding degraded actin (faded phalloidin staining) (Figure 8 and 9).
- SEM of leucocyte preparations shows apoptosis in leucocytes exposed to *P. atlantica* gv. *salmonicida* (Figure 11 and 12).

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7. Appendix

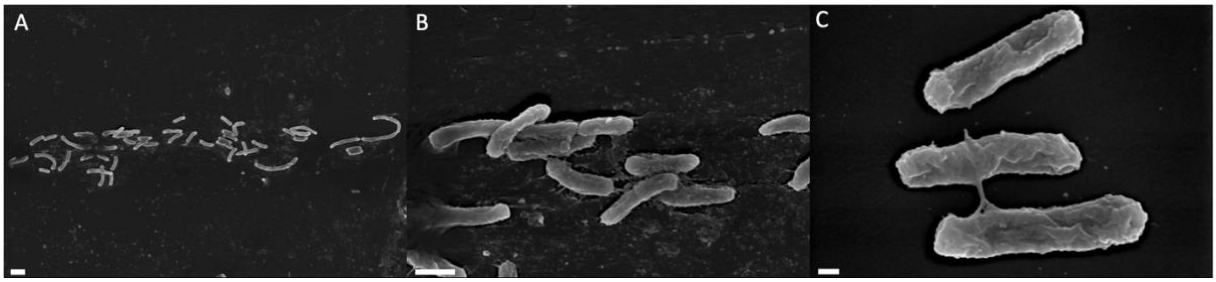

Figure 13: *Pictures of infected (B-D) and non-infected (A) leucocytes harvested after 6h and 24h.* ***A****. 6 h infected group showing bacterial microcolonies. Scalebar = 2µm.* ***B****. picture from 6h infected group displaying possible biofilm formation. Scalebar = 1µm.* ***C****. picture from 6 h infected group displaying possible horizontal gene transfer by conjugation pillus. Scalebar = 200 nm.*

Recipes for solutions used in the study:

7.1 – Liquid growth media (TSB+)

Liquid growth media (TSB+) 1000 ml:

Tropic soy broth (TSB) – BD	30 g
NaCl – Honeywell International	15 g
Fetal calf serum (FCS) - Gibco	105 g
MilliQ® - Merck Millipore, model MilliQ® Advantage A10® System	1000 ml

7.2 – NaOH 10M

NaOH 10M 100 ml:

NaOH – Sigma Aldrich	4 g
MilliQ® - Merck Millipore, model MilliQ® Advantage A10® System	100 ml total volume

7.3 10 % (w/v) SDS

10 % (w/v) SDS 90 ml:

SDS	10 g
MilliQ® - Merck Millipore, model MilliQ® Advantage A10® System	90 ml

7.4 Resolving gel 1.5 M Tris-HCl, pH 8.8

Resolving gel 1.5 M Tris-HCl, pH 8.8, 80 ml:

Tris base (18.5 g/100 ml)	27.23 g
MilliQ® - Merck Millipore, model MilliQ® Advantage A10® System	80 ml
Adjust pH with 6N HCl	pH 8.8

7.5 Stacking gel

Stacking gel 60 ml:

Tris base	6 g
MilliQ® - Merck Millipore, model MilliQ® Advantage A10® System	60 ml
Adjust pH with 6N HCl	

7.6 Electrode (Running) Buffer, pH 8.3

Electrode (Running) Buffer, pH 8.3, 1000 ml:

Tris base	30.3 g
Glycine	144.0 g
SDS	10.0 g
MilliQ® - Merck Millipore, model MilliQ® Advantage A10® System	1000 ml

7.7 Gel formulation 4 % gel

Gel formulation 4 % gel, 10 ml:

DDI H2O	6.4 ml
30 % degassed Acrylamide/Bis	1.0 ml
Gel Buffer	2.5 ml
10 % w/v SDS	0.1 ml

7.8 Gel formulation 12 % gel

Gel formulation 12 % gel, 10 ml:

DDI H2O	4.4 ml
30 % degassed Acrylamide/Bis	3.0 ml
Gel Buffer	2.5 ml
10 % w/v SDS	0.1 ml

7.9 Silver stain solution

Silver stain solution 65 ml:

Silver Complex Solution	5 ml
Reduction Moderator Solution	5 ml
Image Development Reagent	5 ml
Development Accelerator Solution	50 ml

7.10 5 % acetic acid solution

5 % acetic acid solution 1000 ml:

Acetic acid Honeywell International	5 ml
MilliQ® - Merck Millipore, model MilliQ® Advantage A10® System	995 ml

7.11 PBS pH 7,3 solution

PBS solution 1000 ml:

$Na2HPO4{\cdot}2H2O$	7.2 g
$KH2PO4$	0.27 g
NaOH 10 M	pH = 7.3
MilliQ® - Merck Millipore, model MilliQ® Advantage A10® System	1000 ml

7.12 PBS with 0,5 % BSA

PBS+ 0,5 % BSA 100 ml:

Bovine Serum Albumin (BSA) (SIGMA ALDRICH)	0.5 g
PBS	100 ml

7.13 PBS with 2 % BSA

PBS+ 0,5 % BSA 100 ml:

Bovine Serum Albumin (BSA) (SIGMA ALDRICH)	2 g
PBS	100 ml

7.14 PBS (380mOsm) 1000 ml

PBS (380mOsm) 1000 ml:

$Na2HPO4{\cdot}2H2O$	0.854 g
$KH2PO4$	0.254 g
NaCl	11.104 g
MilliQ® - Merck Millipore, model MilliQ® Advantage A10® System	1000 ml total volume

7.15 PBS+E, pH 7.3

PBS+E, pH 7.3, 500 ml:

BSA	5 g (Sigma-Aldrich)
EDTA	4.65 g (Triplex)
PBS	500 ml

7.16 Aceton:methanol (1:1) fixation solution

Aceton:methanol (1:1) fixation solution:

Aceton	1 part
Methanol	1 part

7.17 0,36 M NaCl

0,36 M NaCl 250 ml:

NaCl	5.26 g
dH2O	250 ml

7.18 0,46 M NaCl

0,46 M NaCl 250 ml:

NaCl	6.72 g
dH2O	250 ml

7.19 50x TAE buffer

50x TAE buffer 1000 ml:

Tris Base – Sigma-Aldrich	242 g
0.5M EDTA – Life Technologies Corporation	100 ml
Acetic acid – Honeywell International	57.1 ml
MilliQ® - Merck Millipore, model MilliQ® Advantage A10® System	1000 ml total volume

7.20 1 TAE buffer

1x TAE buffer 1000 ml:

1x TAE buffer	20 ml
MilliQ® - Merck Millipore, model MilliQ® Advantage A10® System	980 ml

7.21 Sample buffer

Sample buffer 9.5 ml:

0.5M Tris-HCl	1.25 ml
Glycerol – Sigma-Aldrich	2.5 ml
Sodium dodecyl sulfate – Sigma Aldrich	200 mg
0.5 % Bromophenol blue – Merck	200 µl
β-mercaptoethanol – Sigma Aldrich	500 µl
MilliQ® - Merck Millipore, model MilliQ® Advantage A10® System	3.55 ml

7.22 Fixative solution

Fixative solution 160 ml:

Methanol – Honeywell International	100 ml
Acetic acid – Honeywell International	20 ml
Fixative Enhancer Concentrate – Bio-Rad	20 ml
MilliQ® - Merck Millipore, model MilliQ® Advantage A10® System	20 ml

7.23 Percoll solutions

Percoll solutions 63.9 ml:

1.060 g/ml:

Percoll	30 ml
0.36 M NaCl	33.9 ml

1.075 g/ml:

Percoll	45 ml
0.46 M NaCl	31.9 ml

7.24 Glucose cushion

Glucose cushion 100 ml:

BSA	3 g (Sigma-Aldrich)
D-glukose	33.9 ml (Sigma-Aldrich)
PBS	100 ml

7.25 Triton 0,1 %

Triton 50 ml:

Triton® X100 (SIGMA ALDRICH)	0,05 g
PBS	50 ml