Pasteurella atlantica in Norwegian lumpsuckers

Characterisation, pathogenicity, vaccine development and immune responses

Rebecca Marie Ellul

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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Scientific environment

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Abstract

Lumpsuckers (*Cyclopterus lumpus* L.) have gained popularity as a cleaner fish to biologically control salmon lice infestations in farmed Atlantic salmon in Norway. Starting in 2012, most producers in Norway have been raising lumpsuckers in captivity from milt and eggs collected from wild-caught fish. Since then, this industry has grown exponentially to meet the demands of Atlantic salmon producers. However, this growth was not without its issues. A number of bacterial diseases have been recorded from farmed lumpsuckers, with *Pasteurella atlantica* being one of the most concerning to farmers, given it is an emerging pathogen first diagnosed in Norwegian lumpsuckers in 2012.

The main objectives of this project were to characterise *P. atlantica*, investigate the pathogenicity and the resulting immune responses in lumpsuckers, develop and test vaccines, and identify vaccine targets for future vaccine development.

The first milestone was the establishment of *P. atlantica* culture in liquid medium. Through this work, bacterial growth was achieved in tryptic soy broth supplemented with foetal calf serum. This meant that bacteria could be produced in large volumes as required for experiments and vaccine development.

Subsequently, a variety of challenge models were tested to study pathogenesis of *P. atlantica* in lumpsuckers. The bath challenge model was identified as the most reliable model, which could be further used during vaccine testing. Through these experiments, it was found that an asymptomatic carrier status occurs following exposure to the bacteria, highlighting the importance of fish health screening programs.

To that end, whole cell inactivated vaccines were developed against *P. atlantica* to investigate their protection potential. However, only limited protection was conferred following experimental challenge of vaccinated lumpsuckers despite serology following vaccination showing high specific antibody levels. Additionally, *in vitro* experiments to investigate interactions between *P. atlantica* and

lumpsucker head kidney leucocytes demonstrated potential intracellular behaviour, as well as auto-aggregation and adherence of the bacteria to host cells.

Whole genome sequencing was then used to investigate the genome of *P. atlantica* to shed light on virulence factors that may be involved in disease and may be suitable as vaccine targets, paving the way for further work in reverse vaccinology. Furthermore, as the pathogen was still unclassified taxonomically at this point, phylogenetic analysis was additionally carried out which suggested the taxonomic position of *P. atlantica* within the Family Pasteurellaceae.

In silico analysis subsequently identified a putative uncharacterised adhesin protein as being a major virulence factor and potential vaccine target and possessed similarities to the adhesins YadA and Hia found in *Yersinia* spp. and *Haemophilus influenzae*, respectively.

Gene expression analysis was then used to assess the regulation of this adhesin in the presence and absence of lumpsucker head kidney leucocytes. It was observed that the gene encoding the putative adhesin is upregulated significantly, both during bacterial culture as well as when bacterial cells are exposed to lumpsucker head kidney leucocytes *in vitro*, complementing the *in silico* analysis.

The knowledge gained from this work has significant implications for vaccine development. Through reverse vaccinology, a potential vaccine target has been identified that, through further research, could be used to develop subunit vaccines such as recombinant or DNA/mRNA vaccines. These promising results provide grounds for further research against a serious emerging disease in aquaculture with the aim of improving lumpsucker health and welfare.

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Abbreviations

Amoebic gill disease	(AGD)
Antigen presenting cell	(APC)
B-cell receptor	(BCR)
Cardiomyopathy syndrome	(CMS)
Cytotoxic T cell	(Tc)
Genomic island	(GI)
Haemophilus influenzae adhesin	(Hia)
Head kidney leucocyte	(HKL)
Heart and skeletal muscle inflammation	(HSMI)
Helper T cells	(Th)
Horizontal gene transfer	(HGT)
Infectious haemopoietic necrosis	(IHN)
Infectious pancreatic necrosis	(IPN)
Infectious salmon anaemia	(ISA)
International Union for the Conservation of Nature	(IUCN)
Intramuscular	(IM)
Intraperitoneal	(IP)
Lipopolysaccharide	(LPS)
Major histocompatibility complex	(MHC)
Melanomacrophage centre	(MMC)
Mucosa-associated lymphoid tissue	(MALT)
Multilocus sequence analysis	(MLSA)
Natural killer	(NK)
Neisseria adhesin A	(NadA)
Non-specific cytotoxic cell	(NCC)
Nucleotide binding oligomerisation domain-like receptor	(NLR)
Operational welfare indicator	(OWI)

Outer membrane protein	(OMP)
Pancreas disease	(PD)
Pasteurella multocida toxin	(PMT)
Pathogen- or danger-associated molecular pattern	(PAMP or DAMP)
Pattern recognition receptor	(PRR)
Photobacterium damselae subsp. *piscicida*	(*Phdp*)
Relative percent survival	(RPS)
Repeats-in-toxin	(RTX)
Retinoic acid inducible gene-like (RIG-I) receptor	(RLR)
Reverse vaccinology	(RV)
Salmonid alphavirus	(SAV)
Skin-associated lymphoid tissues	(MALT)
Skin associated lymphoid tissue	(SALT)
T cells receptor	(TCR)
Toll-like receptor	(TLR)
Type 1 secretion system	(T1SS)
Type 3 secretion system	(T3SS)
Type 5c secretion system	(T5cSS)
Viral haemorrhagic septicemia	(VHS)
Yersinia adhesin A	(YadA)

List of Publications

This thesis is based on the following papers, hereafter referred to in the text by their Arabic numerals:

Paper 1

Ellul, R., Walde, C., Haugland, G. T., Wergeland, H., & Rønneseth, A. (2019a). Pathogenicity of *Pasteurella* sp. in lumpsuckers (*Cyclopterus lumpus* L.). *J Fish Dis*, *42*, 35-46. doi.org/10.1111/jfd.12905

Paper 2

Ellul, R., Bulla, J., Brudal, E., Colquhoun, D., Wergeland, H., & Rønneseth, A. (2019b). Protection and antibody reactivity in lumpsucker (*Cyclopterus lumpus* L.) following vaccination against *Pasteurella* sp. *Fish Shellfish Immunol*, *95*, 650–658. doi.org/10.1016/j.fsi.2019.11.016

Paper 3

Ellul, R., Kalatzis, P. G., Frantzen, C., Haugland, G. T., Gulla, S., Colquhoun, D. J., Middelboe, M., Wergeland, H. I., & Rønneseth, A. (2021). Genomic analysis of *Pasteurella atlantica* provides insight on its virulence factors and phylogeny and highlights the potential of reverse vaccinology in aquaculture. *Microorganisms*, *9*, 1-22. doi.org/10.3390/microorganisms9061215

1. Introduction

Lumpsucker (*Cyclopterus lumpus* L.) is a new farmed species introduced to Norwegian aquaculture in 2012. Lumpsucker production was initially straightforward and successful due to their ability to eat food pellets and fast growth rates, adaptability to the cold Nordic climate, and efficiency as cleaner fish. However, as with most new farmed species, a number of difficulties were encountered related to welfare, nutrition, and diseases and vaccine development. These issues have garnered significant attention, with research efforts making progress to improve the sustainability of lumpsucker production.

1.1 Lumpsuckers in aquaculture

Background

The lumpsucker is a morphologically unique fish species belonging to the Order Scorpaeniformes, Family Cyclopteridae and is the only member of the genus *Cyclopterus*. The body is rounded and compressed anteriorly and posteriorly, with the front dorsal fin forming a crested hump, and the pectoral fins having evolved to form the characteristic muscular sucker used for attachment to substrates (Fig. 1). In the wild, they seem to prefer smooth substrates, such as floating kelp (fry and young juveniles), mussels and stones (older fish). As they grow older, they become more pelagic and tend to use the entire water column (Jonassen et al., 2018). The skin is scaleless, tough, and covered with pointed tubercules along defined ridges (Powell et al., 2018a). Coloration is an additional identifying feature, with immature fish ranging from blue to green to grey while sexually mature males can vary from pink to orange and red (Fig. 1). Females do not change colour upon sexual maturation.

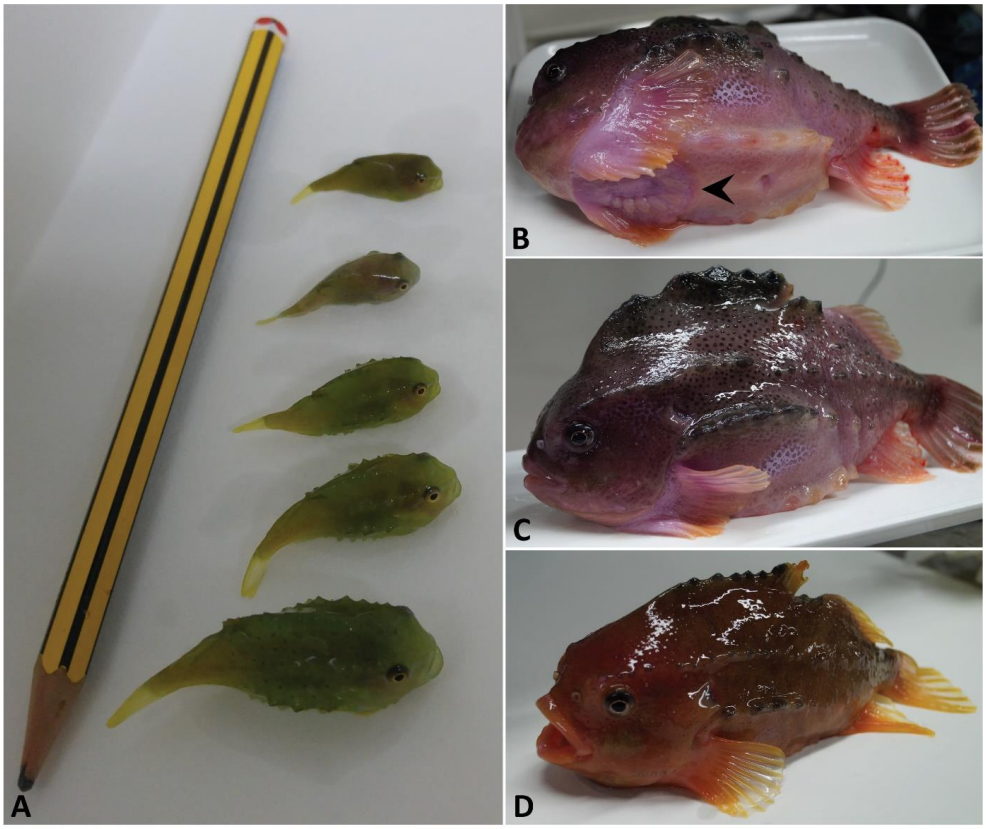

Figure 1. Morphology and coloration variability in lumpsuckers. A: juvenile lumpsuckers. B: ventral muscular sucker (arrow), C and D: sexually mature males.

Geographically, lumpsuckers are native to the northeast and northwest Atlantic Ocean, Greenland, Iceland, over most of the North Sea, the Norwegian Sea, and the western and southern parts of the Barents Sea and as far as 80°N off the north-west coast of Svalbard. Further south, they have been recorded around the British Isles, France, and northern Portugal. Additionally, a small population was also recorded in the Baltic Sea (Davenport, 1985). In Norway, the main populations are estimated to be located around Troms, Nordland and Finnmark (Durif, 2020).

Historically, male lumpsuckers were caught for their flesh, while female lumpsuckers were caught for their roe, which was a cheaper alternative to sturgeon caviar (Davenport, 1985). In recent years, the use of lumpsuckers has also

diversified through use of both male and female fish as so-called 'cleaner fish', i.e. in biological control of salmon lice in farmed Atlantic salmon, as a more environmentally friendly alternative to chemotherapeutic methods of lice control (Powell et al., 2018b). To improve sustainability of the cleaner fish industry, efforts have been made to close the lumpsucker life cycle in captivity. Currently most broodstock is still wild caught and culled to collect eggs and milt which is then used to raise offspring in captivity. Historical and current commercial fishing practices has resulted in a sharp decline in the abundance of wild lumpsuckers across known populations by more than 30% over the past 20 years (Powell et al., 2018b), with the species considered near threatened by the International Union for the Conservation of Nature (IUCN) (Lorance et al., 2015).

Lumpsucker and Norwegian Atlantic salmon production

Current large-scale production of farmed salmon is not without its fair share of challenges, both to the natural environment as well as to fish welfare. Infectious diseases from bacterial, viral, and parasitic agents pose one of the biggest threats to fish welfare, however the introduction of multivalent vaccines and vaccination regimes in the 80s and 90s have helped to eradicate a large proportion of bacterial diseases (Sommerset et al., 2005).

Despite advancements, bacterial diseases such as flavobacteriosis, furunculosis, bacterial kidney disease, winter ulcer disease, and pasteurellosis still pose problems to the industry (Sommerset, 2021a). In addition, viral diseases such as cardiomyopathy syndrome (CMS), heart and skeletal muscle inflammation (HSMI), pancreas disease (PD), infectious salmon anaemia (ISA), and infectious pancreatic necrosis (IPN) (Sommerset, 2021b), as well as ectoparasitic agents such as the amoeba *Paramoeba perurans* which causes amoebic gill disease (AGD), and the salmon louse (*Lepeophtheirus salmonis*) have become more serious (Bornø, 2021). Moreover, salmon lice infections are still both a persistent economic and welfare issue, with the estimated cost of sea lice management reaching USD 525 million in 2019 (Jensen et al., 2020).

The salmon louse is a marine copepod ectoparasite which feeds off skin mucus, blood, and tissue of Atlantic salmon. At low burdens, the parasite causes skin lesions and results in reduced feeding of fish, while at high burdens it leads to high mortalities due to osmoregulatory failure and secondary infections (Grimnes & Jakobsen, 1996; Nolan et al., 1999). Although the salmon louse is a natural parasite of Atlantic salmon, the stocking densities typical of fish farms enable the lice to flourish, thus being detrimental to fish welfare even at low burdens. In addition, due to the vicinity of farms to migratory routes of wild salmonids, the potential of sea lice spilling over from farmed to wild salmonids is high (Serra-Llinares et al., 2014). In Norway, this has led to weekly compulsory lice counts on farmed fish being introduced by regulatory authorities, with corrective measures being required by law if lice numbers exceed an average of 0.2 to 0.5 (season dependent) adult female lice per fish (Lovdata, 2017a).

L. salmonis treatments have traditionally been of a chemotherapeutant nature, with organophosphates, pyrethroids, hydrogen peroxide and avermectins being the most commonly used agents (Overton et al., 2019). However, overuse of these chemicals has led to increased resistance of sea lice, drastically reducing the efficacy of such treatments (Fjørtoft et al., 2020). This, in addition to the deleterious impact of such chemicals on the surrounding flora and fauna (Haya et al., 2005; Parsons et al., 2020), has led to the development and use of more environment-friendly measures such as biological control in the form of cleaner fish (Brooker et al., 2018; Haugland et al., 2020; Powell, et al., 2018b; Treasurer, 2002), and mechanical and thermal de-lousing methods (Overton et al., 2019). The latter have unfortunately been shown to result in high stress levels in salmon, with post-treatment mortalities also being recorded, due to handling and the temperatures at which the treatments occur (Overton et al., 2019). This highlights the need for alternative solutions which are less stressful to salmon, such as biological control through the use of cleaner fish.

Cleaner fish are regarded as the best option to help tackle salmon lice infections, from an environmental and salmon welfare point of view. The most used species

are ballan wrasse (*Labrus bergylta*) and lumpsuckers (*C. lumpus* L.), although other species of wrasse are also employed, such as goldsinny (*Ctenolabrus rupestris*), corkwing (*Symphodus melops*) and cuckoo wrasse (*Labrus mixtus*) (Imsland et al., 2018; Powell et al., 2018b; Treasurer, 2002). Wrasse species are commonly used in climates with sea temperatures of 6°C or higher, where they have a higher feeding capacity compared to lumpsuckers. Wrasses enter a state of hypo-metabolism at lower temperatures, and lumpsuckers are therefore favoured throughout Norway, especially due to the cold winter temperatures.

Lumpsuckers predominantly originate from cold water climates, and they have shown to efficiently eat sea lice at low water temperatures (Imsland et al., 2018). This means that there is no need for introduction of a new species like wrasse in an unfavourable climate, such as northern Norway. In addition, lumpsuckers are more robust, easier to farm, and have a high growth rate which can reach up to 3.65% daily (under optimal conditions) (Nytrø et al., 2014). This enabled more rapid improvements in farming and nutrition for lumpsuckers compared to wrasse, making lumpsucker farming more sustainable. In turn, biosecurity is improved, as introduction of pathogens from imported fish (as is a concern with wild-caught wrasse transported over large distances) is reduced. The high growth rate means that lumpsuckers reach a deployment size of 20g in a third of the time it takes farmed wrasse to reach a deployment size of 40-50g (Brooker et al., 2018; Erkinharju et al., 2021a; Powell et al., 2018a).

In Norway, increasing demand for lumpsuckers from an ever-growing salmon farming industry has led to staggering numbers of lumpsuckers being sold annually (Fig. 2), increasing from 431,000 in 2012 to 36.5 million produced in 2020 (Directorate of Fisheries, 2021).

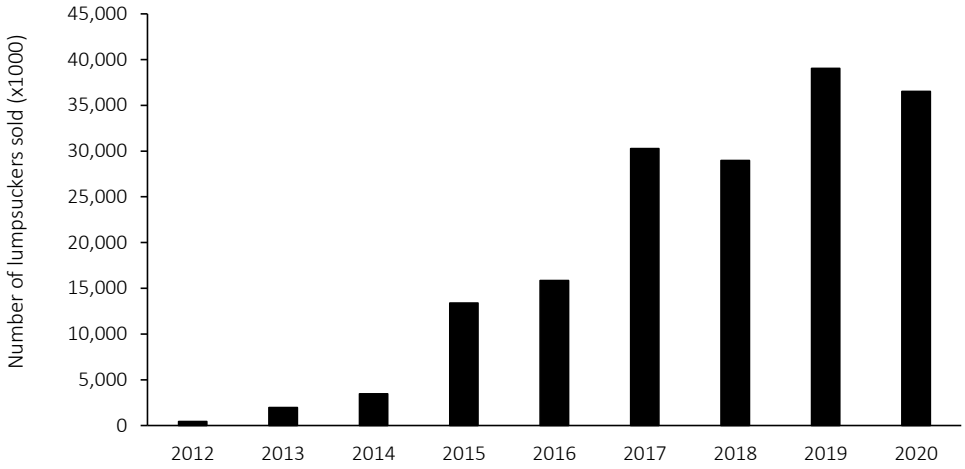

Figure 2. Sale of farmed lumpsuckers to Atlantic salmon producers in Norway. Data for 2020 is preliminary. (Source of data: Directorate of Fisheries, 2021).

Biology and rearing practices

Substrates: Lumpsuckers are diurnal foragers and attach to substrates at night by means of their ventral sucker, which likely represents predator avoidance behaviour. In net pens and hatcheries, farmed juveniles appear to prefer smooth plastic vertical surfaces, such as artificial kelp fronds (Imsland et al., 2015).

Behaviour: Lumpsuckers are typically more active when housed with salmon and wrasse, compared to monoculture. However, as lumpsuckers grow larger and become more competitive, they tend to show aggressive behaviour towards large wrasse and salmon, which negatively impacts salmon growth (Imsland et al., 2014).

Hatchery management: The growth rate of juvenile lumpsuckers is greatly affected by temperature (Haugland et al., 2020). In fact, the optimum temperature for lumpsuckers decreases with increasing weight, from 16°C for juveniles compared to 9°C for adult lumpsuckers. The temperature tolerance, however, increases with increasing body weight (Nytrø et al., 2014). For vaccinations, the minimum size is 7g and a period of 500°D is recommended until protective immunity is achieved (Haugland et al., 2018; Jonassen et al., 2018). At that timeline, fish are 40-60g at the time of deployment to salmon pens (Jonassen et al., 2018).

Feeding: In the wild, lumpsuckers feed mainly pelagically on plankton and seagrass and only visit shallow waters to spawn (Powell et al., 2018a). In hatcheries, lumpsucker larvae are initially fed *Artemia* nauplii then weaned onto appropriate formulated feed. This feeding regime has resulted in improved growth rates and reduced mortality in larval and juvenile stages when compared to lumpsuckers fed formulated feeds only (Jonassen et al., 2018). However, incidences of nutrition-related deformities, such as cataracts, have been recorded (Jonassen et al., 2017). Imsland et al. (2019) also found that in hatcheries, daily feeding of lumpsuckers resulted in too rapid growth rates and increased the risk of cataract development and gut inflammation.

In Norway, hatchery reared lumpsuckers are commonly fed formulated feed pellets as early as 2-5 days post hatch. However, a study by Dahle et al. (2017) found that since functional stomachs develop much later, at around 21-34 dph, such a feeding regime may impact the mucosal linings of the gut and energy storage, increasing the risks of nutritional-related issues and mortalities. An additional study investigating the impact of live first-feeds for lumpsucker larvae indicated that using copepods and enriched *Artemia* nauplii resulted in better growth rates compared to larvae fed only formulated feeds (Rian, 2019). Moreover, it has been suggested that delaying the weaning period from live to formulated feeds by a few days may be beneficial for increased larval survival (Dahle et al., 2017; Rian, 2019).

Once deployed in net pens, lumpsuckers continue to feed and grow at low temperatures, and can be over-wintered in the pens with salmon. Kousoulaki et al. (2018) reported that only around 30% of lumpsuckers actually feed on lice when deployed and suggested that the high incidence of cataracts observed in juveniles may be the cause. Lumpsuckers tend to ignore slow-moving or sessile organisms, which may be an alternative explanation if low delousing activity occurs. Adults also become sexually mature at approximately 500g, at which point their delousing efficacy greatly diminishes. Fish above this size are not recommended for use as cleaner fish (Imsland & Reynolds, 2018).

1.2 Lumpsucker health

Welfare status

Research on lumpsucker welfare indicators has gained momentum, and the Norwegian Food Safety Authority recently published of a report looking into the welfare situation of lumpsuckers in Norway (Mattilsynet, 2020). Treasurer et al. (2018) reviewed morphological, physical, and behavioural indicators that can be used to monitor lumpsucker welfare. Morphological indicators include fin erosion and damage to the suction disc. Behavioural indicators include swimming activity, aggression, and ventilation rate. Physical indicators are linked to stress and allostasis. Lumpsuckers have been documented to show lower cortisol levels immediately following stress episodes, however they are more prone to the chronic effects of stress (Jørgensen et al., 2017). Noble et al. (2019) also compiled a factsheet series on lumpsucker operational welfare indicators (OWIs) based on current scientific knowledge, and Imsland et al. (2020) and Eliasen et al. (2020) suggested a number of OWIs related to the physical condition of the fish. In the most recent study, Gutierrez Raban et al. (2021) developed a Lumpfish Operational Welfare Score Index based on visual assessment of several physical indicators, that can be used by farmers in hatcheries and for employees at fish farms.

Bacterial diseases

As a relatively new species to aquaculture, several bacterial diseases have been documented in farmed lumpsuckers (Fig. 3). Mortality spikes seem to occur soon after hatching, after vaccination, and following transfer to salmon cages (Johansen, 2013). The most commonly encountered bacterial agents are *Vibrio* species (including *V. anguillarum* and a *V. ordalii*-like bacterium), *Tenacibaculum* spp., *Moritella viscosa*, *Pseudomonas anguilliseptica*, atypical *Aeromonas salmonicida* and *Pasteurella atlantica* (Alarcón et al., 2016; Ellul et al., 2021, Paper 3; Erkinharju et al., 2020; Marcos-López et al., 2013; Rimstad et al., 2017; Rønneseth et al., 2017).

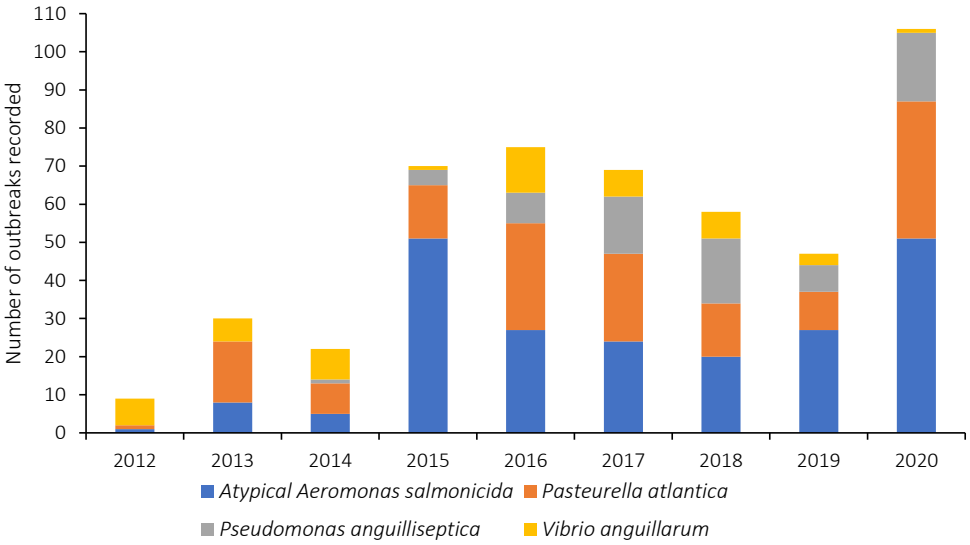

Figure 3. Commonly recorded bacterial infections in lumpsuckers in Norway between 2012 and 2020 (Source of data: Fish health report 2019 and 2020).

Atypical *A. salmonicida* subtype VI, based on A-layer typing (using the *vapA* gene), is the most problematic in lumpsuckers (Gulla et al., 2016a), and infections caused by isolates expressing the A-layer result in 100% mortality (Scholz et al., 2018). The disease can be acute or chronic, with symptoms including skin ulcers, haemorrhaging at the base of fins and internal organs, and darkening of the skin (Scholz et al., 2018). Histopathology typically shows bacterial colonies present throughout the visceral organs and in musculature. As the pathology is similar to that caused by *P. atlantica*, molecular analysis and bacteriology are required to confirm a diagnosis. Antibiotics can be used to treat the disease albeit with limited success, as re-emergence of infection is common (Kverme et al., 2021 [manuscript submitted]; Scholz et al., 2018). Vaccine development is difficult due to antigenic heterogeneity among strains, however preliminary studies have shown that vaccination can provide protective immunity (Rønneseth et al., 2017).

V. anguillarum serotypes O1 is typically isolated from lumpsuckers in Norway (Erkinharju et al., 2020). The disease is mostly acute, and symptoms include haemorrhaging at the mouth, operculum, fins, and vent, ophthalmitis, ascites,

darkening of the skin, and tail rot (Scholz et al., 2018). Histopathology shows bacterial cells present in tissues. Antibiotic treatment is usually effective if the disease is diagnosed at early stages (Scholz et al., 2018), and both florfenicol and oxolinic acid have been used in preliminary work to treat lumpsuckers experimentally challenged with *V. anguillarum* (Kverme et al., 2021[manuscript submitted]). Vaccination is also effective, with outbreaks in salmon pens being rare. A *Vibrio ordalii*-like bacterium (formerly *V. anguillarum* biotype 2) (Lillehaug & Colquhoun, 2020) has recently been recorded in lumpsuckers in Norway (Erkinharju et al., 2020, 2021b) and is associated with white lesions around the eyes (Scholz et al., 2018).

There are two 'types' of *M. viscosa* that affect farmed fish species: the type strain and the variant strain. The latter of the two is most problematic for lumpsuckers in Norway. The disease presents as a systemic infection with superficial lesions that can be secondarily infected by *Tenacibaculum* spp. The variant strain can cross-infect salmon, but this only occurs where the type strain is already an established problem (Scholz et al., 2018). The type strain does not appear to cross-infect lumpsuckers (Einarsdottir et al., 2018). Antibiotics are not effective against this disease, and no data exists on vaccine efficacy in lumpsuckers (Scholz et al., 2018).

Tenacibaculum maritimum infections are encountered at every life stage, including in lumpsuckers cohabitating with salmon in net pens. Symptoms include ulcers in the mouth and skins, fin rot, and may also become systemic. Diagnoses are best made using molecular analysis. Vaccine development is hindered due to antigenic variation between isolates (Scholz et al., 2018; Småge et al., 2016).

P. anguilliseptica was initially problematic for Japanese aquaculture, where it was responsible for red spot disease in Japanese eel. However, it has lately been also recorded in Europe from several species, including Atlantic salmon and lumpsuckers. In the latter case, the isolate responsible for disease is different from that recorded in Japanese eel although outbreaks are linked to high mortalities,

where temperature plays an important factor. In lumpsuckers, the symptoms include haemorrhaging at the vent, mouth and fin bases, skin irritation, and haemorrhagic septicaemia. While there are no commercial vaccines available against the disease, antibiotic treatments are usually successful (Scholz et al., 2018; Walde et al., 2019).

Outbreaks of pasteurellosis caused by *P. atlantica* in lumpsuckers in net pens mostly occur in late summer and autumn and are typically associated with prior stressful events such as handling and delousing treatments (Erkinharju et al., 2021b). In Norway, most outbreaks often result in mortalities of up to 100% (Erkinharju et al., 2021a). Symptoms include haemorrhaging at fin bases, tail rot, and skin lesions (Ellul et al., 2019a, Paper 1). Florfenicol treatment can be used to treat pasteurellosis in lumpsuckers in the field (Alarcón et al., 2016) and under experimental challenge (Larsen, 2019), but infections tend to re-emerge in the field (Alarcón et al., 2016). As histopathology is similar to atypical *A. salmonicida* infections, diagnosis is best confirmed by molecular analysis (Ellul et al., 2019a Paper 1; Scholz et al., 2018).

1.3 *Pasteurella atlantica*

Background and geographical distribution

The first confirmed outbreak of pasteurellosis caused by *P. atlantica* in Norwegian lumpsuckers was reported by the Norwegian Veterinary Institute from a farm in southern Norway in 2012 (Johansen, 2013). Additional cases have since been reported from sites along the western coast of Norway (Erkinharju et al., 2021b). Pasteurellosis has been recorded in fish ranging from juveniles to broodstock, in hatcheries, following deployment in salmon cages and in wild-caught broodstock (Alarcón et al., 2016). *P. atlantica* has also been detected in milt and eggs from lumpsuckers in higher levels than are usually detected during regular tissue screenings (Kui, 2017), suggesting that vertical transmission of disease is theoretically possible. In Norway, this has led to screening programs of lumpsucker broodstock, milt, and eggs prior to entry into facilities to avoid introduction of the

pathogen, especially as no vaccines are yet available. Since the first recorded outbreak, cases of pasteurellosis have been steadily increasing, with 36 sites reporting outbreaks in 2020 (Erkinharju et al., 2021b). *P. atlantica* therefore remains a welfare and economic issue, and as the infection is non-notifiable, under-reporting of outbreaks cannot be excluded.

Taxonomy

The Family Pasteurellaceae is composed of commensals, opportunistic and primary pathogens and includes the genera *Pasteurella*, *Actinobacillus* and *Haemophilus* amongst others (Christensen et al., 2014). The genus *Pasteurella* has a broad host range, but little is known about pathogenic *Pasteurella* spp. in the marine environment.

Based on 16S rRNA gene sequence phylogenetic analysis these three genera are polyphyletic and include five, ten and three species, respectively. However, phylogenies based on single genes such as 16S rRNA may result in incorrect classification due to, amongst others, horizontal gene transfer (HGT) and insufficient information within the gene to resolve relationships between closely related species. For this reason, phylogenies based on multiple genes such as multilocus sequence analysis (MLSA) or whole genome analyses would be more appropriate and may improve the separation of species-like taxa and confirm monophyletic taxa (Christensen et al., 2014).

Protein signatures identified from published genome sequences show that taxa within the Pasteurellaceae can be divided into two groups, with *Pasteurella* and *Haemophilus* in one group, and *Actinobacillus* in a second group (Christensen et al., 2014).

Alarcón et al. (2016) carried out initial phylogenetic analyses, using partial sequences of the 16S rRNA gene and the *rpoB* gene, from a number of isolates collected following outbreaks of pasteurellosis in lumpsuckers and these were found to represent a novel *Pasteurella* species. The authors also included strains of *Pasteurella* associated with 'varracalbmi' ('blood eye' in the Lapp language) in

Atlantic salmon in Norway, and *Pasteurella skyensis* isolates from Scotland causing pasteurellosis in Scottish Atlantic salmon. It was shown that all the Norwegian isolates formed a separate cluster from the Scottish *P. skyensis* isolates and pairwise sequence identity between the two clusters was 97.1–98.0% for the 16S rRNA gene, indicating that the lumpsucker and 'varracalbmi' isolates were a distinct species or sub-species from *P. skyensis*. Following current suggested nomenclature, the lumpsucker *Pasteurella* isolate is termed *P. atlantica* (Nilsen et al., 2021).

In 2018, an additional *Pasteurella* variant was detected following outbreaks of disease in farmed Atlantic salmon in Norway (Colquhoun, 2019). This has since continued to increase in importance and was identified in 57 salmon farming localities in 2020 (Nilsen et al., 2021). The strain has also been identified in a small number of cases in infected lumpsuckers cohabiting with infected salmon. While also belonging to the species *P. atlantica*, this new strain is genetically distinct from the *P. atlantica* strain primarily affecting lumpsuckers in the field. However, lumpsuckers have been found to be susceptible to both strains under laboratory conditions (Sandlund et al., 2021).

Pasteurellosis caused by *P. atlantica* should not be confused with pasteurellosis, commonly termed pseudotuberculosis or photobacteriosis (Romalde, 2002), a disease recorded in warm water climates, characterised by white granulomas on internal organs. This latter disease is caused by *Photobacterium damselae* subsp. *piscicida* (*Phdp*) (previously *Pasteurella piscicida*) now classified under the Family Vibrionaceae. *Phdp* has a wide host range including ayu (*Plecoglosus altivelis*), and yellowtail (*Seriola quinqueradiata*) in Japan, gilthead seabream (*Sparus aurata*), and sea bass (*Dicentrarchus labrax*) in the Mediterranean, and striped bass (*Morone saxatilis*), and white perch (*Morone americana*) in the USA (Romalde, 2002).

Isolation and phenotypical characteristics

P. atlantica is a relatively slow-growing bacterium when cultured on blood agar, needing a 4-day incubation at 15 °C for colonies to be identifiable. In liquid medium, *P. atlantica* flocculates in the early lag phase of growth. These clumps disappear as cell numbers increase (Fig. 4). Alarcón et al. (2016) carried out comprehensive biochemical characterisation of the bacterium. Briefly, the colonies were described as small, pinpoint, non-haemolytic and grey. Blood and NaCl were also documented as requirements for growth of *P. atlantica* on agar. *P. atlantica* cells are Gram negative, non-motile coccobacilli which produce acid from glucose both aerobically and anaerobically and are highly sensitive to the vibriostat 0/129. The authors noted that although *P. atlantica* was phenotypically similar to both 'varracalbmi' and *P. skyensis* isolates, it was biochemically less reactive.

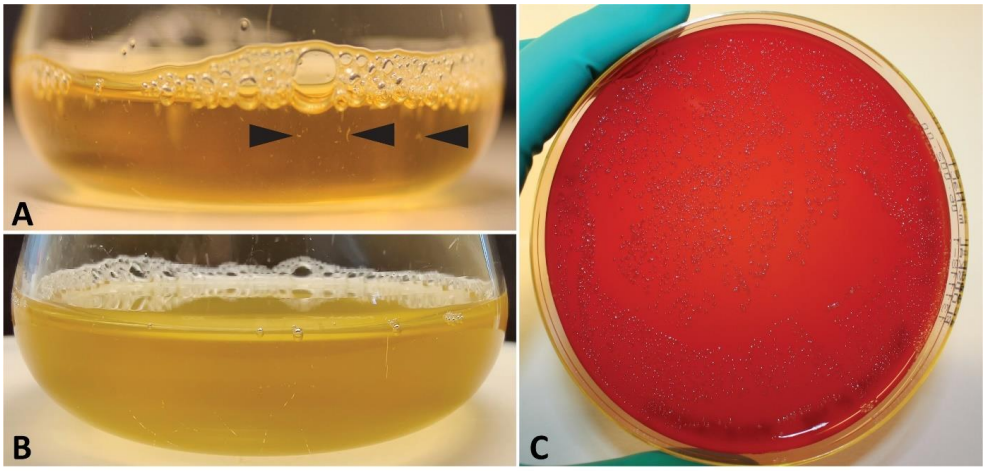

Figure 4. Culture of *P. atlantica*. A: liquid culture in early lag phase with clumps indicated by arrows. B: liquid culture at late exponential growth. C: Colonies on blood agar.

Susceptible species and clinical signs of disease

Symptoms in diseased lumpsuckers (Fig. 5) may vary according to the severity of infection and include characteristic white spots all over the skin and eyes, with frayed fins and haemorrhage at the base of the jaw and fins in more chronic cases (Ellul et al., 2019a Paper 1; Ellul et al., 2019b Paper 2). Alarcón et al. (2016)

additionally recorded tail rot, bleeding from gills, and granulomas on visceral organs.

Histopathology indicates that bacterial cells aggregate into microcolonies within organs, and are often associated with focal necrosis and an influx of inflammatory cells (Alarcón et al., 2016; Ellul et al., 2019a Paper 1). Some similarities were present in histopathological findings between the varracalbmi and *P. skyensis* isolates in Atlantic salmon and *P. atlantica* in lumpsuckers (Alarcón et al., 2016).

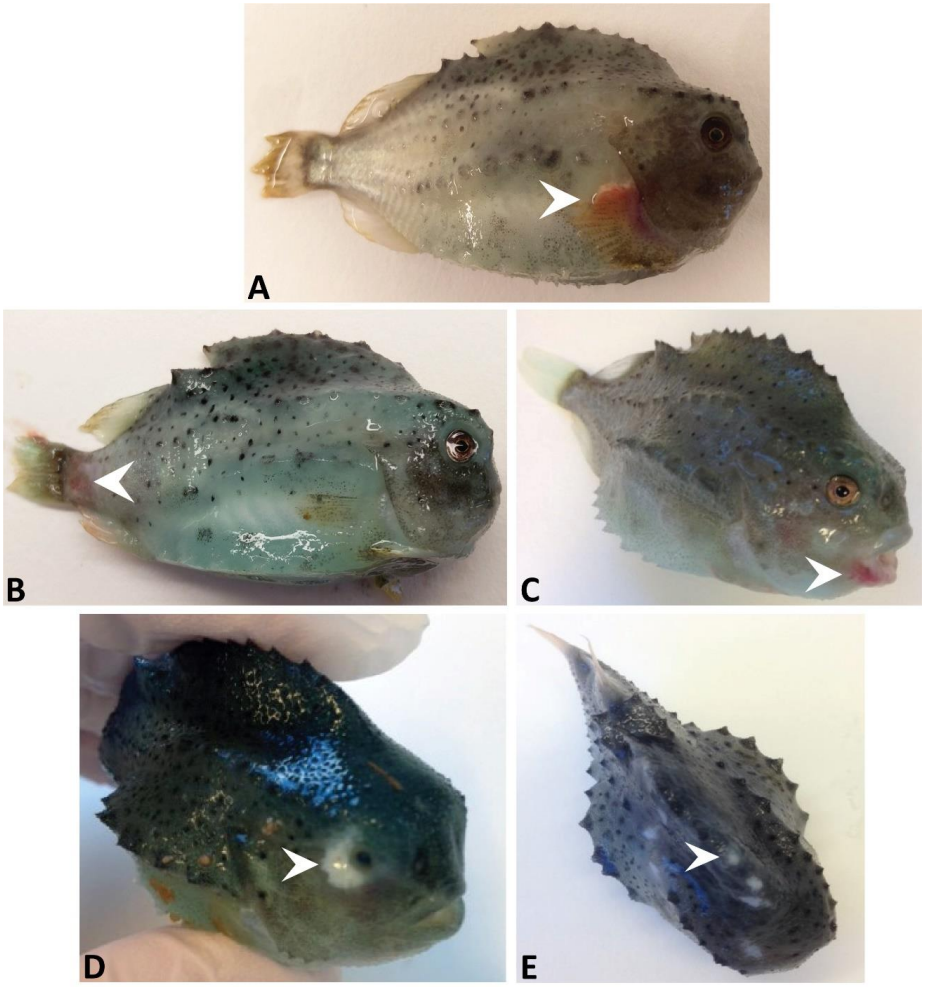

Figure 5. Gross pathology of *P. atlantica* infection in lumpsuckers, shown by arrows. A: haemorrhage at base of fins. B: haemorrhage and erosion of caudal fin. C: haemorrhage around the mouth. D: white spot surrounding the eyes. E: white spots over the skin.

1.4 Virulence factors

The first interaction between pathogenic bacteria and their host involves attachment to the cell surface of the latter, which may lead to colonization followed by infection and disease. The cellular components and structures that serve to evade host defences and mediate bacterial pathogenicity are known as virulence factors. They can be present within the bacteria (endotoxins), on the surface of the bacterial cell (e.g., bacterial capsule, flagella, fimbriae/pili), or even released extracellularly (exotoxins). While endotoxins can elicit antibody responses, these are rarely protective. An exception to this is lipopolysaccharide (LPS) which is a major protective endotoxin found in most Gram negative bacteria (Welch & LaPatra, 2016). Exotoxins, conversely, can be immunogenic, and are typically known to elicit protective antibody responses, although this depends on various factors including size of the toxin (Casadevall & Pirofski, 2001). This property makes exotoxins possible vaccine targets, as they can be used to generate a protective immune response in the host.

Colonisation and infection of a host by pathogenic bacteria is typically coordinated through a class of proteins known as adhesins, which are found on the outer membrane of the bacteria and are considered to be attributes of virulence. Aggressiveness of the pathogen can be conferred through a capsule surrounding the pathogen resulting in resistance to phagocytosis or survival within phagosomes, hence inhibiting or overcoming host defences. Bacterial components can alternatively result in disease indirectly, through damage caused by the host's own reaction to such components, such as an inflammatory response to polysaccharide components of bacterial capsules which results in disease (Casadevall & Pirofski, 2001).

Virulence factors are classified into two categories. They can be requisite, whereby they confer pathogenicity on their own (e.g., bacterial capsules, toxins) or contributory, where they modify the degree of pathogenicity and may not be sufficient alone to cause disease (e.g., some proteases). In the latter case, such

components would form part of a group of components acting synergistically. Perhaps most importantly, bacterial virulence factors rely on antigenic variation to constantly adapt in response to host selective pressures and thereby increase survivability of the pathogen (Casadevall & Pirofski, 2001; Linke et al., 2006). An example of this is resistance to serum components conferred by the YadA adhesin of *Yersinia* species (Linke et al., 2006).

There is little to no information available on virulence factors of *P. atlantica*, as genomic analyses that can provide answers are still sparse. Consequently, annotations of the genome are limited. At this stage, therefore, only extrapolation can be made on virulence factors from other members of the Pasteurellaceae.

Outer membrane proteins (OMPs): Members of the Pasteurellaceae family are known to cause septicaemia, respiratory disease, and mastitis amongst other conditions. OMPs present in *Pasteurella multocida* are immunogenic, and rabbits and turkeys inoculated with these antigens were protected against disease. *P. multocida* toxin (PMT) extracted by sonication and expressed in *Escherichia coli* has also been found to be a potent immunogen. When used as an inactivated toxin, good protection was achieved in rodents and pigs (Confer, 1993).

Lipopolysaccharide (LPS): The LPS O-antigen from *P. multocida* is toxic to mice leucocytes, and vaccination with this antigen resulted in strong opsonising antibody production, but no protection on challenge (Confer, 1993).

Haemagglutinins: Two loci were identified in the genome of *P. multocida* PM70, PfhB1 and PfhB2, which code for haemagglutinins and were found to have strong homology to a haemagglutinin of *Bordatella pertussis*. This protein is involved in adhesion to host cells and is used in the vaccine against whooping cough in humans, and conserved motifs in PfhB1 and PfhB2 indicate that the same properties may be present in *P. multocida*. Some regions of these haemagglutinins are also similar to the serum-resistance protein p76 of *Haemophilus somnus*, which confers resistance to opsonisation and enhances pathogen survival (May et al., 2001).

Repeats-in-toxin (RTX) proteins: RTX proteins are present in *Pasteurella*, *Mannheimia*, and *Aggregatibacter,* as well as *Vibrio* species and *E. coli*, amongst others. They are secreted by the Type 1 secretion system (T1SS) and an ABC transporter system, and typically exhibit cytotoxic pore-forming activity. They also cause tissue damage by eliciting anti-inflammatory responses (Linhartová et al., 2010).

They are produced by a wide range of Gram negative bacteria and are characterised by nonapeptide repeats of glycine and aspartate-rich sequences in the toxin peptide, which are sites of calcium ion binding. RTX proteins may have originated in Pasteurellaceae and spread to other species through HGT (Linhartová et al., 2010). This is supported by the G:C ratio which, at approximately 36-46% G:C, is similar to most Pasteurellaceae genomes, indicating the source of origin (Frey & Kuhnert, 2002).

Despite their antigenicity, they are not essential virulence factors, but rather work synergistically with other virulence factors to cause disease (Frey & Kuhnert, 2002). Another example is LktA, a moderately haemolytic and cytotoxic protein present in *Mannheimia haemolytica* (responsible for bovine and ovine pasteurellosis) which induces histamine release and stimulates neutrophil respiratory burst (Frey & Kuhnert, 2002).

Iron uptake mechanisms: Iron acquisition systems are important virulence mechanisms. Siderophore-mediated systems are used to sequester iron usually from host transferrin which is crucial for bacterial growth. The mechanism for uptake of iron is well conserved, with unique transporters for siderophores found in different species. Bacteria can have more than one different system besides their own. These would typically be acquired through HGT and indicate survival and bacterial protection mechanisms.

In the Pasteurellaceae, iron uptake systems are not particularly notable, and only a few proteins with occasional affinity to iron sequestration systems have been recorded from a limited number of species. This would explain the requirement for

iron enrichment when culturing the bacteria, especially the need for blood and foetal calf serum, which is recorded for a number of members of the family (Christensen et al., 2014), including *P. atlantica* (Ellul et al., 2019a Paper 1). However, despite the limited presence of specific uptake systems, homologs to proteins involved in iron uptake have been recorded in the genome of *P. multocida* PM70. Specifically, genes are present that are homologous to those in *Actinobacillus pleuropneumoniae* and *Yersinia pestis* (May et al., 2001).

Siderophore-independent mechanisms are also present, where haem is used as the source of free iron. This is sequestered via haemolysis and cytotoxins such as RTX proteins. In *P. multocida* strain PM25 a number of haemin- and haemoglobin-binding proteins have been identified. However, despite some of these proteins being immunogenic, these were not protective (Bosch et al., 2004). It is possible that the availability of a wide range of receptors can be manipulated by the bacteria to use different receptors in different hosts, at different stages during the infection process, or even to evade host responses, thus ensuring a supply of iron.

Pili: There are two types of pili recorded in the Pasteurellaceae, Flp and Type IV pili. Flp pili encoded by the *tad* locus are found in *P. multocida*, and *A. pleuropneumoniae*, but not in *Haemophilus influenzae*. They are important for adhesion, auto aggregation and biofilm formation, and are implicated in disease progression. The second type of pili, Type IV pili, have similar functions. They are associated with motility, biofilm formation and colonisation of host cells and are found in *H. influenzae*, *P. multocida*, and *A. pleuropneumoniae* (Christensen et al., 2014).

Adhesins: Adhesins are a class of surface-bound proteins involved in facilitating bacterial attachment to host tissues. They are classed as fimbrial or non-fimbrial based on the absence or presence, respectively, of an outer membrane anchor in the protein. Among the different types of adhesins, bacterial lectins are the most common (Wizemann et al., 1999). The mediation of attachment occurs through recognition of specific carbohydrates, proteins or lipids presented on the host cell

surface. Trimeric autotransporters are a family of non-fimbrial, bacterial adhesins which are secreted by the Type 5c secretion system (T5cSS), and whose main function is attachment of pathogenic Gram negative bacteria to hosts and other abiotic surfaces. *Yersinia* adhesin A (YadA) is the most extensively studied member of this family, and others include *H. influenzae* adhesin (Hia) and *Neisseria* adhesin A (NadA) (Comanducci et al., 2002; El Tahir & Skurnik, 2001; St. Geme & Cutter, 2000).

In the first functional genomic work done on lumpsucker isolates of *P. atlantica*, we have identified several proteins that are promising vaccine targets, which include an as yet uncharacterised adhesin similar to Hia and YadA from *H. influenzae* and *Yersinia* species, respectively (Ellul et al., 2021, Paper 3).

YadA is a potent virulence factor present in *Y. enterolitica* and *Y. tuberculosis* relying on attachment to collagen, fibronectin, and laminin, and colonises the intestine by adhering to the intestinal mucosa (El Tahir & Skurnik, 2001; Spahich & St Geme, 2011; Tsugo et al., 2017). Immunologically, YadA has been shown to be a highly immunogenic antigen, with vaccines using inactivated *Y. enterolitica* resulting in a more comprehensive protective immune response in mice than when using live bacteria (El Tahir & Skurnik, 2001).

Hia is an adhesin found in 25% of clinical non-typable strains of *H. influenzae* and causes respiratory tract infections and meningitis in humans (Winter & Barenkamp, 2009). Like YadA it is highly immunogenic, and opsonophagocytic antibodies were generated in serum in guinea pigs and mice following vaccination (Winter & Barenkamp, 2009).

Adhesins also promote the delivery of bacterial toxins through the upregulation of additional virulence genes that facilitate invasion of the host (Wizemann et al., 1999). An example of this is the triggering of the Type 3 secretion system (T3SS) by bacterial attachment to host cells to secrete effector molecules from the bacteria into the host cell. These effectors modify host cell proteins, typically immune cells, to inactivate them. This can also be used as a strategy by intracellular bacteria to

reside within the inactivate immune cells to evade host immune responses (Hoepelman & Tuomanen, 1992). These interactions can compound the severity of disease by inducing reactions from the host such as cytokine production or lectinophagocytosis (Abraham et al., 2014).

Given this high potential of virulence, adhesins can be utilised as targets for vaccine development through reverse vaccinology (RV) (He et al., 2010). Protective immunity can be achieved in theory by inducing adhesin-specific antibodies targeting and blocking epitopes required for microbial attachment. The development of such vaccine will be of great advantage to combat pasteurellosis in lumpsuckers. Although few in number, successful adhesin vaccines developed through RV have been tested for farmed fish including those against diseases caused by *Vibrio harveyi*, *Edwardsiella tarda*, and *Aeromonas hydrophila* (Fang et al., 2004; Jin & Li, 2021; Maiti et al., 2012; Zhu et al., 2019).

1.5 Teleost immune system

The immune system of teleosts is divided into the innate and adaptive systems, and the major lymphoid organs are the thymus, kidney, spleen, and mucosa associated lymphoid tissues. Recently, Løken et al. (2020) identified a teleost analogue to the bursa of Fabricius in Atlantic salmon with secondary lymphoid functions and age-dependent characteristics, which suggest the functions involved are related to lymphocyte maturation.

Immune organs and cells

In marine teleosts, the ontogeny of lymphoid organs is different from that of freshwater species. In the former, it is the kidney that develops first followed by the spleen, and lastly the thymus. In freshwater teleosts, the thymus becomes lymphoid first, followed by the kidney and spleen (Castro & Tafalla, 2015; Uribe et al., 2011; Zapata et al., 2006).

The kidney is the largest site of haematopoiesis with erythropoiesis, granulopoiesis, thrombopoiesis, monopoiesis and lymphopoiesis taking place in

the anterior kidney. It is the primary site for the production of B cells and contains a large presence of melanomacrophage centres (MMC) which are encapsulated accumulations of pigment-containing macrophages. For this reason, the anterior kidney is a primary lymphoid organ, while the posterior kidney is a secondary lymphoid organ (Uribe et al., 2011).

The structure of the thymus varies among teleosts in terms of differentiation, as it can be one or several pairs of organs, and it is usually difficult to observe differences between the cortex and medulla. As a primary lymphoid organ, it contains an aggregation of macrophages promoting the encapsulated proliferation of T cells (Castro & Tafalla, 2015; Uribe et al., 2011).

The spleen is a secondary lymphoid organ with specific T and B cells and contains lymphoid cells in the red pulp, and MMCs, and splenic ellipsoids in the white pulp. The latter are thick-walled capillaries whose cells are involved in macrophage phagocytosis of antigens. MMCs retain antigens for long periods and increase in size and frequency depending on environmental stress and infections. Furthermore, the spleen is important in adaptive immunity due to the presence of memory cells (Castro & Tafalla, 2015; Uribe et al., 2011).

Other organs (tissues) are also important for immune responses, despite not being primarily lymphoid in function. These include the mucosa-associated lymphoid tissues (MALTs), which are present in the nasopharyngeal tract, gills, intestine, and skin, which are major tissues for antibody secreting cell production through the mucosa. Lymphocytes accumulate at the base of gill filaments (Koppang et al., 2010), and local Ig responses (IgT) can be produced through the mucosa of the intestine. IgT is specialised for mucosal immunity and is almost exclusively produced only in the intestine (Hansen et al., 2005).

MALT, especially skin associated lymphoid tissue (SALT), is a crucial physical barrier for fish. Mucus contains innate humoral factors such as complement proteins, natural antibodies, antimicrobial peptides, lysozymes, and agglutinins that form the humoral components of the innate immune system and are able to

eliminate a range of pathogens and foreign material. For cohabiting lumpsuckers and Atlantic salmon, frequent delousing treatments risk stripping away the SALT leaving the already stressed fish vulnerable to pathogenic attack.

Innate immune system

The innate immune system is also known as the non-specific system and is therefore first involved in attacking invading foreign materials. Due to their non-specific nature, these responses are consistent and are not known to possess immune memory. As it is the first defence system to develop during fish embryogenesis, it provides a broad range of protective functions based on physical barriers such as skin, scales, and mucus, followed by humoral and cellular components. Studies have shown that the innate immune system does not act in isolation; several innate responses result support adaptive immune responses. Furthermore, studies have shown that the innate immune system can be "trained" using immune-modulating compounds such as β-glucans, such that protection can be conferred against a secondary infection independently of T and B cells by relying on non-specific cytotoxic cells (NCC), which are teleost equivalents of natural killer (NK) cells, and macrophages (Petit et al., 2019; Petit & Wiegertjes, 2016).

The major teleost cell types involved in non-specific defence against pathogens are phagocytes and NCC cells. Auxiliary cells such as basophils, eosinophils, mast cells and platelets are also present and are involved in inflammatory responses. The teleost equivalents of NK cells have been identified in channel catfish and are of two classes- NK-like cells which are large, granular, and isolated from blood, and NCC, which are small, agranular and located in lymphoid tissues. These cells can kill stressed or infected cells either directly or by secreting inflammatory cytokines (Jørgensen, 2014).

Phagocytes can be antigen presenting cells (APC) which express MHC class II and include B cells, monocytes, macrophages, and dendritic cells (mononuclear). The latter are phagocytic in the immature form and present antigen-derived peptides

to T-cells in the mature form. Neutrophils (polynuclear granulocytes) are also phagocytic cells and have been shown to express major histocompatibility complex (MHC) class II in some species (Cuesta et al., 2006;) but their role in antigen presentation is still unclear.

While macrophages are always present in the peritoneal cavity, neutrophils are only recruited during inflammatory responses. A subset of macrophages, melanomacrophages, are present in the head kidney and spleen. They are also antigen presenting cells and are recruited during inflammatory responses. During phagocytosis, the pathogen is internalised into a phagosome, which fuse with a lysosome and develops into a phagolysosome where the pathogen may be neutralised by exposure to enzymes and reactive oxygen and nitrogen species such as superoxide anion and nitric oxide (Jørgensen, 2014).

Despite their widespread use, knowledge of the lumpsucker immune system remains limited. Haugland et al. (2012) first characterised the components and functionality of the lumpsucker innate immune system by isolating leucocytes from peripheral blood, spleen, and head kidney, and determined the non-specific phagocytic capacity of lumpsucker leucocytes to be very high. Rønneseth et al. (2015) then characterised B cells from lumpsuckers and found high phagocytic ability among IgM^+ B cells isolated from serum.

Recognition of pathogens occurs through pathogen- or danger-associated molecular patterns (PAMPs or DAMPs) and is mediated by pattern recognition receptors (PRRs) which can either be cell membrane-associated or soluble. PAMPs include bacterial membrane lipids, peptidoglycan, flagellin, as well as bacterial and viral nuclei acids. The most important classes of PRRs include Toll-like receptors (TLRs), of which 12 have been described in lumpsuckers (Eggestøl et al., 2018), retinoic acid inducible gene-like (RIG-I) receptors (RLRs); MDA-5 and LGP2 are two examples described in lumpsuckers, and nucleotide binding oligomerisation domain-like receptors (NLRs), five of which are found in lumpsuckers (Larsen, 2019). C type lectins and complement components are additional classes of PRRs,

although their primary roles are related to phagocytosis. However, they additionally regulate adaptive immunity by mediating antigen recognition by dendritic cells. Most components of the complement pathways are identified in lumpsuckers (Haugland et al. 2018; Eggestøl et al., 2018). PAMP-PRR interactions activate intracellular signalling pathways which result in cytokine expression tailored to neutralise different pathogens (Jørgensen, 2014).

Intracellular bacteria have developed mechanisms to avoid phagocytosis. The most common methods involve modulation of cytokine pro-inflammatory and anti-inflammatory responses to inhibit the elimination of infected cells and promote bacterial survival within the cell, respectively, as well as producing own cytokines that help resist toxic reactive oxygen and nitrogen species. They can also modulate development of the phagosome in order to resist the acidic environment within phagolysosomes (Munang'andu, 2018).

Adaptive immune system

The adaptive immune response is initiated by the innate response, and although it is usually delayed, it is essential for long lasting protection against pathogens, and its activation is the key goal for vaccines. Adaptive immune responses are mediated by B cells (memory and antibody responses) and T cells (helper T (Th) cells that are needed for activation of other immune cells and cell-mediated responses by cytotoxic T (Tc) cells). When naïve B cells are activated by antigens (both T-dependent and T-independent) and stimulated by Th2 cells they proliferate into plasma cells which secrete antibodies, and into memory cells which will ensure a more rapid response upon second encounter with the same antigen (Fischer et al., 2013). Tc cells, activated by Th1 cells, recognise the antigen by the T-cell receptor (TCR) when presented by MHC class I and eliminate infected cells by secretion of cytotoxic compounds (Secombes & Wang, 2012).

MHC proteins are the tools directly involved in activation of T cells. MHC class I is present on the majority of cells and presents viral or intracellular bacterial peptides to CD8+ Tc cells. MHC class II is expressed by APCs and present degraded

pathogen components to CD4+ Th cells. This then results in a complex signalling cascade (Magnadottir, 2010).

Cellular responses

T cells are produced in the thymus, then migrate to tissues to induce responses directly. They can be differentiated into Tc cells that eliminate infected host cells or Th cells that produce cytokines to stimulate cells of the innate system to eliminate the pathogen (Secombes & Wang, 2012). In addition to CD4+ and CD8+, T cells also possess CD3 signalling molecules that facilitate cellular immune activation upon receptor-antigen interaction (Secombes & Wang, 2012). Both CD4 and CD8 have been identified in lumpsucker (Straumsnes, 2018).

Pathogenic peptides presented on the cell surface by MHC class I are usually derived from viruses, intracellular bacteria, or tumours (Secombes & Belmonte, 2016). IL-2 promotes the differentiation of naïve Tc cells into effector Tc cells and, further on during the immune response, into memory Tc cells (Yamaguchi et al., 2019). The differentiated forms can be distinguished from naïve Tc cells by the expression of their surface markers (Laing & Hansen, 2011).

$CD4^+$ T cells (Th) are stimulated by peptides derived from extracellular pathogenic peptides presented on the cell surface by MHC class II on APCs and regulate immune responses via cytokine release. Only TCRs that bind with antigens of sufficient affinity will trigger activation. Th cells may then differentiate into four cell groups which can be distinguished by their unique cytokine profile, transcription factors, and functions. Th1 (express INF-γ and IL-2) mount a cell mediated response against the target cell and activate macrophages and Tc cells. Th2 (express IL-4, IL-13, and IL-20) drive the humoral response, upregulate antibody production through B cell activation, and decrease inflammation. Th17 (express IL-17A, IL-17F, and IL-22) upregulate tissue inflammation to defend against extracellular bacteria that are not cleared by Th1 and Th2. Treg (express TGF-β1, IL-10, and IL-35) control the inflammatory response mounted by Th17 (Secombes & Wang, 2012; Secombes & Belmonte, 2016).

Humoral responses

B cells are produced in the anterior kidney but can be found in a number of tissues even in the absence of infection. They recognise soluble antigens directly via the B-cell receptor (BCR) present on the cell surface and can also form soluble version of the BCR (Ig) which are secreted as antibodies that bind to antigens that triggered their production. Binding of antibodies to targets may have several functions such as inactivation of toxins and prevention of attachment to the host and marking of antigens for destruction by phagocytic cells. These antibodies can be classified as neutralising (act against the pathogen directly) or opsonising (assist phagocytes with internalising the pathogen) (Secombes & Wang, 2012; Secombes & Belmonte, 2016). Furthermore, binding of antibodies to microbes and antigens can lead to agglutination, activation of the complement system and antibody-dependent cell mediated cytotoxicity (ADCC).

Tetrameric IgM is the most predominant antibody in teleosts (Sunyer, 2013) including lumpsuckers (Rønneseth et al. 2015), followed by IgD and IgT/Z (Danilova et al., 2005; Hansen et al., 2005). IgM levels are affected by environmental factors such as temperature, and is found in various locations in the fish, predominantly in plasma, but also in skin, intestine, gills, and bile. While IgT has mainly been recorded from intestinal tissues of rainbow trout (Zhang et al., 2010), IgT (also identified as IgZ in zebra fish) has been recorded in the thymus and head kidney of zebrafish (Danilova et al., 2005).

Teleosts have two major subsets of B cells: ones that express IgM and IgD both in the same cell (IgM^+IgD^+) and those that express IgT only (IgT^+). IgD^+ B cells have also been identified in catfish and rainbow trout (Edholm et al., 2010; Ramirez-Gomez et al., 2012). Exact proportions, however, depend on individual species, highlighting the complexity of B cell composition. The immune response from B cells is dependent on the location of the infection: if this occurs in the intestine, a local IgT response is mounted. Interestingly, IgT levels following an intestinal infection can be high in intestinal mucus but low in serum, while the opposite can

be true for IgM levels (Secombes & Wang, 2012). Rønneseth et al. (2015) found that immunisation of lumpsuckers using a range of inactivated bacterins resulted in the production of specific antibodies in each case, and further demonstrated that such immunisation resulted in vaccine-induced protective immunity following experimental challenge with atypical *A*. *salmonicida* (Rønneseth et al., 2017).

B cell activation occurs following antigen binding to BCR, internalisation, processing, and presentation of antigens by MHC class II to Th cells. Once activated, B cells mature leading to the differentiation to plasma cells (produce and secrete antibodies) and memory cells (remember the pathogen upon a second encounter). Activation depends on complex signalling pathways and communication with Th2 cells.

Once matured, plasma cells differentiate into short-lived plasma cells and long-lived plasma cells. These cells do not proliferate further and can only secrete antibodies for the duration of their lifecycle. Short-lived plasma cells are generated upon a high affinity interaction at the tissue site of infection and typically die off once the pathogen is destroyed. Low affinity B cells migrate to MMCs in the anterior kidney where they slowly mature to long-lived plasma cells and persist for long periods, where they will continue secreting antibodies without needing to be restimulated. These low affinity cells will over time be replaced by higher affinity ones, resulting in a memory immune response (Secombes & Belmonte, 2016).

In the case of immune memory, T-independent antigens are those that activate B cells in the absence of Th cells either through binding of innate receptors or through simultaneous binding of several B cell receptors, and do not result in an immunological memory response, whereas T-dependent antigens are those that can only stimulate B cells in the presence of Th cells and leads to the formation of an immunological memory (Cruse et al., 2004).

The presence of antigen specific antibodies confirms that the entire process from antigen presentation by Th cells and recognition by BCR to B cell expansion has

occurred. This is the key for humoral adaptive responses and the goal for protective vaccines.

1.6 Vaccine status of lumpsuckers

The rapid development of global aquaculture is not without its fair share of challenges. Management and control of diseases poses one of the biggest impacts to welfare and economic viability, and hence sustainability, of the industry. Improvements therefore need to be made to limit and prevent disease outbreaks that lead to significant losses of fish during each production cycle. In the past, antibiotics were the standard method of treating outbreaks of bacterial diseases. Unfortunately, this soon led to antibiotic resistance. Better prophylactic, rather than remedial, measures were therefore required to protect fish against pathogens, and the first vaccines were produced and utilised successfully. Nowadays, vaccination has become the new standard for the industry and for some high value aquaculture species such as Atlantic salmon, tilapia, sea bass, and sea bream, commercial vaccines are available against major diseases. Commercial vaccines to protect against novel emergent pathogens are very few, as are vaccines to protect lower value farmed fish species such as cleaner fish. Advances in vaccine development technologies are therefore vital to ensure improved welfare of aquaculture species.

Vaccines for aquaculture have been developed since the 1940s, to protect fish species all over the world against diseases caused by numerous pathogens, including *A. salmonicida*, *V. anguillarum*, *Yersinia ruckeri*, and *Phdp,* amongst others. A variety of vaccine types are available for fish ranging from whole bacterin inactivated vaccines, subunit vaccines available against ISA in Chile, live attenuated vaccines against disease caused by *Edwardsiella ictaluri* and *Flavobacterium columnare* infections are licensed in the USA (Evensen, 2016), and DNA vaccines against infectious haemopoietic necrosis (IHN) and pancreas disease (PD) in salmonids (Dalmo, 2018).

Bacterial diseases in lumpsucker hatcheries are often treated using antibiotics; however, there are no approved protocols available to provide adequate treatment. Furthermore, in cases of outbreaks in sea cages, treatment is not possible due to their cohabitation with Atlantic salmon. Therefore, prophylactic measures in the form of autogenous or commercial vaccines are of increasing importance to combat diseases and improve the welfare status of farmed lumpsuckers. Currently, the majority of farmed lumpsuckers in Norway are vaccinated against disease caused by *V. anguillarum* and atypical *A. salmonicida*. As *P. atlantica* is a relatively recently discovered pathogen, vaccine development against pasteurellosis is still in its infancy, and *P. atlantica* antigens are currently not included in commercially available lumpsucker vaccines.

Vaccination is the most important prophylactic measure that aims to stimulate the adaptive immune system to respond protectively against particular immunogenic antigens of a pathogen. Immunogenicity occurs when host antibodies bind a pathogenic component (antigen) and raise a humoral or cellular immune response specific to that antigen to neutralise it, thus protecting the host. Conversely, a number of antigens may be bound by host antibodies, but which do not result in a protective immune response generated in the host. Such antigens are not immunogenic.

Through evolutionary forces, host immune responses influence virulence factor expression, as pathogens constantly adapt to try to evade these responses. Therefore, by knowing the antigenic targets to which host protective responses are directed, the bacterial components responsible for disease can be identified. These can then be used as vaccine targets to develop refined vaccines that confer better protection, thus reducing the virulence of the pathogen.

Additionally, the stronger the immune response generated, the better the suitability of the target for vaccine development. In turn, individual targets on their own may not be sufficient for a protective immune response to be generated and may require other immunogenic components to act synergistically to induce a

satisfactorily protective host immune response. This would need to be given due consideration in the process of vaccine development.

Through the principles of RV and using bioinformatic analyses of pathogen genomes, immunogenic antigens can be predicted *in silico* to be utilised as targets for vaccine development. RV is a more rapid process than the conventional approach of vaccine development, and is considered to reduce development time by about 2 years (He et al., 2010). It also ties into the 3Rs with a reduction in the number of fish required for vaccine testing, as well as resulting in a more accurate and effective vaccine being developed. RV has been successful in the identification of vaccine targets and the development of vaccines against disease caused by a number of farmed fish pathogens, including those caused by *A. salmonicida* (Marana et al., 2017), *Phdp* (Andreoni et al., 2013, 2016), *A. hydrophila* (Kaur et al., 2021), and *V. anguillarum* (Baliga et al., 2018).

Technology to aid RV has come a long way since the principles were established and applied. A number of programs and databases are now available that facilitate the prediction of suitable targets. These programs rely on criteria based on physical characteristics from the bacteria, such as sub-cellular localization, adhesin probability, and the number of transmembrane domains present in outer membrane proteins (Ong et al., 2020). In order to ensure reliable targets are predicted, they should be only present in virulent strains and have protein sequences that are dissimilar to host sequences (He et al., 2010).

Vaccine components and categories

Vaccines typically contain an antigen dissolved in water (water-based) or in a stabilising emulsion of water and oil (oil-based). In the latter, the antigen can be present in either phase. Adjuvants are also often present in vaccines to enhance the immune response, with the most common being mineral oil. Water-based vaccines can be administered orally, by immersion or by injection (intraperitoneal (IP) or intramuscular (IM)) whereas oil-based vaccines can only be IP injected. The main categories of vaccines are replicating, inactivated (whole cell or subunit), or nucleic

acid based. Inactivated vaccines are the most commonly encountered vaccines and are generally effective against extracellular bacteria. However, their efficacy against intracellular bacteria may be limited. In subunit vaccines, the antigen is produced recombinantly in an expression system and purified at the end of the production cycle, then used for formulating the vaccine. All these categories of vaccines usually require the addition of adjuvants to prime the host immune system. Alternatively nucleic acid vaccines (mRNA or DNA) can be produced, using only the sequence encoding for the antigen (Evensen, 2016; Knappskog et al., 2014).

Adjuvants

Adjuvants are necessary to improve the efficacy of non-replicating vaccines. They are compounds that modulate the immunogenicity of an antigen and are categorised as Signal 1 or Signal 2 based on the immunological events they induce. Additional roles include promoting the induction of mucosal immunity and through their use, lead to a reduction in antigen doses and antigen competition in multivalent vaccines (Tafalla et al., 2014). Signal 1 facilitators extend the immune availability of the antigen by slow release of the antigen (depot effect) and attracting APCs to the antigen. Signal 2 facilitators provide co-stimulatory signals to increase or skew the immune response towards the antigen. A third class of adjuvants, signal 0, mimic PAMPs to trigger PRRs and in doing so simultaneously function as signal 2 facilitators to induce secondary signals (Dalmo et al., 2016).

Signal 1 adjuvants include oil-based adjuvants such as Freund's complete adjuvant, Freund's incomplete adjuvant, and Montanide ISA, amongst others. FCA is composed of inactivated *Mycobacterium* and mineral oil with surfactant. While it has been shown to elicit Th1 and Th17 responses, use of this adjuvant is very limited due to the resulting severe side-effects including granulomatous peritonitis (Dalmo et al., 2016). Furthermore, an increase in immunogenicity is not always guaranteed, as observed in vaccines against *Phdp* in yellowtail (Kawakami et al., 1998) and *Streptococcus iniae* in rainbow trout (Soltani et al., 2007). However,

protection was conferred in rainbow trout in a vaccine containing *A. hydrophila* (LaPatra et al., 2010) and *F. psychrophilum* (Högfors et al., 2008).

Freund's incomplete adjuvant was then developed which lacks *Mycobacterium* and is still highly effective at priming the immune system. However, peritonitis is still a side effect, for example in cod (Gjessing et al., 2012). This adjuvant provided significant protection and induced innate, humoral, and cellular responses in Japanese flounder vaccinated with an *E. tarda* vaccine (Jiao et al., 2010). Protection was also conferred to vaccinated Atlantic salmon challenged with *T. maritimum* (van Gelderen et al., 2009). Encapsulation of antigens (*vis a vis* oil-based adjuvants) has been shown to improve the depot effect of Signal 1 adjuvants and results in lower antigen doses required in vaccine formulations (Tafalla et al., 2014).

Signal 2 adjuvants boost the immune response to enhance the establishment of protection and can include inflammatory cytokines and DAMPs. These are recognised by innate receptors such as TLRs that in turn activate Th responses. The most commonly used adjuvants for fish are aluminium-containing, β-glucans and Poly I:C (Dalmo et al., 2016).

Aluminium compounds induce Th2 responses and promote protective humoral immunity and lead to a depot effect which is beneficial for long-term immunity. This may not be sufficient however, if cellular immunity is required. Furthermore, potential side effects include melanisation and organ adhesions resulting in degraded flesh quality at slaughter (Dalmo et al., 2016; Tafalla et al., 2014). When tested in Atlantic salmon vaccines against disease caused by *A. salmonicida*, protection was conferred, however not significantly (Mulvey et al., 1995). Conversely, when tested together with inactivated *E. coli* in a catfish *E. ictaluri* vaccine, significant protection was achieved (Tyler & Klesius, 1994).

The effect from β-glucans is dose dependent and short-lived, but may result in increased innate immune responses via a C-type lectin receptor and can provide a degree of protection (Petit & Wiegertjes, 2016). Poly I:C induces type 1 IFNs leading to innate responses. When tested against IHN in rainbow trout and viral

haemorrhagic septicemia (VHS) in Japanese flounder, good protection was conferred (Kim et al., 2009; Takami et al., 2010). Cytokines are potential adjuvants for fish vaccines though few have been explored (Dalmo et al., 2016; Tafalla et al., 2014). IL-8 has been shown to modulate early immune responses in rainbow trout against VHS (Jimenez et al., 2006).

Replicating vaccines

These vaccines can consist of either live attenuated bacteria, or bacterial/viral vectors carrying genes encoding for and expressing the antigen of interest. An extensive knowledge of the molecular biology of the pathogen is required to ensure its attenuation. Live attenuated vaccines carry some of the highest risks due to potential impacts from virulence reversion, effect in non-target species, spread in the environment, persistence in the vaccinated organism, and high documentation costs (Knappskog et al., 2014). However, recent advances have been made where reverse genetics can be used to attenuate the pathogen, greatly reducing the risk of reversion of virulence. On the other hand, this makes the pathogen a genetically modified organism, so legislation surrounding the production and use of the vaccine is stricter. They also require very specific storage conditions as the vaccine requires cold temperatures for stability. Despite this, live vaccines are generally efficacious and require a 1000-fold lower dose than inactivated vaccines. As adjuvants are not required, the administration of these vaccines is much more straightforward, and they have been successful in inducing both humoral and cellular immunity. The drawback with vector vaccines is that an immune response will be generated against the vector, and any pre-existing antibodies against such vectors may counteract the usefulness of the vaccine itself. Live attenuated vaccines for use in farmed fish include those against disease caused by *Renibacterium salmoninarum, E. ictaluri, F. columnare,* and *Piscirickettsia salmonis*, as well as a replicon vaccine based on the salmonid alphavirus (SAV) genome that provided protection against ISA in salmon (Aas-Eng, 2016; Knappskog et al., 2014; Wolf et al., 2013).

Inactivated vaccines

Whole cell vaccines have historically been preferred for safety reasons as they do not cause or spread disease. Bacteria are harvested at late exponential growth phase, then inactivated by physical (temperature, radiation) or chemical (formaldehyde) methods. At low concentrations, formaldehyde maintains the immunogenicity of the bacterin. Additionally, in order to confer effective protective immunity, adjuvants are required along with the inactivated bacterin to prime the immune system, as the bacterins alone often do not generate strong enough responses, despite being immunogenic.

Rønneseth et al. (2015) vaccinated lumpsuckers with inactivated bacterin vaccines containing two strains of atypical *A. salmonicida*, *P. atlantica*, *V. anguillarum* and *V. ordalii*, and found that for each pathogen except the latter, specific antibodies were generated at high levels. A commercially available vaccine against vibriosis (*V. anguillarum* serotypes O1 and O2a) similarly appears to result in protective immunity in lumpsuckers. Protection following vaccination against other pathogens is not always achieved, however, since difficulties may arise, for example due to antigen heterogeneity among different isolates- as is the case for atypical *A. salmonicida*, or due to challenges such as cultivation of the bacterium, in the case for *P. atlantica*.

Subunit vaccines

Subunit vaccines are also considered to be inactivated vaccines and can be produced either directly or indirectly. The direct method is not commonly used and involves purification of the antigen directly from the bacteria in culture. For the indirect method, the antigen is produced in a heterologous expression system, commonly *E. coli* or *Saccharomyces cerevisiae*. Here, the sequence encoding the antigen is inserted in plasmids and introduced into the expression system which is cultured, and the antigens purified following harvest of the expression system at the end of the production cycle (Knappskog et al., 2014). An example of such vaccines against fish pathogens include an ISA vaccine (*S. cerevisiae* plasmid) used

in Chile. Overall, these vaccines can be safer to use than live attenuated or inactivated bacterin vaccines and have the additional advantage of containing a selection of highly immunogenic antigens in the vaccine formulation. This method is also preferable over the purification of antigens directly from bacterial culture as the antigen can degrade during purification (Wizemann et al., 1999).

The choice of plasmid relies on the ability to produce large quantities of immunogen *in vitro*, the ease of manipulation and the ability to express the quaternary structure of the protein. However, despite stimulating both humoral and cellular immune systems, subunit vaccines have been associated with low protective immunity, thus typically require high doses or booster doses, and the addition of adjuvants to boost immune responses (Biering & Salonius, 2014).

Nucleic acid vaccines

DNA and mRNA vaccines rely on genes encoding protective antigens rather than the antigens themselves. In the case of DNA vaccines, the coding sequence are present in bacterial plasmids and provided to the host by IM injection. The constructs within the plasmid additionally contain a suitable promoter that ensure gene expression in the fish.

mRNA vaccines are designed to facilitate the short-term translation and production of the antigen in the host and consist of the mRNA sequence for the protein required, along with additional sequences necessary for correct translation of the protein.

Through IM injection of DNA vaccines, the plasmids are introduced into myocytes and dendritic cells and expression of the protein will persist in the cells, whereas for mRNA vaccines the protein is translated directly from the mRNA sequence presented, which will subsequently be degraded. The goal in either method is for antigens to be exhibited by MHC class I and class II, which induces Th and Tc cells in addition to antibody production (Adams, 2019). This makes them effective against intracellular pathogens. mRNA vaccines are deemed safer than DNA

vaccines as the risk associated with genomic integration is eliminated (Evensen, 2016; Knappskog et al., 2014).

Development of nucleic acid vaccines is much faster compared to traditional inactivated bacterin vaccines, as the only requirement is the gene sequence of the vaccine target. Additionally, this ease of development also facilitates the development of multivalent vaccines (Biering & Salonius, 2014). Production of the plasmids is straightforward, and storage and transport are simpler than for other vaccine formulations since plasmids are more stable. Furthermore, the need for adjuvants added to the vaccine is eliminated as the constructs can include sequences which replicate adjuvant effects: namely those which increase the potency of the vaccine or to improve delivery and uptake of the antigen genes. Additionally, the associated side effects, notably peritonitis, are eliminated (Biering & Salonius, 2014).

Biosecurity is higher in nucleic acid vaccines compared to other methods. There are also no risks or impacts from the pathogen such as reversion of virulence or incomplete inactivation. Despite the risk of potential integration of the construct in the vaccine into the genome, this is approximately 40 times less likely to happen than spontaneous mutation. Moreover, the risk of these constructs being passed on to the consumer is therefore smaller, practically negligible. However, one notable disadvantage is high variability between pathogens in terms of efficacy or stability of the translated protein, and only two commercial DNA vaccines are available for fish: against IHN and PD in Atlantic salmon (Biering & Salonius, 2014; Dalmo, 2018).

Autogenous vaccines produced by commercial companies under controlled conditions are authorised to be used when available commercial vaccines do not offer protective immunity against a field strain, or when no commercial vaccines are available due to emerging pathogens or strains of pathogens causing outbreaks- such as the increasing outbreaks of pasteurellosis in Norway. Such vaccines are usually developed for use during critical situations, and the level of

protection may not be comparable between different strains (Evensen, 2014; Knappskog et al., 2014).

Measures of efficacy

One method of measuring the efficacy of a vaccine is the relative percent survival (RPS). However, it is not an adequate measure for diseases that do not result in mortalities. Such diseases require alternatives such as the determination of the specific mechanisms of protective immunity induced by vaccination. Correlates of protection are biomarkers that serve as thresholds at which a vaccine is deemed to be protective and include antibody production and antigen dose threshold. The antibody titre can be used as an indicator of the level of protective immunity conferred by the vaccine, although this only holds true if the antibodies produced are neutralising or opsonising in nature. As antibodies can be produced that bind to non-immunogenic antigens, the absolute titre may not be accurate in determining the efficacy of a vaccine. Additionally, by increasing the antigen dose in a vaccine until the antibody titre reaches a plateau, a threshold for protective immunity can also be derived (Munang'andu et al., 2014).

Improving the ethics and sustainability of vaccine development

Using experimental challenges to test vaccine development presents an ethical issue with regards to fish welfare, although challenges are currently the only possible route for fish vaccine development in most cases. Possible alternatives include *in vitro* serological tests, such as ELISA. However as explained above, these have practical limitations which need to be overcome in the development stage. An amelioration of experimental challenges would be to introduce early humane endpoints which replace death as an endpoint, in order to reduce the impact on experimental fish welfare. Experiments involving fish are strictly regulated by the European Union (EU Experimental Animals Directive 2010/63/EU; EU, 2010) and Norwegian legislation (Regulation concerning the use of animals in experiments; Lovdata, 2017b), in order that the 3Rs (reduce, refine, replace) are incorporated in

the experimental design. This is to ensure that fish welfare is maintained to the highest standard possible.

Batch potency or efficacy tests are required for each vaccine batch release to ensure consistency and high quality of the vaccine produced and are typically based on challenge experiments or serology tests. Such tests must comply with established and published protocols when these are available for the vaccine and are set by the European Pharmacopeia. At present, protocols are only available for vaccines against furunculosis, cold water vibriosis and classical vibriosis. As most vaccines are multivalent or contain multiple serotypes of a pathogen, challenge models may not be practically adequate to test for all antigens present (van Hulten, 2016).

If serological tests (*in vitro*), such as ELISA can be developed and validated to show good correlation between antibody response and protection, then this will initially refine, and eventually replace, challenges (*in vivo*) when testing vaccine efficacy. This decreases the number of animals use, improving animal welfare, and can also be used to distinguish any sub-standard batches (Figa Bosch, 2016). Effects from variations in disease development present in challenge models are not encountered in serological tests, making them more robust, and multivalent vaccines can be studied more adequately (van Hulten, 2016).

In vitro tests are not without their issues, however. Serological tests require extensive optimisation and refinement, to ensure standardised and consistent results across different labs, individual fish, batches, and vaccines. Adjuvants present in the vaccine may influence serology, and the effect on the immune response resulting from them cannot be distinguished from the antigen-induced effect. There is therefore a need for separate evaluation methods. DNA and mRNA vaccines have more potential options regarding flexibility of testing: as there are no adjuvants, antigen DNA/mRNA can be quantified directly, and functionality can be tested using quantitative tests for protein expression (Cooney, 2016).

2. Aims of the study

The main purpose of this work was to investigate the pathogenicity of *P. atlantica* in Norwegian lumpsuckers, develop and test the efficacy of vaccines and to study the related immune responses. Specifically, the aims of this project were:

- To establish optimal growth conditions of *P. atlantica* in liquid medium.
- To establish a replicable challenge model that can be used to investigate disease progression in lumpsuckers, as well as testing vaccine efficacy.
- To develop R&D vaccines against pasteurellosis and examine the resulting immune responses post-vaccination, and protection upon challenge.
- To carry out phylogenomic and gene expression analyses to determine the taxonomic status of *P. atlantica* and identify virulence factors that may serve as potential vaccine targets.

3. Major findings of the project

- Cultures of *P. atlantica* were established in liquid medium, with peak growth of approximately $1.5x10^9$ cells mL^{-1} obtained at 18-19 hours incubation (Paper 1).
- A bath challenge model was identified as the most adequate to study disease progression and vaccine testing in lumpsuckers (Paper 1).
- Koch's postulates were fulfilled for *P. atlantica* upon re-isolation of bacteria following challenge (Paper 1).
- Characteristic gross pathology of pasteurellosis in lumpsuckers is white spots over the entire skin, especially around the eyes (Paper 1).
- Fish surviving challenge can be carriers of the bacteria, thus re-emergence of disease cannot be excluded (Paper 1).
- Vaccination using whole cell formaldehyde-inactivated bacterins resulted in high levels of specific antibodies (Paper 2).
- Vaccination with inactivated bacterins conferred some protection (approximately 40%) against pasteurellosis upon experimental challenge (Paper 2).
- An uncharacterised putative adhesin protein (named <Hia> in this project) was identified in the *P. atlantica* genome as a potential vaccine target (Paper 3).
- Gene expression analysis showed that the gene encoding the putative adhesin is upregulated significantly when bacterial cells are exposed to lumpsucker head kidney leucocytes, which may indicate regulation by leucocyte activities (Paper 3).
- Comparative phylogenomics resulted in its distinct taxonomic position as a novel species of the Pasteurellaceae family with the suggested name of *P. atlantica* (Paper 3).

4. Methodological considerations

Paper 1:

- 16S rRNA sequencing for differentiation of fish pathogenic *Pasteurella*: 16S rRNA sequencing was used in combination with growth characteristics to confirm the identity of the *Pasteurella* sp. isolate collected from Norwegian lumpsuckers to be used for this work.
- Challenge models: Injection, cohabitation and bath challenge models were tested, to determine which model would result in the most accurate depiction of the pathogenesis of pasteurellosis in lumpsuckers, and which subsequently can be used for vaccine testing. Experimental fish were screened and found to be pathogen free and were monitored closely throughout the duration of the study to ensure that fish welfare was maintained.
- Challenge doses: The doses used were chosen based on previous work carried out by Rønneseth et al. (2014), and bacterial numbers were verified by CASY cell counts and counting of colony forming units from titrations of the challenge material. The aim was to establish different levels of mortalities, ranging from high to low, to better understand the progression and pathogenicity of *P. atlantica* and find an optimal dose for vaccine testing.
- qPCR: The protocol enabling quantification of bacterial load in the challenged fish was adapted from that established by Gulla et al. (2016b). While it cannot be guaranteed that the bacterial load detected by qPCR indicates live bacteria, the survivors from challenges were analysed after three consecutive days of no mortalities and swabs from the head kidney were streaked onto blood agar where *P. atlantica* was successfully grown. It is for this reason that inferences were made regarding carrier status of the challenge survivors. SYBR-green assay for the *sodA* gene from *P. atlantica*, used as reference gene in the qPCR analyses, was developed in this paper.

Paper 2:

- Vaccines: Whole cell inactivated vaccines were preferred at this stage both for ease of production as well as being the most straightforward type of vaccine, when taking into consideration that no work had been done on vaccination of lumpsuckers against pasteurellosis. The two doses considered were formulated, with adjuvant from PHARMAQ, to investigate the effect of the presence or absence of extracellular proteins (based on the inclusion or exclusion of bacterial supernatant in the final vaccine formulation) on the degree of protection conferred, if at all.
- Enzyme-linked immunosorbent assay (ELISA): ELISA was used as the most sensitive technique to measure the antibody levels generated following vaccination. The reasons for choosing ELISA were two-fold: to determine that a specific antibody response was in fact generated, and to confirm that the 500°D timeline for a sufficient antibody level prior to challenge was adequate in this study.
- SDS-PAGE and Western blotting: The high resolution of separated proteins obtained by SDS-PAGE allow for clear analysis of proteins which may be identified as immunogenic through Western blotting. Western blotting provides insight into which proteins sera from immunised fish are reacting to, and to what degree. It may also indicate the level of immunogenicity of the proteins present. A shortcoming of this method is that glycosylated antigens would not be transferred to the membrane, thus any such immunogens may be missed.
- Bath challenge doses: The bacterial doses used for this challenge were based on results from Paper 1, and the range of bacterial concentration for the doses was chosen to determine the limits at which the vaccines would be protective, if at all. The time point for challenge was chosen to be 500°D post-vaccination, following established procedures from literature (Haugland et al., 2018).

- Head kidney leucocyte (HKL) exposure to bacteria: The number of bacteria and leucocytes were chosen to ensure sufficient amounts of each, to allow interactions between the host and pathogen to be followed. The timings of the challenge were such to allow the infection process to be followed from the earliest point of bacteria interacting with leucocytes until establishment of infection. Visualisation of the bacteria was performed by using a rabbit anti-*Pasteurella* antibody for immunofluorescence analysis.

Paper 3:

- Illumina and PacBio sequencing: Two sequencing methods were used as these methods complement each other. Illumina sequencing provides short reads of the genome with high accuracy and PacBio sequencing provides longer reads with slightly lower accuracy. Taken together, both methods provide a clear overall picture of the *P. atlantica* genome.
- Phylogenomics: Whole genome phylogenetic analysis provided insights to the phylogenetic positioning of *P. atlantica* within the *Pasteurellaceae*.
- Virulence factors: The proteome of *P. atlantica* was screened against major virulence factor databases in order to identify virulence factors, including their localisation within the bacterial cell, their secretion systems, and structural properties. The strongest candidates were then selected and analysed for use as vaccine targets.
- Identification of vaccine targets: Vaxign and VaxiJen were used to predict the vaccine targets based on characteristics that are known to be highly immunogenic, such as adhesion potential.
- *In vitro* work: The gene expression analysis on leucocytes exposed to P. atlantica were designed to investigate whether the gene for the putative vaccine target adhesin was expressed only in the presence of host cells, or whether it was also expressed in bacteria during growth, which would benefit vaccine development further on.

5. Discussion

In this study, the pathogenicity of *P. atlantica* was investigated in Norwegian lumpsuckers through development of appropriate challenge models and vaccine testing. Vaccination of lumpsuckers with inactivated *P. atlantica* bacterins provided only limited protection against disease following experimental challenge despite high antibody levels, indicating that cellular immunity and use of strongly immunogenic antigens should be targeted for effective protection. In the first two papers, the *Pasteurella* isolate used in this study was not yet classified and referred to as *Pasteurella* sp. In Paper 3, through phylogenomic analysis, the taxonomic position of the bacteria was identified, and the bacteria was named *P. atlantica*. Furthermore, an uncharacterised putative adhesin was identified as a potential vaccine target for use in vaccine development through RV. *In vitro* investigations on the interactions between *P. atlantica* and lumpsucker head kidney leucocytes showed that bacteria tend to form chains and attach to the surface of the leucocytes, and expression of the adhesin increased significantly in the presence of leucocytes.

Defining the taxonomic position of *Pasteurella atlantica*

At the beginning of this project, it was unclear where *P. atlantica* belonged within the Pasteurellaceae family. Through phylogenomic analysis (**Paper 3**), the distinct taxonomic position of the bacteria was determined, and it clustered with *Ph. uteri* and *P. skyensis*, lying closer to the latter than the former. Phylogenetic analysis carried out by Alarcón et al. (2016) of various isolates collected from outbreaks of pasteurellosis in Norway, showed that *P. atlantica* was found to be genetically distinct from the related species *P. skyensis*.

Until 2020, *P. skyensis* had not been recorded in Norway. The Norwegian Veterinary Institute has since then confirmed two outbreaks of disease caused by *P. skyensis* in Norwegian farmed Atlantic salmon (Nilsen et al., 2021). Additionally, since Spring 2018, a new type of *Pasteurella* has been recovered from several outbreaks of pasteurellosis in Atlantic salmon in a number of farms in Norway,

including cases involving Atlantic salmon in monoculture and cohabiting salmon and lumpsuckers (Nilsen et al., 2021).

Although the route of transmission of the new salmon *Pasteurella* strain between cohabiting salmon and lumpsuckers is still unclear, a study by Sandlund et al. (2021) indicated that lumpsuckers are also susceptible to this new strain isolated from salmon. Furthermore, knowing the taxonomic position of the pathogen is useful to identify similarities between *P. atlantica* and other members of the Pasteurellaceae. In the case of a newly discovered pathogen, this will provide a starting point for investigations into prevention and treatment both in hatcheries and especially in the field.

Pasteurellosis as a novel disease in Norwegian lumpsuckers

As a bacterial disease, pasteurellosis in farmed lumpsuckers is a relative newcomer, with the first cases recorded around 10 years ago (Johansen, 2013). As it was such a new species, Koch's postulates were at the time unfulfilled. The challenges concerning culturing of *P. atlantica* in broth, due to the requirement of foetal calf serum supplementation, additionally complicated studies to investigate the disease, which translated to delayed vaccine development.

The first step, therefore, was to establish growth of *P. atlantica* in liquid medium. It was already known that the bacteria could be isolated and grown on marine blood agar (Alarcón et al., 2016), but large volumes of bacteria would be required for extensive studies, as well as in industrial settings. We tested two different broth compositions, and tryptic soy broth supplemented with foetal calf serum was the most straightforward composition that enhanced bacterial growth in a timely manner (**Paper 1**). Peak exponential growth of bacteria of approximately $1.5x10^9$ cells mL^{-1} was reached after 18h of incubation at 20°C. The ability to grow *P. atlantica* in liquid medium and obtain relatively high cell numbers per mL was an important step forward towards understanding the disease, investigating challenge models and vaccine testing.

Development of a challenge model to study pathogenicity of *P. atlantica* and test vaccine efficacy

A variety of challenge models were tested and evaluated to follow the progress of pasteurellosis and mortality in lumpsuckers (**Paper 1**). Models based on injection of the pathogen (IM and IP) resulted in highly acute mortalities within a very short timeframe, highlighting the toxicity of *P. atlantica*. Similarly, as the cohabitation model relied on IP injected shedders, it is likely, although not confirmed, that only low numbers of bacteria were available to infect the naïve cohabitant fish. These models were therefore considered unsuitable for further work. The bath challenge model however, resulted in a more stable and gradual mortality pattern, although deaths still rose to almost 100% across the dose range used. Having such results from this model was promising, in the process of investigating disease progression and for vaccine efficacy testing. Bath models are a close replication of a natural infection route, as pathogens infect the fish orally and through mucus membranes and no natural defence barriers are artificially breached to aid the infection process. Several studies have employed bath challenges to monitor disease caused by problematic fish pathogens, including *Y. ruckeri* (Ohtani et al., 2019), SAV (Jarungsriapisit et al., 2016), *V. anguillarum* (Gudmundsdóttir & Björnsdóttir, 2007), and atypical *A. salmonicida* (Rønneseth et al., 2017).

As *P. atlantica* was re-isolated from the challenged fish, the criteria required to fulfil Koch's postulates were met (**Paper 1**). The pathognomonic symptom of disease was the appearance of white spots over the skin and around the eyes in the bath challenged fish. Inflammation and haemorrhage at the base of the jaw and fins were observed in both bath and cohabitation challenges, although these symptoms are not unique to *P. atlantica* infections. In fact, Scholz et al. (2018) noted that these latter symptoms may be confounded with those resulting from an atypical *A. salmonicida* infection in lumpsuckers, and recommended molecular tests for diagnostic confirmation. The occurrence of symptoms from the cohabitant fish is still noteworthy, as despite the acute mortalities amongst shedders (and potential low amounts of bacteria available for infection) and low mortalities of naïve

cohabitants, significant symptoms of disease were still observed. No symptoms specific to the disease were recorded from the IP and IM injection challenges, mostly due to the acute mortalities. However, severe tissue damage was recorded at the injection point, highlighting the toxicity of *P. atlantica*. Similar symptoms (white spots, inflammation, and haemorrhage) were also observed following bath challenge of vaccinated fish (**Paper 2**), with the addition of frayed tail fins. The latter symptoms were also reported by (Alarcón et al., 2016) as occurring during a natural outbreak of pasteurellosis in lumpsuckers. Interestingly, the cohabitation model results in symptoms typically observed in chronic infections, while the bath model exemplifies symptoms of acute stages of pasteurellosis.

The remaining survivors from the cohabitation and bath challenges were shown by qPCR to be asymptomatic carriers of *P. atlantica*, several days after mortalities ceased. The lower level of bacteria in the cohabitation survivors compared to the bath survivors is likely due to the lower concentration, or slower replication, of bacteria available from the shedders, although there was no significant difference in bacterial levels from the survivors of the two challenges. Other fish pathogens with known ability to induce latent infections include *Y. ruckeri* (in rainbow trout) and *E. ictaluri* (in channel catfish), which both result in re-emergence of disease in the surviving fish following the introduction of a stressor (Antonio & Hedrick, 1994; Busch & Lingg, 1975; Hunter et al., 1980). This has serious implications for the fish farming industry, as the re-emergence of disease following a stress episode cannot be ruled out (**Paper 1**). The carrier state of lumpsuckers following pasteurellosis outbreaks has also been reported from industrial settings, where the disease re-emerged following outbreaks where the fish had been treated by antibiotics (Alarcón et al., 2016). This highlights an additional issue, that antibiotic treatments for lumpsuckers are not yet optimised at a commercial level to provide an adequate treatment regime in the face of this disease. Despite this, Kverme et al. (2021 [manuscript submitted]) show that florfenicol is efficient against pasteurellosis, at least after experimental challenge. These results highlight the importance of screening fish prior to transport and deployment to limit the risks

of outbreaks. Overall, this initial work served to emphasise the pathogenicity of the bacteria, even at different challenge doses, and its persistence in surviving fish, and indicates the rapid need for vaccine development.

Despite the high pathogenicity of *P. atlantica*, transmission of disease is not always straightforward. In cases recorded in the field from Atlantic salmon, outbreaks of pasteurellosis typically occur in situations where a prior stress event has taken place, such as lice treatments and sea transfers. This could also be the case for outbreaks recorded from lumpsuckers. Since these are housed with salmon and exposed to the same stressors, it is plausible that the increased stress or suboptimal conditions in this environment would lead to decreased immune fitness making lumpsuckers more susceptible to pasteurellosis. Experimental infections, due to their controlled nature may not always result in disease, as reported by Sandlund et al. (2021). In this study, the authors carried out an experimental cross-infection of Atlantic salmon by lumpsuckers through a cohabitation challenge model, where the lumpsucker shedders were infected using the bath model described in **Paper 1** using *P. atlantica* and a recently identified salmon strain of *Pasteurella*. While the lumpsucker "shedders" were infected successfully with both *Pasteurella* isolates, the naïve salmon were not, as evidenced by qPCR, which might support stress theory.

Vaccine efficacy and adaptive immune responses in lumpsuckers challenged with *P. atlantica*

The next step was to evaluate the adaptive immune responses mounted by lumpsuckers on exposure to *P. atlantica* (**Paper 2**). Accordingly, two formalin-inactivated whole cell vaccines were formulated, one containing supernatant from the culture medium, and one with 90% of the supernatant removed, resulting in an approximate 10x bacterin concentration (termed concentrated). Both vaccines resulted in *P. atlantica* specific antibodies at levels comparable to those recorded in literature for other bacterial pathogens (Rønneseth et al., 2015), following triple immunisation of lumpsuckers. The 'concentrated' vaccine also resulted in significantly higher levels of antibodies raised than the non-concentrated one; this

is likely due to the final higher concentration of bacterial cells present per mL in the concentrated vaccine.

Despite the high levels of antibodies, these were only slightly protective in nature and the RPS post-challenge was around 40% in the vaccinated groups. Furthermore, the higher antibody level resulting from the concentrated vaccine was not reflected in increased protection levels. Småge et al, (2018) reported similar results in a study of Atlantic salmon vaccinated and challenged with *Tenacibaculum finnmarkense*.

Such results draw attention to the shortcomings of *in vitro* methods used to measure correlates of protection for some bacterial pathogens. While ELISA is a useful technique to measure antibody levels following vaccination, it cannot always be used as a standalone procedure. This is because it does not specifically detect neutralising or protective antibodies unless a 'neutralising' antigen is used in the test. Non-neutralising antibodies can be present in high levels, but are not involved in protective immunity and therefore do not correlate with protection (Plotkin, 2008).

In order for vaccines to provide protective immunity, they need to elicit the production of opsonising or neutralising antibodies, which in turn lead to cellular and humoral immune responses, including complement degradation. In the process of vaccine development, a number of aspects must be taken into consideration to develop effective vaccines.

Initially, having a robust challenge model is crucial to establish reliable endpoints for determining vaccine efficacy. Subsequently, knowing measures of vaccine efficacy will allow for improved understanding of the immune processes involved. Briefly, such measures can include prevention of adherence, prevention of infection following penetration, prevention of distribution within the host, prevention of pathogen loading, and prevention of pathology in target tissues (Munang'andu & Evensen, 2019). When the measures are identified, the next steps

involve their measurement *in vitro* and determining whether they are correlates of protection.

In the case of prevention of adherence and distribution of the pathogen, mucosal and neutralising antibodies, respectively, can prevent adherence to mucosal surfaces and eventual systemic dispersal, including in intracellular locations. In both cases, high antibody levels can correlate with protection, and the absence of pathogen in host tissues or cells is a measure of efficacy of the vaccine. Vaccines can also be targeted to suppress replication in target organs, thus limiting the spread of the pathogen, and a suppressed pathogen load is a measure of efficacy (Munang'andu & Evensen, 2019). Crucially, antibody levels become correlates of protection if they are able to exceed a threshold at which they confer protective immunity.

Various studies in salmonids have successfully determined abilities of specific antibodies to eliminate pathogens and protect through antibody-mediated opsonophagocytosis (Lamas & Ellis, 1994; Michel et al., 1990; Nikoskelainen et al., 2007; Sakai, 1984). Specifically, the antibodies activated the complement system which in turn also enhanced the phagocytic ability of macrophages and neutrophils and this activity could be quantified by measurement of respiratory burst activity (Nikoskelainen et al., 2007). Opsonising antibodies can therefore be considered a suitable correlate of protection.

However, it is worth noting that a number of facultative intracellular fish bacterial pathogens such as *E. tarda, P. salmonis, Y. ruckeri, Francisella noatunensis,* and *R. salmoninarum* (as reviewed by Munang'andu, 2018) are able to evade respiratory burst activity and survive in macrophages. For that reason, other correlates may be more suitable for the determination of protective immunity against these pathogens.

As cellular immunity is involved in protection against intracellular pathogens, a theoretical stance would be to investigate whether T cell responses correlate with protection in lumpsuckers. It is known that lumpsuckers possess CD4 and CD8

(Straumsnes, 2018), indicative of Th and Tc cells, as well as different subtypes of Th cells (Nelsson & Haugland, 2020; unpublished data). However, the quantification and duration of protection awarded by these cells has not been established to date, and no studies have correlated these responses with protection. Despite the drawbacks, alternatives are possible. Nucleic acid and attenuated vaccines still induce antibody production, and although these vaccines primarily target cellular immune responses, the antibody levels produced can be used as surrogates or proxies of protection as they can still be reliably used to predict efficacy of vaccines (Munang'andu & Evensen, 2019; Plotkin, 2008). A number of studies have employed this mechanism and related the upregulation of cellular immune genes with antibody levels generated following vaccination which was then correlated with protective immunity in rainbow trout against IHN and VHS, and in Japanese flounder against VHS (Kim et al., 2000; McLauchlan et al., 2003; Yong Byon et al., 2005).

Sera from the vaccinated lumpsuckers analysed by immunoblotting at 500°D post-vaccination reacted to only few *P. atlantica* proteins separated by SDS-PAGE, specifically: high molecular weight proteins at approximately 75kDa and 100kDa as well as proteins at 48kDa and 15kDa. These reactions were similar to, although less pronounced, than those observed in lumpsuckers vaccinated three times against *P. atlantica* which reacted to additional bands of mid-range molecular weight. This limited reactivity may indicate that the antibodies generated following vaccination were not directed towards easily accessible cell wall-integrated antigens, thus partly explaining the limited protection conferred.

It is also possible that potentially antigenic components did not transfer to the immunoblot due to glycosylation of proteins. Antibodies reactive to such antigens would have to be detected by other techniques (Wood & Kaattari, 1996). Among such methods is the microarray technique, where antigens (either purified or from inactivated whole bacterins) are covalently bound to silica or glass plate chips and immunofluorescent methods are then used to detect the specific antibody-antigen interactions (Blixt et al., 2004; Thirumalapura et al., 2005, 2006). As the additional

reactive bands resulting from the triple immunised lumpsuckers were visible, it is also possible that vaccination boosts or different adjuvants might be required to generate an appropriate response. Agglutination tests were also carried out, and live *P. atlantica* was agglutinated by lumpsucker immune sera. This could indicate that binding to bacterial surface components such as capsule or loosely associated carbohydrates may occur.

***P. atlantica*: intracellular or extracellular pathogen?**

A closer look at the interactions between *P. atlantica* and lumpsucker HKLs revealed two potential infection routes: intracellular or extracellular. Confocal and transmission electron photomicrographs showed *P. atlantica* likely were present intracellularly within HKL. At early stages of leucocyte infection, the bacteria were observed as individual entities while after 24h, bacteria had aggregated into chains and were attached to the surface of leucocytes. Such bacterial aggregations were also observed in histopathological images of tissues (heart, spleen and head kidney) from experimentally challenged lumpsuckers (**Paper 1**).

Similarities in host-pathogen interactions as those observed between *P. atlantica* and lumpsucker leucocytes have been noted for other pathogens such as uropathogenic *E. coli*. These bacteria are known to form aggregates which enable the 'microcolony' to evade the host immune system physically, rather than by regulating host immune responses (Justice et al., 2004). The large size of such aggregates confers protection and enhanced survivability of the pathogen, as despite any protective responses raised against the pathogen, phagocytosis would not be possible. This in turn leads to impaired antibody functionality and suboptimal bacterial clearance and is a potential explanation for the limited protection conferred to lumpsuckers by the vaccines tested in this work (Paper 2).

The observations of *P. atlantica* possibly present inside lumpsucker HKLs highlighted two avenues for further work. If *P. atlantica* is a facultative intracellular pathogen, further work should focus on stimulating cellular immunity to confer protection in lumpsuckers via cytotoxic T cell mediated responses which eliminate

cells infected with intracellular pathogens. On the other hand, the agglutination results indicated that cell surface proteins may be involved in disease transmission, as immune sera agglutinated live *P. atlantica*, and this would require genomic analysis to identify potential virulence factors.

***In silico* identification of virulence factors and gene expression analysis of a potential vaccine target**

In the final part of the project the genome of *P. atlantica* was sequenced to establish its taxonomic position within the Pasteurellaceae and to enable *in silico* identification of putative virulence factors and immunogenic components that would be suitable as potential vaccine targets by using the principles of RV. Functional analyses were subsequently carried out to investigate *in vitro* the expression of the vaccine target with highest adhesion potential (**Paper 3**).

Through the *in silico* analyses, it was found that the genome of *P. atlantica* contains 11 genomic islands (GIs) and five prophages. Of the 17 identified virulence factors, five were found in GIs and/or prophages, and accounted for 10% of the total genome. RV was used to identify these virulence factors, and specific criteria were applied to narrow the list to identify those with the highest virulence potential and suitability as vaccine targets. The criteria for the bioinformatic analyses centred mainly around subcellular localisation of the protein and predicted adhesion functionality. From these analyses a putative uncharacterised protein with high adhesin probability ranked highest both as a putative virulence factor as well as a likely vaccine target, with an adhesin probability of 92.5% (**Paper 3**). The protein is very similar to the adhesins Hia and YadA, which are characteristic adhesins from *H. influenzae* and *Yersinia* species, respectively (El Tahir & Skurnik, 2001; St. Geme & Cutter, 2000).

Adhesins are used by pathogenic bacteria to attach to host tissue surfaces, commonly by binding to lectins which enables colonisation, followed by aggregation and infection. This underlines the observations made in previous work (**Paper 1** and **Paper 2**) where bacteria were found to aggregate to each other

as well as attach to HKLs. Aggregation may serve a protective function for the bacteria against host defences and could explain the limited protection conferred by the vaccines tested in this project (**Paper 2**).

Gene expression analysis determined that <*hia*> expression is significantly upregulated (compared to the reference gene used, *gyrA*) both in the absence and presence of leucocytes and confirmed the *in silico* results, as a potential target for subunit protein antigen, mRNA, or DNA vaccine development. The expression of <*hia*> in the absence of host (lumpsucker) leucocytes correlates with increases in bacterial growth rates (**Papers 1 and 3**) and indicates that the adhesin is expressed during bacterial growth. Upon exposure of the bacteria to lumpsucker leucocytes (where bacterial growth is likely inhibited by the L-15 medium used for maintaining leucocyte functionality), expression of <*hia*> was significantly upregulated following an initial period of acclimation of 6 h post exposure, suggesting that the presence of leucocytes triggers the increased expression of adhesins, possibly due to cell adherence.

Since most of the virulence factors listed in **Paper 3** as the most promising vaccine candidates are as yet uncharacterised, it is difficult to predict their functionality. However, suggestions can be made based on the protein class they likely belong to. Three of the listed virulence factors are linked to haemolysins, with one (PfhB1) identified as a filamentous hemagglutinin protein which is a known *P. multocida* virulence factor involved in colonisation and adherence (Fuller et al., 2000).

Potential alternatives to adhesins for consideration as vaccine targets include extracellular proteins, outer membrane proteins, and lipopolysaccharides. However, further work on annotation of the *P. atlantica* genome is initially required to identify more potential virulence factors and vaccine targets.

Implications for vaccine development

The collective results from this PhD project provide good foundation for further work on development of a vaccine against pasteurellosis in lumpsuckers.

Currently, lumpsuckers are vaccinated using vaccines licenced under special exemption against atypical furunculosis (caused by atypical *A. salmonicida*) and vibriosis. However, due to limited protection conferred by some vaccines in particular situations, autogenous vaccines are also used to protect the fish against disease. These are formulated by commercial vaccine producers under strict guidelines by regulatory authorities when new pathogens or strains of pathogens are not covered by commercially available vaccines, or in the case of new fish species being farmed.

Based on the results from the vaccination trial carried out in this project, bacterin-based vaccines against *P. atlantica* may be unlikely to develop sufficient protection, showing the need for investigation into different vaccine formulations. Using the information obtained from our genomic and functional analyses, subunit vaccines can be developed and tested. These can be produced either by using a heterologous expression system or native protein can be purified directly from a *P. atlantica* liquid culture. This latter method might be possible as results (**Paper 3**) show high upregulation of the <*hia*> transcript at peak exponential growth phase. Alternatively, DNA or mRNA vaccines can be manufactured for a more reliable and cost effective vaccine.

In either case, the antigen or antigen sequence used in the vaccine, the PAMP, will be recognised by host immune cells upon vaccination. Depending on whether a recombinant or nucleic acid vaccine was used, this may trigger different signalling pathways or phagocytosis that activate cytokine expression. In turn, this will stimulate adaptive immune responses through Th (humoral) and/or Tc (cellular) responses.

Although subunit vaccines are often straightforward to produce, their efficacy can be lower than that of vaccines containing inactivated or live attenuated microorganisms, or nucleic acids from these pathogens. This could be due to various reasons, such as limited induction of a protective immune response, low antigen repertoire, lack of glycosylation, or issues with folding of the protein antigen used in the vaccine, if recombinantly produced. In the latter case, environmental cues or host responses may impact the natural expression of the adhesin, rendering it different from the recombinant antigen. Moreover, if allelic variations of the adhesin are present across or within isolates (Klemm & Schembri, 2000) or if multiple adhesins are involved in adherence, this will impact vaccine efficacy, and hence, development.

Despite the potential drawbacks with subunit vaccines, some of these issues can be counteracted by developing vaccines that use the conserved region of an adhesin (Ofek et al., 2003). In fact, recombinant protein production has been used to develop vaccines containing major adhesins as the protective immunogens, which successfully conferred protection to farmed fish against bacterial disease by *V. anguillarum*, *V. harveyi*, *E. tarda*, *A. hydrophila*, *A. salmonicida* subsp. *salmonicida* and *Francisella orientalis* (Fang et al., 2004; Hamod et al., 2012; Jin & Li, 2021; Khushiramani et al., 2012; Maiti et al., 2012; Marana et al., 2017; Shahin et al., 2020; Zhu et al., 2019).

Due to their rather low immunogenicity, subunit vaccines require adjuvants to boost the immune response. On the other hand, nucleic acid vaccines are more favourable since they combine the advantages of live vaccines of antigen presentation, without the risk of reversion of virulence. Furthermore, they can overcome the issues related to incorrect folding and incomplete glycosylation encountered in subunit vaccines. They are also more versatile since cellular immune responses can be targeted more specifically and in the case of DNA vaccines, antigen multivalency can be achieved through the development of complex or multiple plasmids (Liu, 2011).

Additionally, coating DNA plasmids with compounds such as Poly (D, L-lactic-co-glycolic acid), chitosan nanoparticles, or alginate microparticles for use as oral vaccines could provide a less stressful alternative administration route to IM injections for DNA vaccines (Adomako et al., 2012). The compounds protect the vaccine from degradation by stomach acid and proteases, and allow for improved uptake compared to traditional oral vaccines where vaccines are coated onto feed. Adomako et al. (2012) found that it was possible to coat an IHN DNA vaccine for rainbow trout in PGLA, which in turn indicated slightly improved survival following challenge, in preliminary tests. Furthermore, Ahmadivand et al. (2017) coated an IPN DNA vaccine, also for rainbow trout, in chitosan-based nanoparticles and alginate microparticles, and observed good protection (up to 70%) conferred from the vaccine following challenge.

Despite the benefits, only two DNA vaccines have been licenced for use in farmed Atlantic salmon as yet, one against IHN in 2005 and one against PD (Biering & Salonius, 2014; Dalmo, 2018). A number of studies have investigated DNA vaccination of fish using adhesins or membrane associated proteins and conferred protection following vaccination and challenge, including FliC from *E. tarda* (Jiao et al., 2009), OmpK from *Vibrio* spp. (Xu et al., 2019), and Omp38 from V. *anguillarum* (Kumar et al., 2007). These results are promising evidence for further investigation of the *P. atlantica* adhesin protein identified in Paper 3 in terms of DNA/mRNA vaccine development.

One drawback of DNA vaccines is that genomic integration remains a potential risk that leads to development of DNA vaccines being a highly scrutinised process by regulatory bodies (Biering & Salonius, 2014). For that reason, mRNA vaccines would be preferable, as following expression of the antigen, the mRNA sequence itself is degraded within a short period of time, minimising risks to the host and the environment.

mRNA used in a vaccine acts as a template of the protein to be produced in the cell cytoplasm, with multiple copies being made from the template before this is

degraded. However, mRNA vaccines have been accompanied by a number of issues which have held back vaccine development, including instability and possible high immunogenicity which could lead to toxicity in the host. Improvements in the form of using modified nucleosides have stabilised the immunogenicity of mRNA (Liu, 2019). Contrary to DNA vaccines, research into mRNA vaccines for fish is hard to come across. However, recent successful advances in mRNA vaccine development as observed for SARS-CoV-2 vaccines provide a promising foundation for future development of mRNA vaccines against fish diseases.

Concluding remarks

In the first phase of this project, the growth of *P. atlantica* was established in tryptic soy broth enriched with sodium chloride and foetal calf serum. This enabled a number of challenge models to be tested, and bath challenge was found to be the most adequate to investigate the disease progression and vaccine testing in lumpsuckers. These experiments also exposed an asymptomatic carrier status that occurs on exposure of the fish to the pathogen.

Subsequently, R&D vaccines were then developed for lumpsuckers using formalin-inactivated *P. atlantica* bacterins. Immune responses were measured post-vaccination, and the level of protection conferred was evaluated post-challenge. Despite high *P. atlantica*-specific antibody levels present following vaccination, the vaccines did not confer satisfactory protective immunity against the pathogen, indicating that inactivated bacterin based vaccines may be insufficient. *In vitro* exposure of *P. atlantica* to head kidney leucocytes indicated the bacteria may potentially be facultatively intracellular and may auto-aggregate and adhere to host cells. In turn this indicated the possible involvement of adhesion-involved virulence factors.

This was the first time the genome of *P. atlantica* was fully sequenced, through which the taxonomic position of *P. atlantica* within the Family Pasteurellaceae was established. Moreover, through this work, further research in RV will be facilitated. *In silico* analysis identified several virulence factors, predicted to be adhesins. The

highest ranking protein (<Hia>) is still uncharacterised, however found to be similar to Hia from *H. influenzae* and YadA from *Yersinia* spp.. Gene expression analysis revealed that expression levels of <*hia*> in *P. atlantica* increased significantly both in bacteria in liquid culture as well as following exposure to lumpsucker leucocytes. This supports *in silico* analysis that <Hia> is a virulence factor, which is additionally triggered by the presence of and possible adherence to host immune cells.

Future perspectives

The work carried out in this project investigating pasteurellosis in Norwegian lumpsuckers is by no means exhaustive, and a number of avenues remain to be explored.

Arguably the most important next step will be structural analysis of <Hia> and the development and testing in lumpsuckers of a recombinant protein or nucleic acid vaccine using this antigen. Elucidating the structure of the protein will ensure that the recombinant protein produced will be stable and contain relevant epitopes, which will increase the likelihood for an efficient vaccine. Moreover, *in silico* analysis will be further looked into to identify other potential vaccine candidates in addition to <Hia>. The generation of protective immunity in this species following vaccination will be an important step in the direction of tackling the pressing issues of cleaner fish welfare and sustainability.

A recently identified salmon *Pasteurella* strain has been documented to cause disease in lumpsuckers both in the field as well as under experimental challenge (Nilsen et al., 2021; Sandlund et al., 2021), however outbreaks caused by other species in the same genus (such as *P. skyensis*) have not been recorded in lumpsuckers. Genomic analyses will need to be undertaken to predict differences in pathogenicity and the most important virulence factors involved in disease, across the various isolates/groups. Furthermore, work also needs to be done to determine whether the Hia-like protein identified in Paper 3 is also present in the isolates mentioned above, as this may streamline vaccine development against

pasteurellosis in Atlantic salmon and lumpsuckers. If <Hia> is not present in the different isolates, this can highlight the need to develop different vaccines to confer protective immunity in lumpsuckers and salmon.

Genomic analyses carried out in this project have identified five intact and inducible prophages present in the genome of *P. atlantica*. Pangenomic analysis could be the way forward to investigate how widespread and conserved these genes are among the various field isolates of *P. atlantica*. This in turn will shed light on new, additional potential routes for the treatment of pasteurellosis, through the use of bacteriophages.

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ORIGINAL ARTICLE

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Pathogenicity of *Pasteurella* sp. in lumpsuckers (*Cyclopterus lumpus* L.)

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Abstract

The incidence of disease caused by *Pasteurella* sp. in farmed lumpsuckers in Norway has been steadily increasing in recent years, causing significant economic losses and fish welfare issues. The disease affects all life stages, both in hatcheries and after release into salmon cages. Therefore, it is important to establish robust challenge models, to be used for vaccine development. Exposure experiments via intramuscular and intraperitoneal injection underlined the high virulence of the bacteria, whereas the cohabitation and bath models allowed the chronic symptoms of the disease to be studied more accurately. Skin lesions and haemorrhage at the base of fins were observed in the more acute cases of the disease. Symptoms including white spots over the skin, especially around the eyes, characterized the chronic cases. The latter were most prominent from the bath challenge model. Histopathology indicated a systemic pattern of disease, whereas qPCR analysis from head kidney showed that bacteria may be present in survivor fish at the end of the challenges. In all the challenge models investigated, *Pasteurella* sp. was re-isolated from the fish, thus fulfilling Koch's postulates. These findings highlight the importance of screening of lumpsuckers prior to transfer to minimize the risks of carrying over asymptomatic carriers.

KEYWORDS
challenge, cleaner fish, infection, lumpfish, pasteurellosis, pathology

1 | INTRODUCTION

Prior to their recent introduction as cleaner fish, the biology and immune system of lumpsuckers were not very well known or not well studied. As is common with most fish species introduced in aquaculture, bacterial diseases encountered during farming were rapidly recognized. The mortality spikes seem to occur soon after hatching, after vaccination procedures and often following transfer to salmon cages (Johansen, 2013). So far, the most common bacterial species identified from disease outbreaks include *Vibrio anguillarum* (Marcos-López, 2013), atypical *Aeromonas salmonicida*, *Tenacibaculum* spp., *Moritella viscosa*, and a *Pasteurella*-like isolate (Alarcón et al., 2016; Bornø and Gulla, 2016; Rønneseth, Haugland, Colquhoun, Brudal, & Wergeland, 2017).

The first confirmed outbreak of pasteurellosis caused by *Pasteurella* sp. in lumpsuckers was reported by the Norwegian Veterinary Institute from a farm in southern Norway in 2012 (Johansen, 2013). Additional cases have since been reported from sites along the western coast of Norway (Alarcón et al., 2016). Pasteurellosis has also been recorded in production facilities where the affected fish ranged from juveniles (6–8 g) to broodstock (340 g), as well as in lumpsuckers deployed to salmon cages where disease occurred within the first few weeks following transfer (Alarcón et al., 2016). More recently, pasteurellosis was diagnosed in lumpsuckers from 14

different sites in 2015 (Bornø & Gulla, 2016). *Pasteurella* sp. has also been detected several times in milt and eggs from lumpsuckers in higher levels than are usually detected during regular tissue screenings (Kui, 2017). This may suggest that vertical transmission of disease is theoretically possible.

Bacterial diseases in hatcheries are currently treated using antibiotics; however, there are no approved protocols available to provide adequate treatment. Furthermore, in cases of outbreaks in sea cages, treatment is not possible due to their cohabitation with Atlantic salmon. Vaccine development against this disease is still in its infancy, and *Pasteurella* sp. antigens are currently not included in commercially available lumpsucker vaccines, due to lack of culture protocols for this bacterium.

The *Pasteurella* sp. isolate from lumpsuckers in Norway is serologically distinct from both *Photobacterium damselae* subsp. *piscicida*, and *Pasteurella skyensis*—a pathogen of Atlantic salmon in Scotland, when analysed using rabbit antisera. In addition, partial sequencing of the 16S rRNA gene of *P. skyensis* and that of Norwegian *Pasteurella* sp. isolates showed that they may represent different species or subspecies (Alarcón et al., 2016).

Phenotypically, lumpsucker *Pasteurella* sp. isolates appear similar to a *Pasteurella* associated with "varracalbmi," which is a systemic infection characterized by severe ophthalmitis in farmed Atlantic salmon in Norway (Valheim, Hastein, Myhr, Speilberg, & Ferguson, 2000). As the characteristics of varracalbmi have not been recorded in lumpsuckers suffering from pasteurellosis, it is possible that the *Pasteurella* sp. isolate from lumpsuckers may differ in pathogenicity from the varracalbmi isolate. However, histopathological similarities between the two infections exist and include skin, fin, and gill lesions and visceral organ granulomas (Alarcón et al., 2016).

Alarcón et al. (2016) found that lumpsucker *Pasteurella* sp. isolates were Gram-negative, nonmotile coccobacilli that produced acid from D-glucose both aerobically and anaerobically. Very weak acid production was observed form lactose, mannose, and galactose under aerobic conditions. All strains were highly sensitive to the vibriostatic compound 0/129. They were weakly oxidase positive, did not produce indole, catalase, gelatinase, or urease, and a combination of sodium chloride and blood were recorded to be strict growth requirements (Alarcón et al., 2016). In this paper, we explain how *Pasteurella* sp. was successfully cultured in liquid medium. We also tested the most commonly used challenge models to identify which would be the most appropriate to investigate pasteurellosis caused by *Pasteurella* sp. in lumpsuckers. These results were subsequently used to describe how Koch's postulates were fulfilled for this disease.

2 | MATERIALS AND METHODS

2.1 | Fish and rearing conditions

Farmed, unvaccinated lumpsuckers were obtained from Vest Aqua Base AS, Norway. The fish stock had a disease-free history and displayed no signs of infection or mortality and were health screened and certified free from atypical *A. salmonicida*, *Pasteurella* sp., *V. anguillarum*, and lumpfish flavivirus (PHARMAQ Analytiq). The fish were acclimated during the quarantine period for 15 days at 12°C in 500 L tanks at a photoperiod of 12L:12D, a salinity of 34 ppt and oxygen saturation of >77% in the outlet water, at the holding facilities in the Industrial Laboratory (ILAB) at the Bergen High Technology Centre. The fish were fed Amber Neptun (Skretting) dry feed by automatic feeders and fasted for 36 hr prior to challenge. The fish were transferred to the challenge unit 3 days prior to the challenge and split into 150 L tanks, with 50 fish per tank. The same water parameters were used as for the holding tanks.

2.2 | Bacterial isolation and characterization

The *Pasteurella* sp. isolate used in the challenge experiments was the same as described by Alarcón et al. (2016). This isolate originated from a site on the southwest coast of Norway. For these experiments, a second passage of the bacteria was used. The bacteria were cultured in Tryptic Soy Broth (TSB) (Becton Dickinson) or Brain and Heart Infusion Broth (BHIB) (Becton Dickinson). These media were supplemented with 1.5% NaCl and a series of foetal calf serum (FCS) (Gibco, Lot no. 1633098) concentrations, and cultures were incubated in an orbital incubator, at 200 rpm and 20°C. An additional *Pasteurella* sp. isolate (named *Pasteurella* sp. P2) originating from the central western coast of Norway, and found to be 100% similar by 16S rRNA analysis to the isolate mentioned above, was used for the intramuscular (IM) and intraperitoneal (IP) injection trials.

Growth curves were compiled for bacteria grown in both media by measuring bacterial cell numbers using a cell counter (CASY Model TT (Innovatis) and CASY worX V1.26). Metabolic characterization was done using API 20NE and API ZYM (Bio Merieux). For the challenge models, only TSB supplemented with sodium chloride and FCS was used to culture the bacteria. The bacterial cultures to be used in the challenge experiments were harvested at the late exponential phase, centrifuged at 2,500 *g* (Beckman Coulter Allegra X-15R) for 15 min at 4°C. Cells were washed once with sterile phosphate-buffered saline (PBS) (Lonza, Lot no. 7MB031), followed by centrifugation and resuspension in PBS prior to use.

2.3 | Sequencing of the 16S rRNA gene

The 16S rRNA gene of the bacteria used for challenge was amplified from a single colony from TSA supplemented with 2% NaCl and 10% FCS, and from another colony isolated on blood agar (BA) with 1.5% NaCl. The 16S rRNA gene was amplified using universal primers 27F: 5′-AGAGTTTGATCMTGGCTCAG-3′, 1525R: 5′-AAGGAGGTGWTCCARCC-3′ (Collins et al., 1991). The template reaction mixture had a final volume of 50 μl and this consisted of 5× HF buffer, 0.5 U of Phusion DNA polymerase (Thermo Scientific), 10 mM dNTP, 2.5 μl of 10 μM of each primer and 2 and 10 μl template from each of the two media types, respectively.

The cycle conditions were as follows: 1 cycle at 98°C for 3 min; 30 cycles- 98°C for 30 s, 58°C for 30 s and 72°C for 1 min; and a

final cycle at 72°C for 10 min. The PCR products were then visualized on a 1% agarose gel. The PCR products were purified using a GenElute™ PCR Clean-Up Kit (Sigma Aldrich) and sequencing was performed by the DNA sequencing facility at the High-Technology Centre, Bergen, Norway.

2.4 | Challenge models

For the cohabitation and bath challenge models, three bacterial doses were tested, and duplicate tanks were used for each of the three doses, as well as for the nonchallenged control. The groups, bacterial doses, and fish numbers for the cohabitation and bath challenge experiments are shown in Table 1. Water temperature was maintained at 12°C.

The average weight of the fish was 20.1 g, ±4.0 (mean ± *SD*) for the intraperitoneal and intramuscular injection challenge, 18.6 g, ±0.62, (number of fish weighed = 80) for the cohabitation challenge and 37.2 g, ±8.7, (number of fish weighed = 50) for the bath challenge. Harvested bacteria were washed twice with PBS and centrifuged at 2500 *g* for 15 min prior to use for challenge experiments.

TABLE 1 Challenge model details showing fish number and bacterial doses

Tank number	Challenge	Dose (bacteria/ml)	*n* fish
1	Cohabitation (IP)	1×10^7	25
1	Cohabitation (Co)	—	25
8	Cohabitation (IP)	1×10^7	25
8	Cohabitation (Co)	—	25
2	Cohabitation (IP)	1×10^6	25
2	Cohabitation (Co)	—	25
7	Cohabitation (IP)	1×10^6	25
7	Cohabitation (Co)	—	25
3	Cohabitation (IP)	5×10^5	25
3	Cohabitation (Co)	—	25
6	Cohabitation (IP)	5×10^5	25
6	Cohabitation (Co)	—	25
4	Cohabitation (IP-NC)	PBS	25
4	Cohabitation (Co-NC)	—	25
5	Cohabitation (IP-NC)	PBS	25
5	Cohabitation (Co-NC)	—	25
9	Bath	1×10^7	50
16	Bath	1×10^7	50
10	Bath	5×10^6	50
15	Bath	5×10^6	50
11	Bath	1×10^6	50
14	Bath	1×10^6	50
12	Bath (NC)	PBS	50
13	Bath (NC)	PBS	50

Note. Co: naïve cohabitant fish; IP: IP-injected shedders; NC: nonchallenged fish.

Confirmation of infection was performed by growth of bacteria isolated from head kidney samples on blood agar (with 2% NaCl) from all the dead fish from every challenge experiment. Presence of *Pasteurella* sp. was also confirmed by quantitative PCR (qPCR) analyses using head kidney samples from the challenged fish.

2.4.1 | Cohabitation challenge

For each tank under the same dose, the challenge was performed by IP injection of 25 fish with 50 µl washed bacterial suspension in PBS. These fish were then VIE tagged and housed with 25 naïve fish (cohabitants). The control fish were treated similarly, but IP injected with 50 µl sterile PBS, and VIE tagged.

2.4.2 | Bath challenge

The fish were transferred to the aerated static challenge tanks (25 L) containing the respective bacterial doses (Table 1) for 1 hr, after which they were returned to their experimental holding tanks. In the control tanks, the fish were treated similarly to the fish in the challenge tanks except that sterile PBS only was used instead of the bacterial suspension.

2.4.3 | Intraperitoneal and intramuscular challenge

Both isolates of *Pasteurella* sp. isolated from lumpsuckers (Alarcón isolate and P2 isolate) were used in this small-scale study to compare the effects of injection site and microbial culture medium on pathogenicity. For each isolate, fish were challenged by IM injection above the lateral line below the dorsal fin or by IP injection. Challenge material was either 50 µl of bacterial suspension in enriched TSB harvested at late exponential growth phase (see Figure 1) or 50 µl of bacterial suspension collected from blood agar plates and

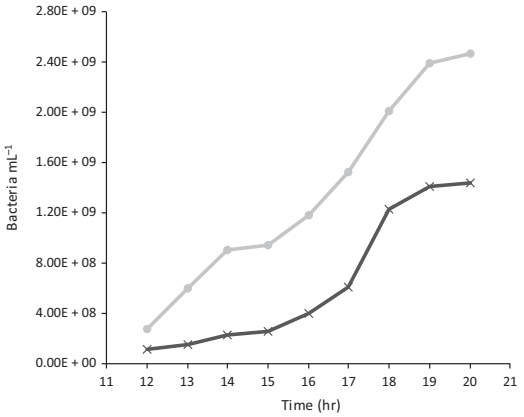

FIGURE 1 Growth curve for *Pasteurella* sp. in TSB (×) and BHIB (●) supplemented with 10% FCS for confirmation of growth medium composition used for bacterial culture for the challenge experiments

transferred to enriched TSB using 1 μl inoculation loops. Challenge by the two isolates (Alarcón isolate and P2 isolate) for comparison was performed in separate tanks. Separate tanks were also used for fish challenged by IP and IM injection. This resulted in four tanks each containing sixteen fish, eight injected with bacterial suspension harvested from enriched TSB and eight injected with suspension of bacteria harvested from agar plates.

2.5 | Histopathology

Samples for histopathology were taken from heart, kidney, spleen, liver, pancreas, intestine, musculature, skin, and gills of moribund fish only from the bath challenge and from cohabitants from the cohabitation challenge experiment. Tissues were fixed in 10% neutral buffered formalin (Sigma) and kept at 4°C until processing. Formalin-fixed tissues were processed, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin at the Norwegian Veterinary Institute, Bergen. Samples from a natural *Pasteurella* sp. outbreak provided by the Norwegian Veterinary Institute were used for comparison.

2.6 | Quantitative PCR

The protocol used for this study was validated using the method of Gulla, Duodu, Nilsen, Fossen, and Colquhoun (2016), with a few modifications as follows. A late exponential phase culture of *Pasteurella* sp. was used to prepare 10-fold serial dilutions in sterile PBS (1.5×10^{10} to 1.5×10^{5} bacteria/ml). For each dilution, 25 μl were added to 20 mg samples of head kidney tissue from noninfected lumpsuckers that were cut into very small pieces (concentration of bacteria in the head kidney samples was approximately 3×10^{8} bacteria/ml). A negative control sample was included where PBS was used instead of bacterial dilution. These were then incubated for 1 hr at room temperature to allow bacteria to be adsorbed to the head kidney cells in the case of the spiked samples, before proceeding with DNA extraction and qPCR.

Kidney samples from all the dead fish, survivors, and control fish from the cohabitants from the cohabitation challenge experiment and from the bath challenge experiment were stored in RNAlater® stabilizing solution at −20°C. DNA was extracted from all samples using the DNEasy Kit (Qiagen), according to the manufacturer's instructions for animal tissue samples. The levels of *Pasteurella* sp. were determined by qPCR using SYBR Green (Sigma). The primers used were RK-Past F: 5′-TTCACCATTCAAAGCACCATCAAG-3′ and RK-Past R1: 5′-CTTCTAAAGCAGCATTGGCATTAT-3′ targeting the superoxide dismutase (sodA) gene. Each qPCR reaction contained a volume of 25 μl and consisted of 12.5 μl 2× SYBR, 1 μl each of the forward and reverse primers (10 μM), 0.5 μl of RNAse and DNAse-free water, and 10 μl of genomic DNA (50 ng). A C1000 Touch thermal cycler (Bio-Rad) was used for qPCR, with the following cycle conditions: (a) 94°C for 5 min (b) 40 cycles of 94°C for 15 s, 60°C for 1 min and (c) an increase from 60 to 92°C at a rate of 1°C/5 s. Data was analysed using Bio-Rad CFX Manager 3.1 software (Bio-Rad). The sizes of the qPCR products were analysed by electrophoresis on 2% agarose gels containing GelRed (Biotium).

2.7 | SDS-PAGE

Bacteria were harvested in late exponential growth phase and centrifuged as described in Section 2.2. Other species of *Pasteurella/Photobacterium* were also included for comparison. Protein profiles from whole bacteria were obtained by SDS-PAGE (10% acrylamide) according to the method of Laemmli (1970) with minor modifications as follows. Electrophoresis was performed using a Mini Protean Tetra Cell (Bio-Rad). Antigens were heat-treated (96°C for 5 min) in sample buffer containing 5% β-mercaptoethanol. Samples (5 mg in 10 ml) were loaded onto each well, electrophoresed at 190 V for 45 min, followed by oxidation with a periodic acid solution (0.7% periodic acid (w/v), 2.7% ethanol, 0.3% acetic acid, 97% distilled water) and stained using Silver Stain Plus kit (Bio-Rad) based on the method of Gottlieb and Chavko (1987).

2.8 | Statistics

The qPCR data on presence of bacteria in samples from challenged fish were $\log_{10}$ transformed and analysed using the Kruskal–Wallis test (nonparametric test for comparison of the means of two or more independent samples). Differences were considered significant when $p < 0.05$. All statistics were carried out in GraphPad Prism V5.

3 | RESULTS

3.1 | Growth and characterization of bacteria

Growth of *Pasteurella* sp. to be used for challenge experiments was obtained in liquid medium when using both enriched TSB and BHIB, under a variety of FCS concentrations ranging from 2% to 10% (v/v). No growth was registered when FCS was excluded. The concentration of FCS required for optimal growth was 6%–10% for BHIB and 10% for TSB. The growth curves showed that an increase in FCS concentration was proportional to an increase in numbers of bacteria (Figure S1a,b). The maximum bacterial concentration obtained in TSB was approximately 1.6×10^{9} bacteria/ml and was reached after 20 hr, at 10% FCS. In contrast, a slower growth rate was observed when using BHIB and the stationary phase was not reached after 20 hr. The initial growth phase shows that the bacteria grow rather slowly for several hours. Cultures in TSB reached late exponential phase at approximately 18–19 hr postinoculation (Figure S1b). Growth curves were repeated with 10% FCS only, for confirmation of the final chosen medium composition for bacterial culture, with similar results as obtained previously (Figure 1).

We attempted metabolic characterization of *Pasteurella* sp. using API 20NE and API ZYM kits, but only very weak or no reaction could be observed up to 48 hr at 20°C. Using universal bacteria primers, the 16S rRNA gene sequence analysis confirmed that the bacteria used in this study were *Pasteurella* sp.

3.2 | Mortalities from the different challenge models

All challenge models induced mortalities caused by *Pasteurella* sp. The cohabitation challenge experiment, however, showed differences when compared with the other challenge experiments, with low mortalities when compared with the IM and IP injection challenges and the bath challenge.

In the IM and IP challenge small-scale study, the two isolates tested resulted in similar mortality profiles for IM and IP injection, both from the agar plate as well as from the broth culture methods. For that reason, only results from the Alarcón isolate cultured by broth method are presented.

Acute mortalities were observed in the IM and IP challenges, with 100% mortality being reached by day 5 post challenge for the IM challenge and 90% by day 7 post challenge for the IP challenge (Figure 2). The rather high bacterial dose used for both types of injection and the similar acute mortality profiles from IP and IM challenge indicated a very potent pathogenicity under these conditions.

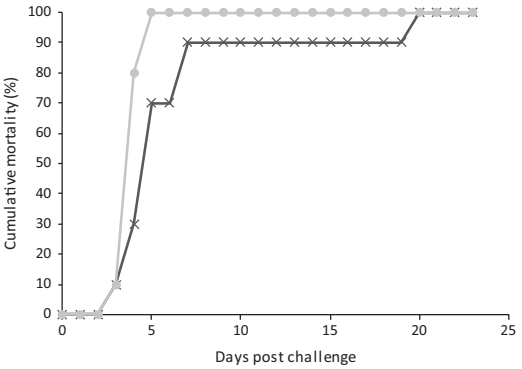

FIGURE 2 Mortalities resulting from challenge of lumpsuckers with *Pasteurella* sp. by intraperitoneal (×) and intramuscular (●) injection

To obtain a more gradual mortality profile, the cohabitation experiment was performed. The IP injection part of this experiment involved testing three doses of bacteria (1×10^7, 1×10^6, and 5×10^5 bacteria/ml). The IP-injected fish (shedders) in the cohabitation challenge experiment followed a similar pattern to the IM and IP challenged fish previously tested. The only difference was the onset of mortality which was delayed by a few days at lower doses of bacteria and all shedder fish were dead by day 12 post challenge. Thus lowering the dose did not induce a more gradual mortality in the group (Figure 3).

In contrast to the acute mortalities after IP and IM challenge, the first mortalities from the cohabitant fish were recorded on day 18 post challenge. The total mortalities in this case were 4% for the highest dose, 14% for the intermediate dose and 10% for the lowest dose tested (Figure 3).

When testing the bath challenge model a gradual mortality was observed. The onset of the mortalities was day 10 post challenge (Figure 4) and gradually increasing mortalities were observed until day 42 post challenge. Here, the high and intermediate doses had a similar profile with mortality at 96% and 99%, respectively, and the low dose also resulted in 91% mortality. This experiment therefore indicates that a clear dose response was not observed by these bath challenge exposures.

No mortalities were observed in any of the nonchallenged control groups in any of the experiments.

3.3 | Re-isolation of *Pasteurella* sp. from challenged fish

Pasteurella sp. was re-isolated from all fish sampled across the different challenges when grown on blood agar supplemented with 2% sodium chloride. Samples from all dead and surviving cohabitants were analysed, as well as from all dead and surviving fish from the

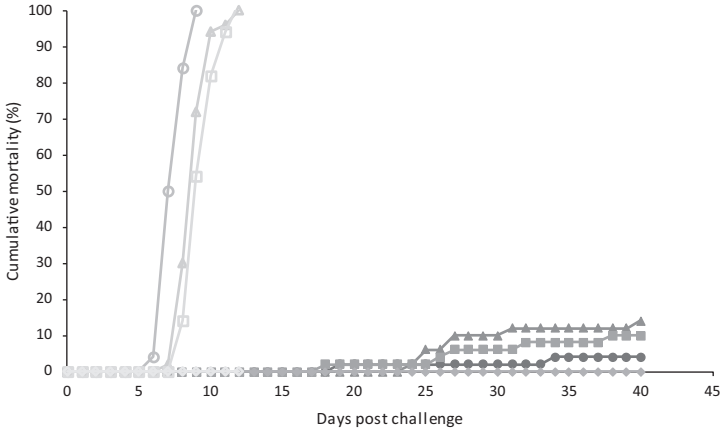

FIGURE 3 Mortalities resulting from challenge of lumpsuckers with *Pasteurella* sp. by cohabitation. Curves with empty symbols represent intraperitoneal (IP)-injected fish (shedders); curves with filled symbols represent cohabitant fish. Doses for IP injection: (○) 1×10^7 bacteria/ml, (△) 1×10^6 bacteria/ml, (□) 5×10^5 bacteria/ml, (◇) nonchallenged control

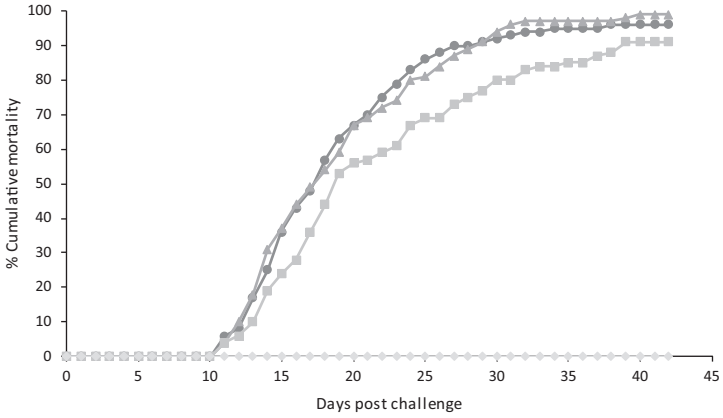

FIGURE 4 Mortalities resulting from challenge of lumpsuckers with *Pasteurella* sp. by bath. (●) 1×10^7 bacteria/ml, (▲) 5×10^6 bacteria/ml, (■) 1×10^6 bacteria/ml, (♦) nonchallenged control

bath challenge. *Pasteurella* sp. was also re-isolated from IP and IM challenged fish.

Quantitative PCR with *Pasteurella* sp. specific primers was also used investigate the concentration of *Pasteurella* sp. present in head kidney samples. This was done by comparing the values obtained from challenge experiment samples with the values from a spiked standard curve. Samples were collected from the cohabitants from the cohabitation challenge experiment and from the bath challenge experiment. Samples were analysed by qPCR to confirm presence of bacteria as cause of disease. The reason was to confirm the presence of bacteria in fish that were not IP infected with bacteria.

The spiked standard curve, obtained by adding known amounts of bacteria to noninfected head kidney samples, showed good correlation (r^2 = 0.9998) between Ct value and bacterial numbers. The limit of detection for *Pasteurella* sp. in kidney samples was found to be 3×10^3 bacteria/ml, which corresponds to a C_t value of 33.97 (Figure S2). Lower counts are not included as the resulting C_t values did not maintain linearity.

Pasteurella sp. was found in high concentrations in the dead fish, but also in low concentrations in the surviving fish (Figure 5). While there were significant differences in bacterial load between the dead cohabitants and surviving cohabitants, this was not the case in the bath challenge. There were few surviving fish in the bath challenge and there was high variation in amounts of bacteria. Comparisons between the survivor groups across the two challenges could not be made reliably as the low number of survivors resulted in no significant differences ($p > 0.05$). The bacterial load in dead fish across the two challenges was not significantly different, meaning that all dead fish were highly infected. The nonchallenged control fish samples were not positive for *Pasteurella* sp. by qPCR.

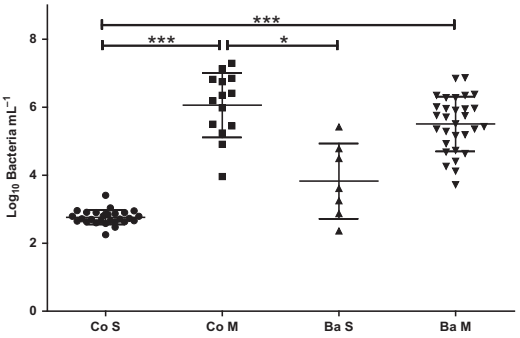

FIGURE 5 Comparisons of bacterial loading (bacteria/ml) between the cohabitation and bath challenge models based by qPCR analysis. (●) Cohabitation survivors, (■) Cohabitation mortalities, (▲) Bath survivors, (▼) Bath mortalities. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

3.4 | Pathology

Redness which indicates inflammation was observed around the site of injection with *Pasteurella* sp. in the IM-challenged fish (Figure 6e, f), whereas IP-challenged fish did not show any lesions at the injection site. Gross pathology in the cohabitant fish from the cohabitation challenge included cataracts, erosion of the lower jaw, and haemorrhage and redness which indicates inflammation at the base of the fins (Figure 6a,b). Symptoms in the bath challenged fish were different compared to those of the cohabitants from the cohabitation challenge. In the bath challenged fish, white spots were observed all over the skin (Figure 6c), especially around the eyes (Figure 6d) and darkening of the skin occurred in most fish. No internal symptoms other than ascites were observed, in any of the challenge experiments.

Histology was carried out on three representative moribund cohabitant fish and one healthy control fish from the cohabitation challenge (one from each dose). From the bath challenge, one moribund fish from the lowest challenge dose was used. Additionally, a dead fish with a severe reaction around the eye was sampled for histology. Since we did not include an additional tank for histological sampling, moribund fish were not encountered more frequently.

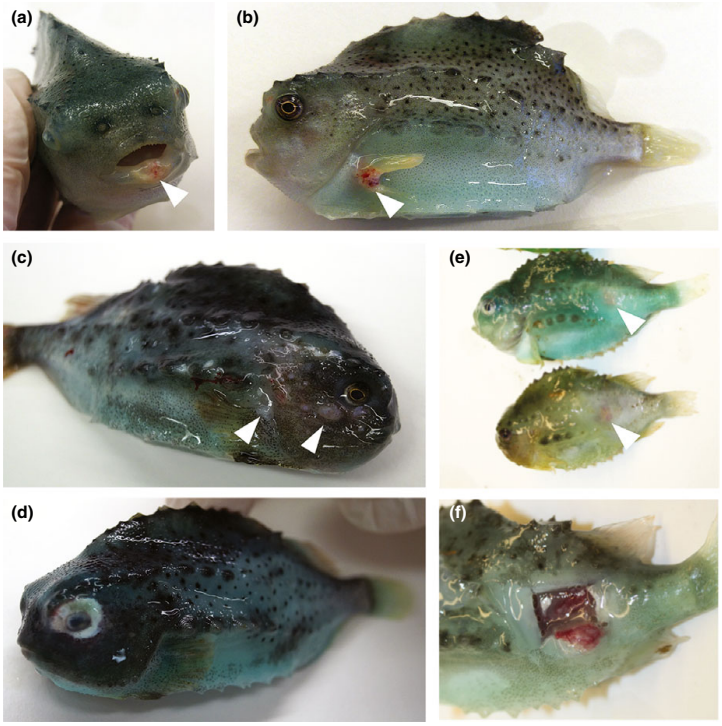

FIGURE 6 Gross pathology of *Pasteurella* sp. infection shown by samples from the cohabitation challenge (a and b), bath challenge (c and d), and IM challenge (e). Panel f shows the internal morphology of the localized reaction seen in panel e. Lesions are marked by arrows [Colour figure can be viewed at wileyonlinelibrary.com]

Histopathological examination showed variable amounts of short rod-shaped bacteria often in loosely shaped aggregates in heart, kidney, spleen, liver, peritoneum, and gills and eye. The tissue response was acute to subacute varying from just a few necrotic cells around bacteria, to large necrotic areas, haemorrhage, eosinophilic exudate, and leukocyte infiltration. There were no differences observed between cohabitant fish from the cohabitation challenge, and fish from the bath challenge.

In the lumen of the ventricle and atrium of the heart there were multiple aggregates of bacteria (Figure 7a). In the kidney the bacteria occurred in sparse amounts in the sinusoids and interstitium (Figure 7b). In the spleen there were abundant amounts of bacteria throughout the tissue (Figure 7c). Both in the spleen and kidney there were scattered necrotic cells mostly around bacteria and increased amounts of circulating leukocytes. In the pancreas some of the exocrine pancreas cells were degenerated. In the perivisceral peritoneum there were multifocal aggregates of bacteria and infiltration of leukocytes. In the gills there were sparse amounts of bacteria in the lamellar capillaries.

The eye and surrounding tissue from a dead bath challenged fish was sampled due to the severity of the lesion around the eye. Histopathological examination of the skin, connective tissue and fatty tissue around the eye showed that the fish suffered from keratitis. There were also large areas with necrotic cells, leucocytes, eosinophilic exudate, haemorrhage, and abundant amounts of bacteria (Figure 7d). The sclera was multifocally rich in leucocytes and some bacteria. The histopathology described above is also observed in histopathological examination of fish tissue samples from natural outbreaks of *Pasteurella* sp. in lumpsuckers (Figure 8a–c).

3.5 | SDS-PAGE protein profiles of *Pasteurella* sp. and *Photobacterium*/*Pasteurella* species

The protein profile of *Pasturella* sp. isolate from lumpsucker was compared to other fish pathogenic bacteria, known to cause pasteurellosis. The *Pasteurella* sp. isolate from lumpsuckers is different from the other *Pasteurella* and *Photobacterium* species (Figure 9). The protein band between 21.5 and 45 kDa for the lumpsucker isolate is not present in the other species, and the majority of bands common to *P. damselae* subsp. *piscicida* are not visible in the lumpsucker isolate. However, a weak band at approximately 21.5 kDa is visible for the lumpsucker isolate which is also present for *P. damselae* subsp. *piscicida*.

4 | DISCUSSION

Pasteurella sp. isolated from Norwegian lumpsuckers has been very fastidious to grow, and to date has not been cultured in liquid media.

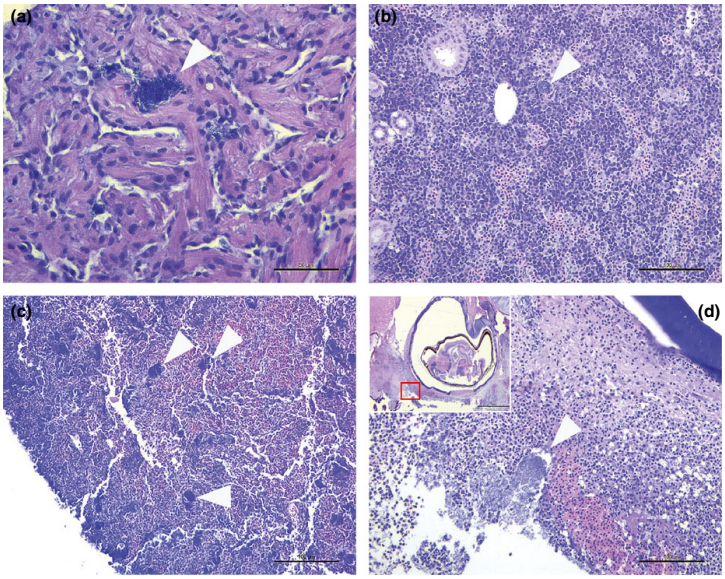

FIGURE 7 Distribution of *Pasteurella* sp. in heart (a), kidney (b), spleen (c), and in the tissue surrounding the eye (d) from challenged fish. Bacteria are seen in loosely shaped aggregates (arrows). Scale bar: a: 50 μm, b–d: 100 μm. a–d (HE). a: 40×, b: 20×, c: 10×, d: overview 2.5× and 20× [Colour figure can be viewed at wileyonlinelibrary.com]

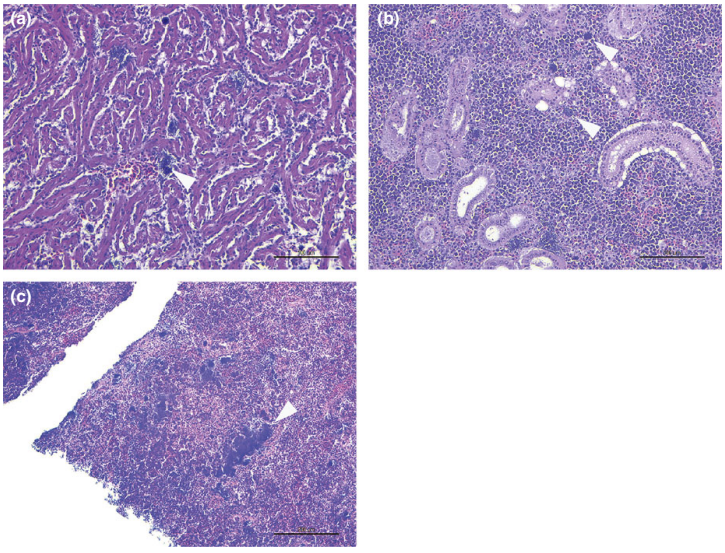

FIGURE 8 Distribution of *Pasteurella* sp. in heart (a), kidney (b), and spleen (c) from fish from a natural outbreak of *Pasteurella* sp. Bacteria are seen in loosely shaped aggregates (arrows). Scale bar: a,b: 100 μm, c: 200 μm. a–c (HE). a and b: 20×, c: 10× [Colour figure can be viewed at wileyonlinelibrary.com]

Until now, this bacteria has only been grown on blood agar, and it has been found to have strict requirements for blood and sodium chloride (Alarcón et al., 2016). This has resulted in only a few studies being carried out on pasteurellosis in lumpsuckers and also delayed vaccine development. In this study, growth of *Pasteurella* sp. in liquid media was established, with relatively high numbers of bacteria being harvested. Based on growth curves, bacteria were harvested in the late exponential growth phase, ensuring standardized bacterial samples for testing of different challenge models.

Foetal calf serum added to the media provided the necessary growth factors required by the bacteria to grow well, with the most optimal growth rates obtained at a FCS content of 10% in TSB. Slightly higher numbers of bacteria were obtained in BHIB, but this medium is not appropriate for growth of bacteria

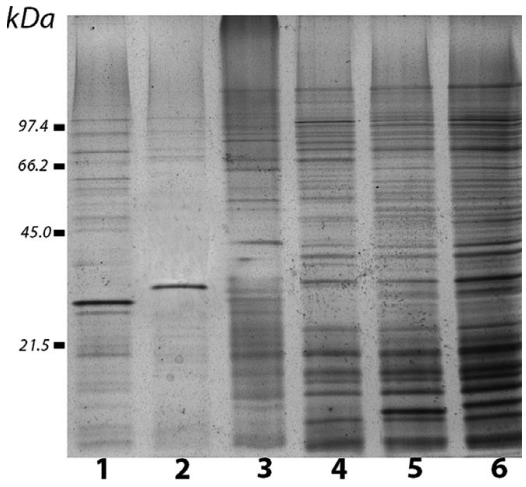

FIGURE 9 Silver stained SDS-PAGE showing protein profiles of various *Pasteurella* and *Photobacterium* species. 1: *Pasteurella* sp. isolated from lumpsuckers; 2: *Pasteurella skyensis* (Scottish Atlantic salmon); 3–6: *Photobacterium damselae* subsp. *piscicida*, 3: fnr 2289/NCIMB, 4: from Cobia, 5: from European sea bass, 6: from Senegalese sole

intended for vaccine production. Therefore, bacteria grown in TSB were used since the challenge models are intended to be used for vaccine testing.

The commonly used API characterization methods did not give satisfactory results for this isolate, under the test conditions used, indicating that more adequate tests are required. The biochemical profile of *Pasteurella* sp. from lumpsuckers has previously been described and found to be similar but weaker to that of a *Pasteurella* strain known to cause ophthalmitis in Atlantic salmon (Alarcón et al., 2016). This may be due to the growth requirement of the bacteria which might not be optimal in these tests and therefore low growth will influence the results.

Pasteurella sp. isolated from lumpsuckers is still uncharacterized on the functional level with regards to virulence components and antigenic properties. The protein profiles of whole bacteria compared to other relevant bacterial isolates known to cause pasteurellosis showed that this lumpsucker isolate is not similar to *P. skyensis* or *P. damselae* subsp. *piscicida*. These two species are the commonly encountered agents of pasteurellosis in aquaculture. *Pasteurella skyensis* has been isolated from Atlantic salmon in Scotland (Birkbeck, Laidler, Grant, & Cox, 2002) and *P. damselae* subsp. *piscicida* has been extensively studied and has caused severe infectious problems in fish worldwide, such as in Japan including flounder (Fukuda, Matsuoka, Mizuno, & Narita, 1996) and amberjack (Kawakami, Yamashita, & Sakai, 2007) and in Europe particularly in Mediterranean species like gilthead seabream (Moriñigo et al., 2002).

The observed differences in protein profiles might indicate that antigenic differences can also exist. Therefore, protein profiles, when combined with immunoblotting, will provide important information on immunoreactive antigens in tests using lumpsucker sera. Lumpsucker sera should be used in analyses to identify the proteins involved in lumpsucker immune responses. Anti-sera from other animals provides information on differences obtained by the actual serum but does not necessarily identify the immunogenic components to lumpsuckers.

Since 2013, there has been a significant increase in the number of *Pasteurella* sp. outbreaks in lumpsuckers from sites across Norway. These have been recorded in both juvenile and sea transferred fish, with mortalities often reaching 100%. According to Johansen (2013), clinical infection can be triggered from subclinical infection by stress-inducing events such as vaccination, transfers, and transport. Some success has been reported when using florfenicol for treatment of the disease, however, recurrence of infection has been reported from many sites. Furthermore, since pasteurellosis in lumpsuckers is not a notifiable disease in Norway, it is possible that the number of cases is under-reported. Lumpsucker vaccines are currently commercially available against atypical *A. salmonicida* and *V. anguillarum*. Development of vaccines for other pathogens is underway, requiring further optimization (Gulla & Bornø, 2018).

The results from the presented challenge experiments in this study have shown that Koch's postulates have been fulfilled with respect to *Pasteurella* sp. causing disease in lumpsuckers under experimental conditions and with respect to pure cultures of bacteria being reisolated from diseased fish.

The IM and IP challenges underlined the high virulence of the bacteria, with acute and high mortalities obtained very early into the challenge period. This suggests the presence of extracellular proteins as virulence factors implicated in disease progression. As the disease onset was too rapid, the fish died before any characteristic symptoms like the typical white skin spots, appeared. A severe local reaction was obtained in the musculature at the injection site when IM injection was used. Due to the severe tissue damage, this challenge model is not recommended for use. The specific reasons for these severe tissue reactions is not known, but bacterial virulence factors, including toxins, might be involved in tissue damage and the resulting damage can also influence the host immune response.

The cohabitation and the bath challenge models allowed the more chronic symptoms of the disease to be studied more accurately. In the cohabitation model experiment, the IP-injected fish followed a similar mortality pattern to that observed in the IM and IP challenged fish, with acute mortalities and no external lesions being recorded. Results from different IP-injected doses showed that decreasing the dose of bacteria only delays the onset of disease, but not the total mortality. This acute mortality of the shedders thus led to a reduced level of shedding, and this was evident from the very low mortalities in the cohabitant group. The highest bacterial dose tested resulted in the lowest cohabitant mortality, indicating that not enough shedding had occurred to result in infection of the cohabitants. The middle dose resulted in slightly higher cohabitant mortalities as the longer period of shedding from the shedders allowed the cohabitants to be exposed to the bacteria for a longer period of

time. Despite this, lesions typical of *Pasteurella* sp. infections were still observed in these dead fish, and included haemorrhage at the base of fins and jaw. Interestingly, no white skin spots or internal symptoms were observed here, possibly due to the low bacterial exposure and subsequent low bacterial concentration in the fish.

Concerning the acute mortalities obtained from the shedders in the cohabitation challenge, one might have tested lower injection doses to determine if this would have provided a more gradual mortality. However, taking into consideration the low mortality in cohabitant fish, this indicates that evaluating the dose for IP infectivity seems to be complicated due to the progression of disease. In a former study, Alarcón et al. (2016) reported an outbreak of pasteurellosis in lumpfish occurring 2 weeks post transfer to holding facilities and this indicated that under "natural infection" the disease progress might be slower, and that stress can induce onset of disease if bacteria already are present in the fish. In artificial media bacterial growth is slow, but this might not be the case in infected fish. Furthermore, it is not known whether toxic extracellular products or other virulence factors are involved in disease development as is the case for *P. damselae* subsp. *piscicida* (Magariños et al., 1992, 1996; Romalde, 2002).

The bath challenge was found to be the most adequate challenge model, as mortalities in this case were more gradual than the cohabitation challenge. The onset of mortality was the same for all the doses, but a more prolonged and lower total mortality was seen with the lowest dose. The potency of *Pasteurella* sp. was again demonstrated as all the doses tested exceeded 90% mortality. The results from the bath challenge indicate that this model can be used for testing of vaccines against pasteurellosis, based on the evaluation of the mortality profile and the experimental period. However, the doses to be tested may need to be refined when carrying out such trials.

The qPCR analysis indicated that *Pasteurella* sp. was present in the survivor fish sampled at termination of the cohabitation and bath challenge experiments. These fish were sampled at day 40 post challenge and day 42 post challenge, respectively and at that point they did not show any external or internal symptoms of disease. This implies that there may be an asymptomatic carrier status, and re-emergence of the disease cannot be excluded if these fish were to have been stressed, particularly the surviving cohabitants. The low and closely grouped qPCR values of bacteria for the cohabitation challenge survivors compared to those from the dead fish seem to corroborate this. The qPCR values from the bath challenge survivors and corresponding dead fish on the other hand, did not show a similar pattern, as the values were overall higher and showed more variation. This might indicate that given a longer period of time, the bath survivors might have succumbed to disease.

Additionally, the presence of bacteria in low numbers in surviving cohabitants from the cohabitation challenge several days after mortality ceased indicates that lumpsuckers can be asymptomatic carriers and can possibly harbour *Pasteurella* sp. intracellularly, but this needs to be verified by analysing bacterial virulence factors. Histopathology findings show that the disease may cause a systemic infection, with bacteria in loosely shaped aggregates. The white spot around the eye of the examined fish is likely due to a subacute bacterial inflammation in the tissue surrounding the eye.

The histopathological findings from this study show similarities to findings obtained from field cases and to findings reported by Alarcón et al. (2016), with respect to redness at the base of fins and the mouth as well as the white spots around the body. However, granulomas were not encountered in this study. Typically, granulomas would require a more latent and chronic form of the disease, as well as the stressors encountered by the fish in the sea cages to develop. Such conditions were not encountered in the facility where the study was being carried out, which explains the lack of granulomas. Furthermore, the fish studied by Alarcón et al. (2016) were obtained from a production facility and likely harboured the bacteria at subclinical levels, allowing granulomas to develop.

It is worth noting that the bacteria that seem to be particularly potent in causing immediate and severe mortalities in short periods when injected, can also cause low mortality and consequent harbouring of bacteria in cohabitants. This might indicate that a sufficient number of bacteria must be present before mortality occurs or that at low levels the bacteria might be under some control by lumpsucker immune activities. It has been previously shown that both phagocytosis and respiratory burst are highly active in lumpsuckers and immunocytochemical analyses of enzymes and oxygen independent mechanisms support the importance of innate immune functions (Haugland et al., 2012). Such mechanisms may help to control bacterial infection in lumpsuckers.

The possible carrier state of *Pasteurella* sp. has an important implication for the lumpsucker farming industry, since the point of infection or its ability to survive in a more dormant stage in fish is not clear and cases of pasteurellosis have been recorded at different life stages. The "carrier state" has been encountered previously in a natural outbreak of pasteurellosis in lumpsuckers (Alarcón et al., 2016). In that study, it was shown that the fish were asymptomatic carriers and that the disease re-emerged following a stress-inducing event in the form of routine handling of the fish. The carrier state and re-emergence has also been encountered in other fish species and diseases such as *A. salmonicida* in Atlantic salmon (Hiney, Kilmartin, & Smith, 1994), *Yersinia ruckeri* in rainbow trout (Busch & Lingg, 1975), *Edwardsiella ictaluri* in channel catfish (Klesius, 1992) *Francisella piscicida* in wild and farmed Atlantic cod (Ottem, Nylund, Isakse, Karlsbakk, & Bergh, 2008) and *P. damselae* subsp. *piscicida* in gilthead seabream (Magariños et al., 2001). For the above reasons, screening of lumpsuckers before transfer into hatcheries, ongrowing facilities, and onto sea cages is of utmost importance, in order to minimize the transfer of asymptomatic carriers. Hopefully vaccines against pasteurellosis caused by *Pasteurella* sp. can be developed, but the mode of action by the bacteria on lumpsuckers seen from these experiments, and a possible intracellular existence, might make this a challenging task.

In summary, growth of *Pasteurella* sp. isolated from Norwegian lumpsuckers was established in TSB enriched with sodium chloride and FCS resulting in high numbers of bacteria harvested. These bacteria were then used to test a number of challenge models, where

the bath challenge model was found to be the most adequate to investigate the disease progression in lumpsuckers. qPCR analysis indicated that an asymptomatic carrier status may be possible, and this highlights the importance of fish health screening both of broodstock and fry before vaccination and transfer to sea. Further work on the possibility of an intracellular mode of action of *Pasteurella* sp. and its implications on lumpsucker immune cells and functions should be performed. The presented results will contribute to progress in vaccine production to provide lumpsuckers with enhanced protection against pasteurellosis.

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CONFLICT OF INTEREST

The authors state that there are no conflicts of interests to declare regarding the presented work.

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Full length article

Protection and antibody reactivity in lumpsucker (*Cyclopterus lumpus* L.) following vaccination against *Pasteurella* sp.

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ABSTRACT

Two monovalent vaccines against pasteurellosis were developed and tested for efficacy using a previously established bath challenge model. High levels of specific antibodies were detected following vaccination. While the vaccine efficacy trial indicated that some level of protection was obtained, high mortality was still observed. qPCR analysis of head kidney tissues from surviving fish post challenge showed no difference in bacterial numbers in vaccinated and non-vaccinated fish. Clinical symptoms observed in moribund and diseased fish included white spots on the skin and around the eyes, frayed fins and redness around the mouth and fin bases. Despite production of specific antibodies, the protection against experimental challenge was relatively weak. A reason for this could potentially be that the specific antibodies produced are not alone enough to provide complete protection against pasteurellosis in lumpsuckers. Confocal and scanning electron microscopy of head kidney leucocytes exposed to *Pasteurella* sp. *in vitro* gave indications of the interactions between the pathogen and leucocytes. The results indicate that parts of the immune system other than humoral antibodies could be important for protection against pasteurellosis. Our combined results highlight the need for further work on host-pathogen interaction between *Pasteurella* sp. and lumpsuckers.

1. Introduction

Lumpsucker (*Cyclopterus lumpus* L.) is a marine species of cleaner fish used for biological control of sea lice infecting farmed Atlantic salmon. In Norway, wild populations as well as farming operations can be found all along the entire coastline, from Troms county in the north, to Vest-Agder county in the south. Due to their ease of culture and relatively high delousing activity at lower water temperatures, lumpsuckers are ideally suited and preferred over various species of wrasse for use as cleaner fish. For this reason, the demand for farmed lumpsucker has been steadily increasing, with approximately 40 million fish used in Atlantic salmon production in Norway in 2018 [1].

As a relatively new species to aquaculture, several bacterial diseases have been documented for farmed lumpsuckers. The most commonly encountered bacterial agents from outbreaks are *Vibrio* species (including *V. anguillarum* and *V. ordalii*), *Tenacibaculum* spp., *Moritella viscosa*, *Pseudomonas anguilliseptica*, atypical *Aeromonas salmonicida* and an as yet unnamed *Pasteurella*-like species [2–4].

Outbreaks of pasteurellosis have been steadily increasing since the first case was recorded in 2012, with 28 sites reporting outbreaks in 2016 [5]. Despite the decrease by half in reported cases to 14 in 2018 [1], *Pasteurella* sp. remains a problematic pathogen for fish farmers, and under-reporting of outbreaks cannot be excluded. The bacteria affect all life stages of lumpsucker, and to date there is no adequate treatment for this disease. The *Pasteurella* sp. pathogenic for lumpsucker is closely related to, albeit serologically distinct from, *Pasteurella skyensis* [6], when analysed using rabbit antisera [3]. It is also phenotypically similar, but genetically distinct from a group of *Pasteurella* isolates pathogenic for farmed Atlantic salmon in Norway, which causes "varracalbmi", a systemic infection characterised by severe ophthalmitis [7].

Prophylactic measures including vaccines have been commercially developed for several lumpsucker diseases. Currently, the majority of farmed lumpsucker are vaccinated against *V. anguillarum* and atypical *A. salmonicida*. As *Pasteurella* sp. is a relatively recently discovered

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pathogen, and is difficult to culture in liquid media, vaccine development against pasteurellosis remains at an early stage. A *Pasteurella* component is, therefore, not currently included in commercial lumpsucker vaccines.

Despite their widespread use, knowledge of the lumpsucker immune system remains limited [8]. Haugland et al. (2012) first characterised the components and functionality of the lumpsucker innate immune system by isolating leucocytes from peripheral blood, spleen and head kidney, and determined the phagocytic capacity of lumpsucker leucocytes to be very high. Rønneseth et al. (2015) [9] then characterised B cells from lumpsuckers and found high phagocytic ability among IgM^+ B cells isolated from blood. Further, it was found that immunisation resulted in the production of specific antibodies and vaccine-induced protective immunity against experimental challenge with atypical furunculosis. In previous work [10], we confirmed that *Pasteurella* sp. causes significant mortalities in lumpsucker, and determined that a bath challenge model is the most adequate to study progression of the disease.

In the current study, we investigated the adaptive immune response of lumpsucker following immunisation against, and subsequent exposure to *Pasteurella* sp.. We also studied the effects of *in vitro* exposure of isolated lumpsucker head kidney leucocytes to *Pasteurella* sp. through immunofluorescence and confocal microscopy, as well as scanning electron microscopy. This was done to shed light on the mechanisms of infection of the pathogen, and in turn clarify how the lumpsucker immune system needs to be stimulated to prevent infection and disease.

2. Materials and methods

2.1. Bacterial isolates and culture

The *Pasteurella* sp. isolate described in previous studies [3,10] was identified from a natural *Pasteurella* sp. outbreak in lumpsucker, and was used in this work for vaccine preparation, challenge and *in vitro* exposure of leucocytes. Briefly, bacteria were grown in tryptic soy broth (TSB) (Becton Dickinson supplemented with 1.5% NaCl and 10% foetal bovine serum (Gibco, Lot no. 1739464) at 20 °C with shaking (200 rpm). For challenge, the cultures were harvested in the late exponential growth phase and centrifuged at 2500 *g* (Beckman Coulter Allegra X-15R) for 15 min at 4 °C. Cells were washed once with sterile phosphate buffered saline (PBS) (Lonza, Lot no. 8MB014), followed by centrifugation and resuspension in PBS prior to use.

2.2. Vaccine preparation and vaccination

The vaccines were formulated as water-in-oil emulsions by PHARMAQ AS, Norway. Two different monovalent vaccine preparations were tested, both based on formalin killed *Pasteurella* sp. emulsified in adjuvant. Prior to inactivation, bacterial cell numbers were measured using a cell counter (CASY Model TT (Innovatis) and CASY worX V1.26) to be approximately 2×10^9 bacteria mL^{-1} and 150 mL culture volumes were used to prepare the vaccines. The antigen concentration in one of the vaccines was concentrated by centrifuging a formalin-killed culture of bacteria and removing 90% of the supernatant. The other vaccine was not concentrated. In addition, control groups were vaccinated using a monovalent *V. anguillarum* O1 vaccine (control vaccine) and phosphate buffered saline (PBS), respectively.

Vaccination was performed by intraperitoneal injection of vaccine (50 μL) using Socorex self-refilling syringes. The antigen dose per fish was $> 2 \times 10^7$ and $> 2 \times 10^8$ cells $fish^{-1}$ for the non-concentrated and concentrated vaccine respectively. Vaccination was carried out six weeks before bacterial challenge. At the time of vaccination, the fish had an average length and weight of 5.1 ± 0.3 cm (n = 50) and 9.8 ± 1.8 g (n = 50) respectively. Vaccine groups were identified by Visual Implant Elastomer (VIE tags, Northwest Marine Technology Inc.) placed subcutaneously on the forehead.

2.3. Fish and rearing conditions

Farmed, unvaccinated lumpsuckers were obtained from Vest Aqua Base AS, Norway. The experimental population had a disease free history, displayed no signs of infection or mortality and were screened and certified free from atypical *A. salmonicida*, *Pasteurella* sp., *Vibrio anguillarum* and lumpfish flavivirus (PHARMAQ Analytic). The fish were acclimated for 15 days at 12 °C in 500 L tanks at a photoperiod of 12 L: 12D, a salinity of 34 ppt and oxygen saturation of > 77% in the outlet water, at the holding facilities in the Industrial and Aquatic Laboratory (ILAB) at the Bergen High Technology Centre. The fish were fed Amber Neptun (Skretting) dry feed by automatic feeders and fasted for 36 h prior to vaccination and challenge. The fish were then transferred to the challenge unit 3 days prior to the challenge and split into 150 L tanks, with 100 fish per tank. The same water parameters were used as for the holding tanks.

2.4. Lumpsucker immune sera

Blood samples were collected post-vaccination from individual fish vaccinated with the test vaccines every 100° days for a period of 600° days and stored overnight at 4 °C. Sera were then obtained by centrifugation at 1300*g* and stored at −20 °C.

2.5. Quantification of lumpsucker-specific antibodies to Pasteurella sp.

Analysis for specific antibody production was carried out by enzyme-linked immunosorbent assay (ELISA). Freeze-dried *Pasteurella* sp. was used as the antigen to coat 96-well plates (Nunc). The antigen was dissolved in 0.01 M PBS, pH 7,3 and sonicated for 2 min at 20 kHz and diluted to a concentration of 10 μg mL^{-1} before being used. Lumpfish sera were diluted (1:50) in PBS containing 0.05% Tween 20. Specific antibodies were detected using rabbit anti-lumpfish IgM serum (1:1500) [4] and peroxidase conjugated goat anti-rabbit immunoglobulin (Dako) (1:2000).

The optical density (OD) was read at 492 nm in a Sunrise microplate reader (Tecan Group Ltd.) using Magellan software. Each dilution was carried out in parallels of two. The blank control employed was wells containing PBS instead of lumpfish serum. For an internal positive control, serum from lumpfish immunised with *Pasteurella* sp. antigens used by Ref. [9] was included.

Agglutination tests were performed using sera from lumpsuckers vaccinated against *Pasteurella* sp. in this work. The tests were carried out by inoculating live *Pasteurella* sp. onto glass slides, followed by the addition of the sera and observing for flocculation. Serum from lumpsucker triple immunised against *Pasteurella* sp [9]. were used as a positive control, while pre-vaccination serum from this study was used as a negative control.

2.6. SDS-PAGE silver staining and western blots of Pasteurella sp.

Whole protein profiles were analysed by SDS-PAGE (12% acrylamide) according to established methods [11] with minor modifications as described previously [12]. Electrophoresis was performed using a Mini Protean Tetra Cell (Bio-Rad). *Pasteurella* sp. prepared as described in Section 2.5 was heat-treated (96 °C for 5 min) in sample-buffer containing β-mercaptoethanol. Samples of 10 μL were loaded onto each well, electrophoresed at 190 V for 45 min, followed by staining of proteins using Silver Stain Plus kit (Bio-Rad) [13].

Western blotting was performed using whole bacteria after fractionation on a 12% SDS-polyacrylamide gel as described above and electro-blotted onto 0.45 mm nitrocellulose membranes (Bio-Rad) as described in Ref. [14] with some minor modifications. Briefly, analysis was performed using serum collected from the vaccinated lumpsucker diluted 1:100, and rabbit anti-*Pasteurella* diluted 1:10,000. Sera from non-immunised fish were used as negative controls while sera from

Table 1
Challenge details showing tank group, total number of fish used and challenge doses. There were two tank parallels for each vaccine group (25 fish per vaccine group per tank).

Tank	Dose (bacteria mL^{-1})	Vaccine group	n fish
1	1×10^5	PBS control	50
		Control vaccine	50
		Non-concentrated vaccine	50
		Concentrated vaccine	50
2	5×10^5	PBS control	50
		Control vaccine	50
		Non-concentrated vaccine	50
		Concentrated vaccine	50
3	1×10^6	PBS control	50
		Control vaccine	50
		Non-concentrated vaccine	50
		Concentrated vaccine	50
4	1×10^7	PBS control	50
		Control vaccine	50
		Non-concentrated vaccine	50
		Concentrated vaccine	50

three times immunised lumpsuckers were also used for comparison [9]. For the production of rabbit and lumpsucker anti-*Pasteurella* immune sera, Freund's Complete Adjuvant and Freund's Incomplete Adjuvant were used respectively. Anti-lumpfish IgM serum was diluted 1:1000, polyclonal goat anti-rabbit immunoglobulins/HRP (Dako) diluted 1:2000. These were developed with HRP Conjugate substrate kit (Bio-Rad) or Clarity Western ECL substrate (Bio-Rad) and visualized in Bio-Rad molecular image chemi Doc XRS þ Imaging system. The molecular weight of the SDS-PAGE fractions and immune reactive bands were identified using SDS-PAGE low range standard (Bio- Rad) and Kaleidoscope pre-stained standard (Bio-Rad), respectively.

2.7. Challenge of vaccinated fish

Six weeks (500° days) post-vaccination fish were bath challenged as described in previous work [10]. Each of the four bacterial doses used for challenge were tested in duplicate, resulting in eight tanks used. Each tank contained 25 fish for each of the two test vaccine groups and control groups, giving 100 fish in each tank (Table 1). Briefly, fish were transferred to aerated static challenge tanks (25 L) containing the respective bacterial doses for 1 h, after which they were returned to clean holding tanks. The fish were then checked twice daily and dead and moribund fish removed accordingly.

Confirmation of infection was performed by re-isolation of bacteria from head kidney samples on blood agar (with 2% NaCl) from all the dead and moribund fish. Kidney samples from the dead fish were also stored in RNAlater stabilising solution at −20 °C to confirm presence of *Pasteurella* sp. by qPCR analysis.

2.8. Quantitative PCR

The qPCR analysis was performed as described previously [10]. DNA was extracted from head kidney samples using the DNEasy Kit (Qiagen), according to the manufacturer's instructions for animal tissue samples. The amount of *Pasteurella* sp. for each sample was determined by qPCR using SYBR Green (Sigma). The primers used were RK-Past F: 5′-TTCACCATTCAAAGCACCATCAAG-3′ and RK-Past R1: 5′-CTTCTAA AGCAGCATTGGCATTAT-3′ targeting the superoxide dismutase (sodA) gene. Each qPCR reaction contained a volume of 25 μL and consisted of 12.5 μL 2X SYBR, 1 μL each of the forward and reverse primers (10 μM), 0.5 μL of RNAse and DNAse-free water, and 10 μL of genomic DNA (50 ng). A C1000 Touch thermal cycler (Bio-Rad) was used for qPCR, with the following cycle conditions: (1) 94 °C for 5 min (2) 40 cycles of 94 °C for 15 s, 60 °C for 1 min and (3) an increase from 60 °C to 92 °C at a rate of 1 °C/5 s. Data was analysed using Bio-Rad CFX Manager 3.1 software (Bio-Rad).

2.9. Isolation and exposure of head kidney leucocytes to Pasteurella sp.

Six lumpsucker were quickly netted and killed by a sharp blow to the head. Leucocytes were isolated from the head kidney on discontinuous Percoll gradients as described previously [8] with the following modifications. The supplemented L-15 medium did not contain gentamicin sulphate, since the cells were to be exposed to viable *Pasteurella* sp. Additionally, resuspension of the isolated leucocytes was done in L-15 supplemented with 5% foetal calf serum (L-15/FCS). The leucocytes were counted in a CASY-TT Cell Counter TM (Innovatis AG) and viability and aggregation factor determined.

The concentration of the isolated leucocytes was then adjusted to 1×10^7 cells mL^{-1} in L-15/FCS and 500 μL volumes were added to each well of 4-chambered chamber slides (5×10^6 cells per well) (Thermo Scientific Nunc) and incubated overnight at 15 °C. The next morning, non-adherent cells were removed by removing the growth medium.

An overnight culture of *Pasteurella* sp. was centrifuged and re-suspended in L-15/FCS and adjusted to 2×10^5 bacterial cells mL^{-1}. 500 μL volumes were then added to each well (1×10^5 bacteria per well). Sterile L-15/FCS medium was used for the non-challenged controls. The cells were then incubated at 15 °C on a shaking incubator set at 60 rpm for 6 h before washing and supplementing with sterile L-15/FCS medium.

2.10. Immunofluorescence staining of bacterial exposed leucocytes

Leucocytes were sampled at 6 and 24 h post bacterial exposure. The L-15/FCS medium including suspended bacteria was discarded and the cells fixed in 3.7% freshly prepared paraformaldehyde for 10 min. Permeabilisation was carried out using 0.1% Triton-X 100 in PBS (v/v) for 5 min.

The wells were then blocked using 2% BSA in PBS (w/v) for 1 h, then incubated for 1 h in a humidity chamber in the dark with primary antibodies (rabbit anti-*Pasteurella*) diluted 1:10,000 in PBS containing 0.5% (w/v) BSA. This was followed by incubation in darkness with Alexa Fluor™ 555 F(ab')2 fragments of goat anti-rabbit IgG (H + L) (2 mg mL^{-1}) (Invitrogen) diluted 1:400 in PBS containing 0.5% (w/v) BSA for 1 h.

The cells were then incubated in darkness with Alexa Fluor™ 488 phalloidin (Invitrogen) diluted 1:40 in PBS containing 1% (w/v) BSA for 20 min and stained with Hoechst 33342 diluted 1:2,000 in PBS for 10 min before mounting with Vectashield® antifade mounting medium (Vector). Wells were washed three times using PBS between each incubation, and further rinsed with distilled water prior to mounting.

Samples were imaged using a Leica TCS SP8 STED 3X confocal microscope at the Molecular Imaging Centre (MIC), Bergen.

2.11. Scanning electron microscopy

Leucocytes were prepared and infected as described in Section 2.7 and sampled at 6 and 24 h post infection. The medium was then removed, and cells washed three times using PBS. The cells were then fixed in 2% glutaraldehyde diluted in 0.1 M sodium cacodylate for 2 h at 4 °C. These were then washed three times in 0.1 M sodium cacodylate for 15 min. Post-fixation was then done in 1% osmium tetroxide diluted in sodium cacodylate for 1 h. Cells were then washed twice in buffer and dehydrated in an alcohol series as follows. 35%, 50% 70% 96% alcohol each for 20 min, respectively, followed by 100% ethanol, three times for 20 min each.

Samples were then submitted to critical point drying (Quorum K850), mounted on aluminium stubs and sputter coated (Jeol JFC-2300HR) with 10 nm Au/Pd and imaged with a Jeol JSM-7400F (Tokyo, Japan) using 5 KV and LEI detector at the Molecular Imaging

Centre (MIC), Bergen.

2.12. Statistics

All statistical analyses were carried out using the software R 3.5.1 (R Core Team 2018). In order to avoid false positives, the p values resulting from multiple comparisons carried out on the same data set were adjusted using the method of Benjamini & Hochberg (1995). The level of statistical significance for all analyses was set at $p = 0.05$. The whiskers shown on box plot figures were scaled with factor 1.5, as the default setting in R.

2.12.1. ELISA

The ELISA data is of hierarchical structure, with within-subject repeated measures. Therefore, linear mixed-effects models were used to examine these effects. In order to evaluate which model is most appropriate for the data, various model specifications were considered. Following Pinheiro & Bates (2000), these data were compared using the model selection criteria Akaike Information Criterion (AIC), Bayesian Information Criterion (BIC), and Likelihood Ratio Tests (LRT). The parameters of the finally selected models were re-estimated via REML. When a significant effect was found, post hoc-type comparisons were performed within the mixed effects framework.

2.12.2. Survival analysis

The results from the vaccine trial were analysed by classical methods from survival analysis, specifically the non-parametric Kaplan-Meier framework. Different Kaplan-Meier curves were compared by the log-rank test. For each bacterial dose used in the bath challenge, a test for an effect of any vaccine type on the hazard function was first carried out. If an effect was found, post-hoc tests were carried out to determine which of the vaccine types was significant.

Differences in mortalities between the non-concentrated and concentrated vaccines at the end of the trial were analysed using Chi square tests for 2×2 contingency tables.

The qPCR data on presence of bacteria in fish sampled from the vaccine trial was $\log_{10}$ transformed and analysed using the one-way ANOVA test.

3. Results

3.1. ELISA

Analyses of sera collected from vaccinated lumpsucker showed that specific antibody production was induced by vaccination and that the levels of antibodies produced increased over time. Significantly different levels of specific antibodies were detected when comparing sera from fish vaccinated with the two test vaccines, where the fish vaccinated with the concentrated vaccine produced significantly higher levels of specific antibodies. For both vaccines, the highest levels of specific antibodies were detected at 500° days post vaccination.

The optical density (OD) of antibody levels induced by vaccines at different degree days are shown in Fig. 1. The OD values increase with time, with a strong effect at 300° days (for the concentrated vaccine) and 400° days (for the non-concentrated vaccine); the OD values for the non-concentrated vaccine seem to rise less steeply than for the concentrated vaccine.

Statistically, the results from post-hoc tests confirmed that the OD values are significantly different to the baseline from 300° days onwards for the concentrated vaccine and from 400° days onwards for the non-concentrated vaccine. Furthermore, from 400° days onwards, OD values for the concentrated vaccine are significantly different from those corresponding to the non-concentrated vaccine.

3.2. Vaccine efficacy trial

A range of bacterial challenge doses was investigated in order to determine the optimal level to test the efficacy of the vaccines being developed. As shown in Fig. 2, the lowest challenge dose resulted in very low mortalities (below 40% in all groups) and for this reason, this dose was not used for further statistical analysis. The other challenge doses all resulted in mortalities higher than 60% for the two control groups. The mortalities from the two vaccine groups follow a similar pattern to each other; however, in the case of the highest challenge dose, mortalities were considerably higher than 60% mortality.

The concentrated vaccine resulted in the best survival rates of all the vaccines tested. However, statistical analysis failed to verify any significant difference in survival rates found between the two *Pasteurella* sp. vaccines tested, at any bacterial dose. For the highest three challenge doses there was a significant difference ($p < 0.05$) in survival rates between the PBS controls and the fish immunised with the concentrated vaccine, overall. The survival rates from the fish immunised with the non-concentrated vaccine differed significantly from those of the control vaccinated fish only in the 1×10^6 and 5×10^5 bacteria mL^{-1} challenge doses.

Chi square analysis of the relative percent survival at the end of the challenge (Table 2) however indicates that a significant difference can be observed between the two vaccines.

Clinical symptoms observed in the challenged fish included those reported previously [10], such as haemorrhage and redness at the base of fins and on the lower jaw, as well as white spots along the body and around the eyes. In addition, frayed tail fins were also encountered, as recorded in other work [3].

The antisera raised against *Pasteurella* sp. in lumpsucker contained antibodies reactive towards proteins of high molecular weight (more than 75 kDa) (Fig. 3 B, C, D). Sera from triple immunised lumpsuckers (Fig. 3D) additionally contained antibodies reactive to proteins of approximately 37 kDa and 48 kDa, which may correspond to major protein bands identified by silver staining (Fig. 3A). The sera from the vaccinated fish also reacted to the protein at approximately 48 kDa, albeit not as strongly as the serum from triple immunised lumpsuckers. The sera from lumpsucker vaccinated with concentrated and non-concentrated vaccine contained antibodies reactive towards proteins of similar molecular weight. The sera produced in rabbit contained antibodies reactive towards several proteins of varying molecular weight, including a band at just below 20 kDa (Fig. 3E) which was not identified by the other sera. By comparison, sera from the triple immunised lumpsucker (Fig. 3D) did not contain the same variety of antibodies as the serum from immunised rabbits. All sera, including the control serum, reacted to components at approximately 15 kDa.

3.3. Re-isolation of Pasteurella sp.

Pasteurella sp. was re-isolated from all fish sampled following bath challenge when grown on blood agar containing 2% sodium chloride. qPCR also confirmed the presence of *Pasteurella* sp. in the head kidney samples of dead fish (Fig. 4). This was done as previously described [10], where the values obtained in this challenge were compared to those from a spiked standard curve. Statistical analysis did not detect any significant differences in bacterial load between the two test vaccines and the PBS-vaccinated control.

3.4. In vitro experiments

Confocal microscopy of leucocytes exposed to *Pasteurella* sp. showed that bacteria were possibly present inside the cells both at 6 h and at 24 h post infection (Fig. 5). In SEM images (Fig. 6), bacteria were visible as individual cells on the surface as well as possibly underneath the surface of the leucocytes at 6 h post infection.

This is also seen in the confocal images (Fig. 5), where bacteria are

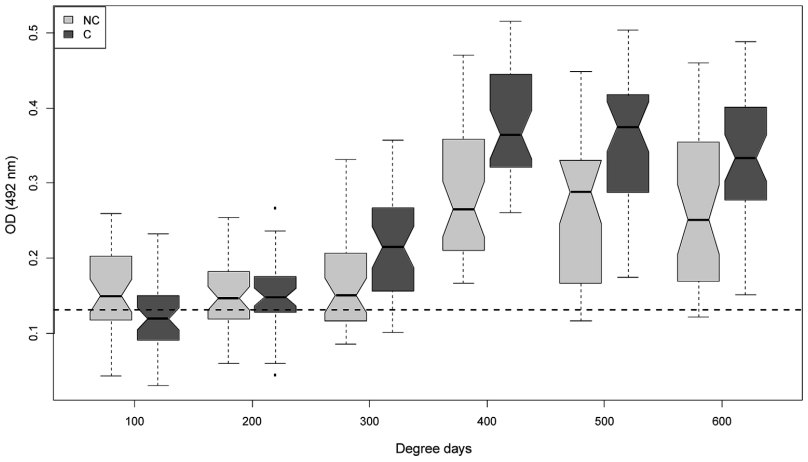

Fig. 1. Antibody levels (OD) post-vaccination measured by ELISA. The horizontal dashed line represents the non-vaccinated baseline control; the boxes represent the first and third quartiles, while the thick solid horizontal line represents the median. The whiskers represent the range of values recorded. (NC: Non-concentrated vaccine, C: Concentrated vaccine).

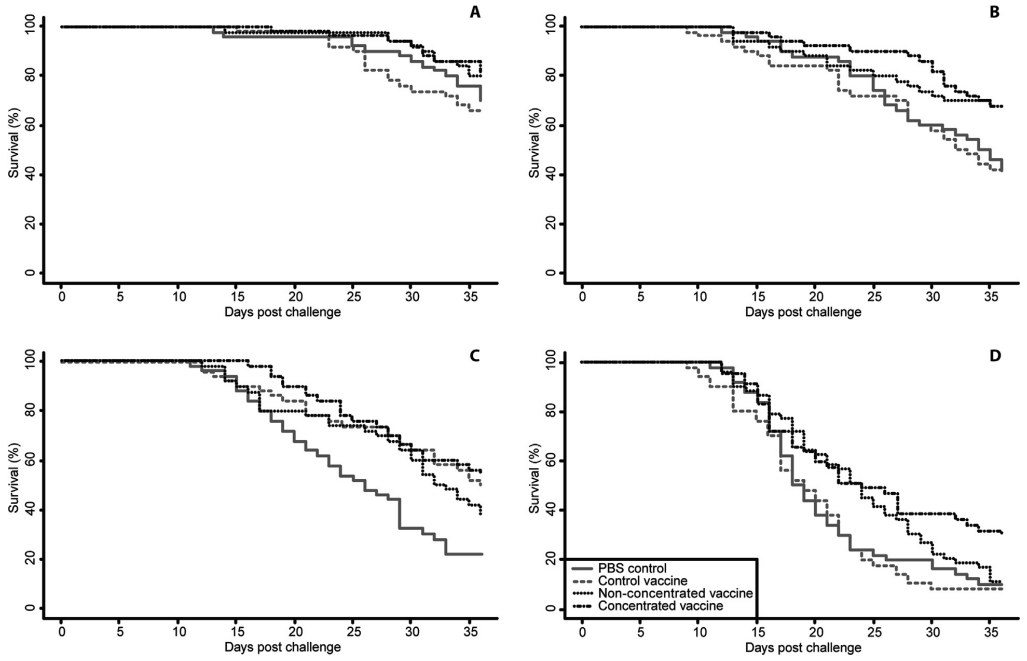

Fig. 2. Survival curves resulting from the vaccine efficacy trial using different bath challenge doses. A: 1×10^5 bacteria mL^{-1}, B: 5×10^5 bacteria mL^{-1}, C: 1×10^6 bacteria mL^{-1}, D: 1×10^7 bacteria mL^{-1}. n = 100 per challenge dose. The results from identical duplicate tanks are combined prior to presentation.

Table 2
Relative percent survival (RPS) at the end of the vaccine trial. *p-value: comparison of mortalities from the two vaccine groups at the end of the trial and set at p = 0.05. The lowest challenge dose is not included in the analysis due to low mortalities observed.

Challenge dose (bacteria mL^{-1})	Concentrated vaccine RPS (%)	Non-concentrated vaccine RPS (%)	p-value*
5×10^5	44.83	44.83	1
1×10^6	41.03	20.51	0.39
1×10^7	21.82	1.27	**0.03**

seen as red dots within the cell, in and around the nucleus of the leucocyte. The bacteria seem to aggregate into chains which gradually become coiled, as can be seen from the SEM and confocal images at 24 h. They also appear to be attached to the surface of the cell. This coiling effect was also observed from Gram stains of the bacteria kept in L-15/FCS and TSB for 24 h, but not in Gram stains done after 6 h of the bacteria in L-15/FCS or TSB (data not shown), and in confocal photomicrographs. As can be noted in Fig. 5B, the bacteria become slightly more elongated and narrower with time as they accumulate to form the coiled chains. The effect of the bacteria on the leucocytes can also be observed with time as these become apoptotic with time but appear

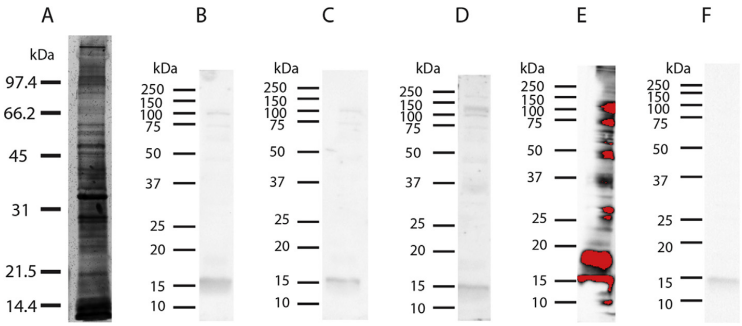

Fig. 3. SDS-PAGE silver stained protein profile of *Pasteurella* sp. bacterial components and serum antibody specificity analysed by western blots. A: silver stained protein profile of *Pasteurella* sp., B: Western blot using serum from fish vaccinated with non-concentrated *Pasteurella* sp. vaccine, C: Western blot using serum from fish vaccinated with concentrated *Pasteurella* sp. vaccine, D: Western blot using serum from fish triple immunised with *Pasteurella* sp. E: Western blot using rabbit anti-*Pasteurella* serum, F: Western blot using serum from non-vaccinated lumpsuckers.

morphologically intact and healthy at 6 h post infection.

4. Discussion

Vaccination of lumpsucker using *Pasteurella* sp. bacterins has, to our knowledge, not been carried out. This has mostly been due to difficulties in culturing the newly encountered pathogen in liquid media. In this work, we used results obtained from previous experiments in order to enhance our understanding of *Pasteurella* sp. and to establish preventative measures against pasteurellosis in lumpsuckers.

ELISA analysis of sera collected from vaccinated fish showed that specific antibodies are generated in relatively high amounts following vaccination with *Pasteurella* sp. bacterins. This is supported by earlier findings showing that *Pasteurella* sp. is highly immunogenic in lumpsucker, providing high levels of specific antibodies after triple immunisation [9]. Moreover, the concentrated vaccine resulted in higher levels of specific antibodies generated, which was likely related to the increased antigen dose. In the concentrated preparation of this vaccine, the bacteria were centrifuged and approximately 90% of the supernatant was discarded prior to further processing. Therefore, the majority of any extracellular proteins (ECPs) produced by the bacteria were not included, unlike the non-concentrated vaccine. However, the number of inactivated bacterial cells per mL were approximately 10-fold higher for the concentrated vaccine, and the slightly higher protection after challenge observed for the concentrated vaccine was likely due to the increased antigen content per dose.

The vaccines did not provide complete protection against the disease. Although the onset of mortality was delayed and the total levels of mortality somewhat reduced, the protection provided by the vaccines was relatively weak. Quite high mortalities still occurred for the vaccinated groups, with approximately 40% relative protection (RPS) at the end of the trial at best. Although the infection pressure in the highest challenge dose may have masked the effect of the vaccine, resulting in the lack of protection observed, this was also seen in the next weaker dose, where mortality in the vaccinated groups was higher than 40%.

The clinical signs of diseased fish observed in this experiment were similar to those reported previously [10]. Of interest was the observation of an additional clinical sign in this study i.e. the frayed fins in the early stages of the infection in the higher two challenge doses used in the trial. This was also observed during a natural outbreak of pasteurellosis in lumpsuckers [3]. Development of these clinical signs together could be due to the different challenge pressure and dynamics present in this trial.

Both experimental vaccines induced significantly increased levels of specific antibodies. A weak trend was detected where the concentrated vaccine consistently provided slightly better protection compared to the non-concentrated vaccine and this might be related to the increased antibody levels. However, significant differences in RPS between the two groups at the end of the challenge could only be proven for the

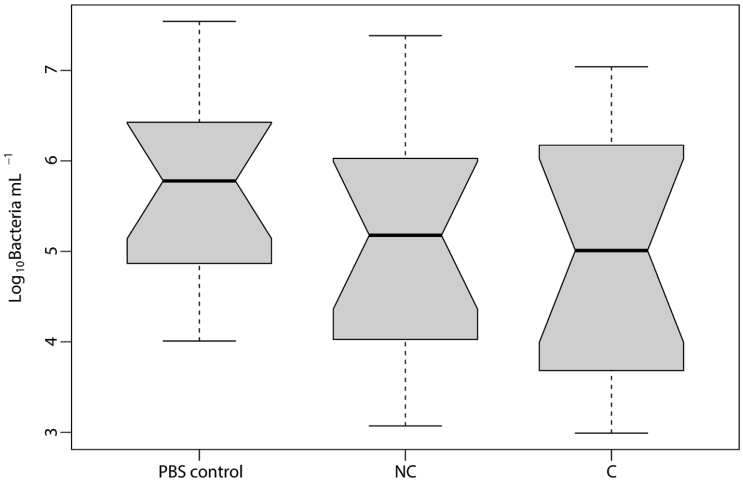

Fig. 4. qPCR confirmation of presence of *Pasteurella* sp. in dead challenged vaccinated and control fish. The boxes represent the first and third quartiles, while the thick solid horizontal line represents the median. The whiskers represent the range of values recorded. (NC: Non-concentrated vaccine, C: Concentrated vaccine).

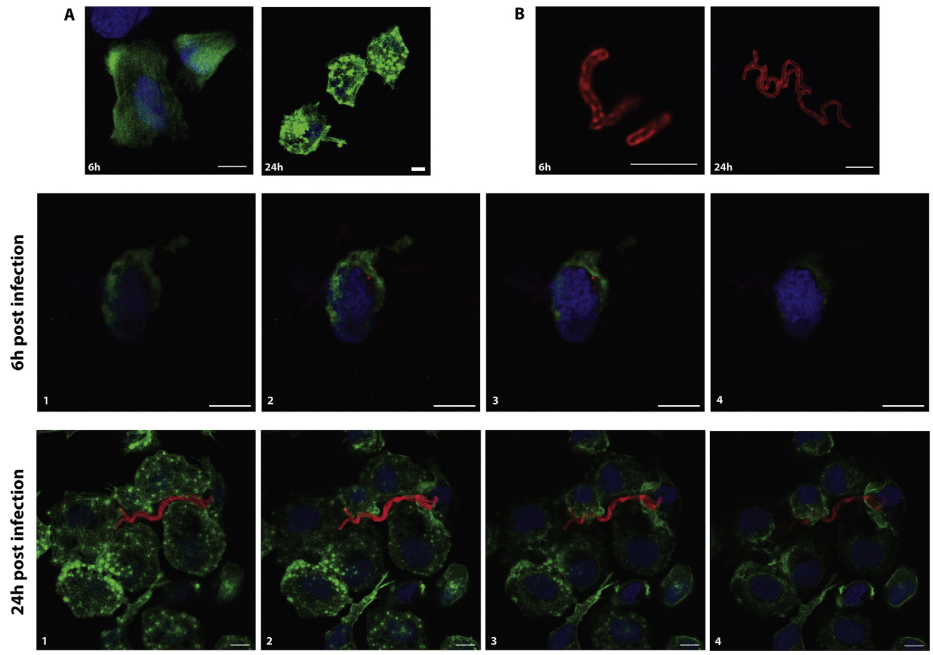

Fig. 5. Confocal micrographs after *in vitro* infection of head kidney leucocytes (HKL) with *Pasteurella* sp. Leucocyte actin filaments and nuclei are stained green and blue, respectively, while bacteria are stained red. A: Non-infected HKL. B: Isolated *Pasteurella* sp. Top series: sections through an infected HKL, 6 h post infection. Bottom series: sections through infected HKL, 24 h post infection. All scale bars: 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

highest challenge dose.

Host-pathogen interactions between *Pasteurella* sp. and lumpsucker leucocytes as observed in this study have also been noted for other pathogens that interfere with the immune system of the host. *M. viscosa* eludes protective immune responses of the host by suppressing the production of IL1β, hence delaying bacterial elimination [15], while *Yersinia ruckeri* [16] and *Francisella noatunensis* subsp. *noatunensis* [17–19] reside and replicate within macrophages.

The immunoblot showing only a few highly antibody-reactive bands from the SDS-PAGE separated bacterial components might suggest that the specificity and reactivity of the antibodies is not directed towards major cell wall-integrated components. This might partly explain the limited protection conferred. The reason for the low number of reactive bands on the immunoblot can be that the proteins are glycosylated and not well transferred to the membrane. If any loosely attached carbohydrate antigens are present in the antigen preparations of the vaccines,

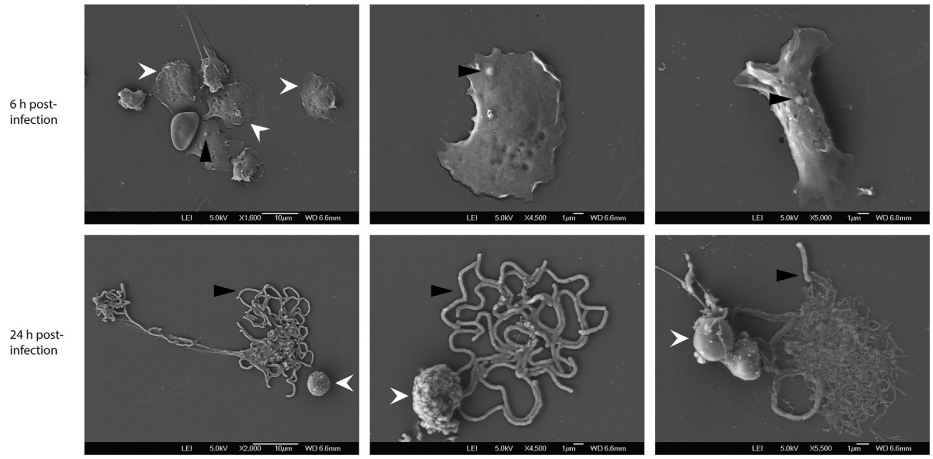

Fig. 6. Scanning electron micrographs (SEM) of head kidney leucocytes at two time points post *in vitro* infection. Scale bar = 10 μm for the first image in each series, 1 μm for the two following images. White arrows indicate leucocytes, while black arrows indicate bacteria.

they can significantly contribute to the high levels of antibodies observed in ELISA.

The presence of carbohydrate and protein reactive fish antibodies to bacterial cell surface components can be detected by other methods [20]. However, there is an obvious difference observed using rabbit serum where many antibody-reactive bands are seen in the immunoblot. Thus, bacterial components are well transferred to the nitrocellulose membrane, and the differences observed between lumpsucker sera and rabbit sera might be species dependent and also dependent on immunisation procedure and adjuvants, since there are more bands from the triple immunised lumpsucker serum, than from the serum from the vaccinated lumpsuckers.

Previous studies on the adaptive immune responses of lumpsucker indicated that IgM^+ B cells abundant in blood were highly phagocytic, and provided a specific antibody response following immunisation [9]. Further work [14] showed that lumpsucker vaccinated with atypical *A. salmonicida* developed a strong humoral immune response to the bacteria, which resulted in significant protection of the fish against bacterial disease tested by challenge. This was also investigated in another study [21], where it was found that specific antibody responses to *V. anguillarum* serotype 01 and *M. viscosa* were lower than towards atypical *A. salmonicida*. The levels of antibodies to the various bacteria recorded in these studies are in the same range as those identified in the current work. However, in contrast to the previously mentioned studies for other bacteria, protection against *Pasteurella* sp. in the present study was relatively weak.

The potency of commercial vaccines must be tested by means of correlates of protection. For fish vaccines, this is usually performed by vaccination-challenge experiments using the pathogens the vaccine is intended to protect against. However, it is also acceptable to use appropriately validates alternative methods not involving challenge of fish. One example of such correlates are specific immune responses to antigens present in a vaccine that elicit protection against an infection or disease [22]. Therefore, vaccines should ideally induce opsonophagocytic and/or neutralising antibodies and should activate immune functions mediated by $CD4^+$ and possibly also $CD8^+$ cells [22]. Moreover, antibody levels measured post-vaccination *in vitro* (such as through ELISA) will not distinguish between neutralising and non-neutralising antibodies, and typically the latter would be antibodies that are not involved in opsonophagocytosis [22] or complement mediated killing. Such antibodies would be surrogates of protection, since they are straightforward to measure, but not directly related to the correlate of protection.

From confocal microscopy of *in vitro* infected leucocytes, one can see aggregated bacteria interacting with the leucocytes. In a previous study [10], diffuse bacterial aggregates were observed in tissue samples from challenged lumpsucker. Whether this take place in natural infections is not clear, but bacterial aggregates would possibly have an impact on the antibody functionality and cause suboptimal bacterial clearance. These factors could possibly explain the results obtained from our vaccine trial with findings of bacterial specific antibodies, but a lack of protection in challenge experiments. Thus, despite the vaccines being capable of eliciting B cell production of specific antibodies, these did not provide sufficient protection from disease and mortality.

The possibility of *Pasteurella* sp. being intracellular, may be indicated from the immune detection of bacteria through confocal microscopy and to an extent, through SEM photomicrographs, as bacteria are possibly found inside leucocytes. Whether this bacterium can reside or even replicate within cells remains to be studied. In that case, improved activation of cellular immune responses could be important to achieve better protection against pasteurellosis. The aggregation of bacteria seen during *in vitro* assays may be a survival strategy to protect against either leucocytes activities, or the incubation medium itself, or other factors including changes in pH. However, considering the results from SEM and confocal microscopy, it cannot be excluded that *Pasteurella* sp. may be a facultative intracellular pathogen and possibly, therefore, stimulation of cellular immunity may be vital to achieve protection of lumpsucker against *Pasteurella* sp.

Agglutination tests carried out showed that the immune sera raised against the bacteria agglutinated live *Pasteurella* sp. indicating that binding to bacterial surface components does occur. It is tempting to speculate, however, considering the low protection obtained by the tested vaccines, that an extracellular capsule, proteins or toxins may be involved in disease progression. If toxic extracellular proteins were involved, extensive genome analysis would be required in order to identify the specific virulence factors and consider if antibodies with antitoxin properties can be obtained.

5. Conclusion

In summary, despite lumpsuckers responding to vaccination with monovalent inactivated *Pasteurella* sp. vaccines by producing specific antibodies, the protection against experimental challenge was relatively weak. A reason for this could potentially be that a specific antibody response is not enough to provide complete protection. This indicates that parts of the immune system other than the humoral part could be important for protection against pasteurellosis, highlighting the need for further work on the mechanism of infection of *Pasteurella* sp. in lumpsucker. Additionally, there might be pathogen specific virulence factors involved in disease development such as intracellular localisation, extracellular and toxic proteins, as well as aggregation. Considering all these results and observations, we think the virulence factors of *Pasteurella* sp. isolate from lumpsuckers should be thoroughly investigated.

Declaration of competing interest

The authors state that there are no competing interests to declare regarding the presented work.

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Genomic Analysis of *Pasteurella atlantica* Provides Insight on Its Virulence Factors and Phylogeny and Highlights the Potential of Reverse Vaccinology in Aquaculture

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Abstract: Pasteurellosis in farmed lumpsuckers, *Cyclopterus lumpus*, has emerged as a serious disease in Norwegian aquaculture in recent years. Genomic characterization of the causative agent is essential in understanding the biology of the bacteria involved and in devising an efficient preventive strategy. The genomes of two clinical *Pasteurella atlantica* isolates were sequenced (≈2.3 Mbp), and phylogenetic analysis confirmed their position as a novel species within the *Pasteurellaceae*. In silico analyses revealed 11 genomic islands and 5 prophages, highlighting the potential of mobile elements as driving forces in the evolution of this species. The previously documented pathogenicity of *P. atlantica* is strongly supported by the current study, and 17 target genes were recognized as putative primary drivers of pathogenicity. The expression level of a predicted vaccine target, an uncharacterized adhesin protein, was significantly increased in both broth culture and following the exposure of *P. atlantica* to lumpsucker head kidney leucocytes. Based on in silico and functional analyses, the strongest gene target candidates will be prioritized in future vaccine development efforts to prevent future pasteurellosis outbreaks.

Keywords: *Pasteurella atlantica*; lumpsucker; aquaculture; pathogenicity; phylogeny; in silico analysis; virulence factors; mobile elements; vaccine

1. Introduction

The prevalence of pasteurellosis in farmed lumpsuckers, *Cyclopterus lumpus*, in Norway has increased in recent years since the first case was recorded in 2012 [1]. Despite a decrease in the number of affected localities [2], *Pasteurella atlantica* [3] remains a significant problem, and as the disease is non-notifiable, under-reporting of outbreaks cannot be excluded. Furthermore, while farmed lumpsuckers are vaccinated against vibriosis and atypical furunculosis, there are no commercially available vaccines against pasteurellosis.

The family *Pasteurellaceae* is composed of commensals, opportunistic- and primary-pathogens and includes the genera *Pasteurella*, *Actinobacillus*, and *Hemophilus* amongst others. The genus *Pasteurella* has a broad host range, but little is known regarding the ecology of marine species. *P. atlantica* isolated from diseased Norwegian lumpsuckers is related to but serologically distinct from *Pasteurella skyensis*, which causes disease in Atlantic salmon (*Salmo salar* L.) in Scotland, and a *P. atlantica* isolate first detected in 2018, which causes disease in Norwegian farmed Atlantic salmon [1,3,4]. Pasteurellosis affects all

life stages of a lumpsucker, from fry to fish deployed in salmon cages [4]. As *P. atlantica* has been detected in lumpsucker eggs and milt, vertical transmission may also be possible [5].

In a previous work [6], we showed that whole cell-inactivated bacterin-based vaccines do not provide adequate protection against the disease, despite the high titers of specific antibodies raised. Such a situation highlights the need for a deeper characterization of the bacterium and the identification of immunogenic and protective antigens.

Most pathogenic bacteria have several tools in their genetic arsenal to avoid host defenses and to enhance their survival. Genetic determinants including pathogenicity islands, antibiotic resistance genes, toxins, and adhesins are often shared between bacterial populations via horizontal gene transfer and are commonly associated with plasmids, prophages, and other mobile genetic elements. As adhesins are involved in the early stages of colonization, they can be utilized as targets for vaccine development via reverse vaccinology (RV).

Using in silico bioinformatic analyses, immunogenic antigens for vaccine development can be predicted [7,8]. RV is a rapid process and can reduce vaccine development time by up to 2 years [9]. It is also a more ethical approach due to a reduction in the number of experimental animals required for vaccine testing and results in effective vaccines. In human medicine, RV has been successful in the development of vaccines against several bacterial pathogens reviewed by Sharma et al. 2021 [10]. Pathogens of significant concern such as *Neisseria meningitidis* [11,12], *Mycobacterium tuberculosis* [13], *Chlamydia pneumoniae* [14], *Streptococcus pneumoniae* [15], *Helicobacter pylori* [16], *Porphyromonas gingivalis* [17], and *Bacillus anthracis* [18,19] have been investigated using RV.

RV technologies have improved considerably since the principles were initially developed and applied. The main criteria required for reliable prediction of vaccine targets include subcellular localization, the probability of adhesion functionality, and the number of transmembrane domains [20]. Additionally, using targets that are present only in virulent strains and having protein sequences that are dissimilar to host sequences results in more reliable vaccine targets that also induce strong immune responses [9].

Adhesins are a class of surface-bound proteins involved in facilitating bacterial attachment to host tissues. They are classed as fimbrial or non-fimbrial based on the absence or presence, respectively, of an outer membrane anchor in the protein. Among the different types of adhesins, bacterial lectins are the most common [15]. The mediation of attachment occurs through the recognition of specific carbohydrates, proteins, or lipids presented on the host cell surface.

In addition to attachment, adhesins also promote the delivery of toxins through the upregulation of virulence genes leading to invasion of the host. Furthermore, such interactions can trigger cytokine production or lectinophagocytosis by the host, compounding the severity of disease [21]. For this reason, subunit vaccines that hinder microbial attachment could be of great advantage in combating pasteurellosis in lumpsuckers. Examples of successful adhesin vaccines include those against enterotoxigenic *E. coli* (ETEC) strains in farm animals using the K88 fimbriae [22] as well as uropathogenic *E. coli* (UPEC) known to cause urinary tract infections (UTIs) using the FimCH complex [23]. For the aquaculture industry, vaccines have been tested by targeting major bacterial adhesins from *Aeromonas hydrophila* [24,25], *Vibrio harveyi* [26], and *Edwardsiella tarda* [27].

As research on *P. atlantica* in lumpsuckers is still in its infancy, no commercial vaccines are available against pasteurellosis in lumpsuckers. Simple phylogenetic analyses using the *rpoB* and 16S rRNA genes have shown that NVI-9100 is similar to other *Pasteurella* sp. isolated from lumpsuckers [4]. Whole genome analysis can, however, determine more accurate phylogenetic relationships. The purpose of this study is, therefore, to determine the taxonomic position of the species through whole genome sequencing and to predict potential vaccine antigens via in silico analysis. To further assess the predicted vaccine targets, their expression levels during exposure to lumpsucker leucocytes were analyzed in vitro.

2. Materials and Methods

2.1. Bacterial Culture, Genome Isolation, and Sequencing

Two *P. atlantica* isolates described in previous studies (NVI 9100 and UiBP1-2013) [4,6,28] collected from clinically sick lumpsuckers were whole-genome sequenced. Briefly, bacteria were grown in tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD, USA) supplemented with 1.5% NaCl and 10% fetal bovine serum, Australian origin, (Gibco, Waltham, MA, USA) at 20 °C with shaking (200 rpm).

Total genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following manufacturer instructions. Briefly, 3 mL of an overnight culture of *P. atlantica* containing a maximum of 2×10^9 cells was centrifuged at $2500\times$ *g* for 15 min and the pellet was resuspended in 180 µL of ATL buffer. Twenty microliters of proteinase K were then added, and the bacteria were incubated at 56 °C in a rotating heat block for 1 h. Two hundred microliters of AL buffer and two hundred microliters of 96% ethanol were added, vortexing well between additions. The mixture was then loaded onto DNeasy Mini Spin columns and washed with the appropriate buffers (AW1 and AW2) prior to elution using buffer AE. The eluted DNA was then purified and measured for concentration before storing at −20 °C until further processing.

Extracted DNA was sequenced using either an Illumina (UiBP1-2013) or PacBio (NVI 9100) platform. Illumina libraries were made using the Nextera DNA Flex Sample Prep kit (Illumina, San Diego, CA, USA) according to the manufacturer instructions and sequenced with Illumina MiSeq (Illumina, San Diego, CA, USA) using V3 chemistry. PacBio libraries were prepared with the Pacific Biosciences 20 kb library preparation protocol, and size was selected using BluePippin (Sage Science, Beverly, MA, USA), with subsequent sequencing performed on a Pacific Biosciences RS II instrument using P4-C2 chemistry and employing three SMRT cells (Pacific Biosciences, Menlo Park, CA, USA).

Raw Illumina sequences were adapter trimmed, quality filtered (Q > 20), de novo assembled using [29,30]. Contigs shorter than 1000 bp or with <5 times coverage were removed from each assembly prior to gene annotation. The genes of the Illumina sequenced genome were predicted by Glimmer 3, version 3.02b; Johns Hopkins University: USA, 1999 [31].

PacBio sequences were de novo assembled using HGAP version 3 (Pacific Biosciences, SMRT Analysis Software version 2.2.0) and circularized with the Minimus2 software version 2 (Amos package). For post-circularization correction of bases, reads were subsequently mapped to the circularized sequences using RS_Resequencing.1 software (SMRT Analysis version version 2.3.0).

This whole genome project has been deposited at GenBank under the Accession Number PRJNA721926 (UiBP1-2013) and Accession Number CP074346 (NVI 9100).

2.2. Phylogenetic Analysis

Twenty-three *Pasteurellaceae* genomes (Table 1) were included for phylogenetic analyses.

In order to evaluate similarity among whole genomes, Orthologous Average Nucleotide Identity Tool using OrthoANI [43] was used to provide reliable and fast assessment of average nucleotide identity (ANI) for taxonomic classification purposes. A Similar Genome Finder with high similarity parameters (max hits: 50; *p*-value threshold: 1; distance: 0.5) utilizing Mash, a fast genome distance estimation tool [44], mounted on the Pathosystems Resource Integration Center (PATRIC) platform (https://patricbrc.org/ (accessed on 3 June 2021)) [45,46] was also used to assess the genomic similarity of *P. atlantica* to the bacterial database.

PATRIC [45,46] was used to construct a whole-genome codon-based tree built on 500 single-copy genes [47] present in all genomes studied (Table 1). Both amino acid and nucleotide sequences were aligned using MUSCLE [48] and the codon align function of Biopython [49], respectively. The concatenated alignments of protein and nucleotide sequences were used in order to generate a RAxML method-based phylogenetic tree with

branch support values determined by 100 rounds of rapid bootstrapping [50,51]. The tree was visualized using iTOL online platform [52].

Table 1. Twenty-three *Pasteurellaceae* genomes used to evaluate the phylogenetic position of *P. atlantica*.

Bacterial Species Name (GenBank)	Animal Host	Country	Genome	Ref.
Pasteurella strains				
Pasteurella multocida strain ATCC43137	Pig	n/a	Assembly	NCBI
Pasteurella dagmatis strain NCTC11617	Human	n/a	Assembly	[32]
Pasteurella testudinis strain DSM23072	Desert tortoise	USA	WGS—69 contigs	[33]
Pasteurella bettyae strain CCUG2042	Human	USA	WGS—47 contigs	NCBI
Pasteurella langaaensis strain DSM22999 Ga0215636	Chicken	Denmark	WGS—29 contigs	[32]
Pasteurella caecimuris strain NM44_TS2-9	Mouse	Canada	WGS—53 contigs	NCBI
Pasteurella canis strain NCTC11621	Human	n/a	WGS—20 contigs	[32]
Pasteurella oralis strain WCHPO000540	Human	China	WGS—30 contigs	[34]
Pasteurella skyensis strain DSM24204	Atlantic salmon	Scotland	WGS—56 contigs	[35]
[Pasteurella] aerogenes strain NCTC13378	n/a	Finland	Assembly	NCBI
Pasteurella pneumonotropica strain DSM21403	Mouse	USA	WGS—22 contigs	NCBI
Non-*Pasteurella* strains				
Phocoenobacter uteri strain NCTC12872	Harbor porpoise	Scotland	WGS—5 contigs	[36]
Hemophilus hemolyticus strain 65117 B Hi-3	Human	Australia	WGS—16 contigs	NCBI
Hemophilus hemolyticus strain M19346	Human	USA	Assembly	[37]
Hemophilus influenzae strain 156_HINF	Human	USA	WGS—41 contigs	[38]
Hemophilus influenzae strain NCTC8143	Human	UK	Assembly	NCBI
Hemophilus parainfluenzae strain 155_HPAR	Human	USA	WGS—61 contigs	[38]
Hemophilus parainfluenzae strain T3T1	n/a	n/a	Assembly	NCBI
Aggregatibacter aphrophilus strain C2008001782	Human	USA	WGS—16 contigs	[39]
Aggregatibacter aphrophilus ATCC33389 strain NCTC5906	n/a	UK	Assembly	NCBI
Glaesserella parasuis strain F9	Pig	Spain	WGS—182 contigs	[40]
Actinobacillus indolicus strain AIFJ1607	Pig	China	Assembly	[41]
Mannheimia hemolytica strain M42548	Ruminants	n/a	Assembly	[42]

2.3. Analysis of Genomic Regions and Reverse Vaccinology Approach

The novel genomes were screened against major virulence factor databases. Specifically, the proteome of *P. atlantica* was blasted against VFDB [53] and VICTORS [54] using an e-value of 1×10^{-10}. A moderate e-value was applied to avoid false positive hits while identifying as many presumptive virulence factors as possible. The prediction was complemented by screening against PATRIC_VF [55], the integrated virulence factor database inside the PATRIC platform (https://patricbrc.org/ (accessed on 3 June 2021)) [45,46]. Subcellular localization for all presumptive virulence factors was examined by PSORTb 3.0 [56] to evaluate putative interaction with the extracellular environment (localization score threshold >7.5). Proteins with both extracellular and outer membrane subcellular localization were imported to SignalP server [57] in order to further assess any known types of signal peptides as well as the pathways in which they are involved during their secretion or anchoring process (threshold >90%). The detection of possible adhesion-related functionality was tested in SPAAN [58]. A Protein Fold Recognition Server, Phyre2 [59], was applied to obtain insights on structure and folding properties of presumptive virulence factors using confidence level of above 97%.

Clusters of genes of probable horizontal origin, known as genomic islands (GIs), were identified by IslandViewer 4, version 4; Simon Fraser University: Canada, 2017 [60]. Inducible prophages were detected using the prophage finder tool, PHASTER [61], while VIRFAM [62] was used to classify intact prophages.

In line with reverse vaccinology principles, the identification of the strongest virulence candidates was deduced through Dynamic Vaxign Analysis, Vaxign [63], a software platform that has been developed and dedicated to vaccine design. The pipeline of Vaxign has integrated PSORTb [56] and SPAAN [58] so it can also be used to validate the previously identified virulence factors. Vaxign-ML [20] was also included in the reverse vaccinology analysis as a machine learning model to improve the prediction of bacterial protective antigens. VaxiJen [64] complemented the analysis to corroborate that the finally selected candidates for vaccine development also can function as protective antigens, which is a prerequisite for vaccine development.

2.4. Functional Studies

2.4.1. Processing Bacteria for qPCR Analysis

Cultures of *P. atlantica* (UiBP1-2013) used for the in vitro exposure experiment (Section 2.4.5) were cultivated as described in Section 2.1, harvested in the late exponential growth phase (18 h post inoculation) and centrifuged at 2500× *g* (Beckman Coulter Allegra X-15R) for 15 min at 4 °C. For the gene expression analysis during bacterial cell proliferation in growth medium, 1 mL samples were harvested 14, 16, 18, and 20 h after inoculation. Bacterial cell counts were measured at harvest using a cell counter (CASY Model TT (Innovatis) and CASY worX version 1.26) followed by centrifugation at 4000× *g* (Beckman Coulter Allegra X-15R) for 10 min at 4 °C. For both methods, the growth medium was then discarded, and the samples were stored on ice.

Total RNA was immediately extracted using the Bacterial RNA Kit (E.Z.N.A., Norcross, GA, USA) according to manufacturer instructions. The RNA was then DNase-treated (Sigma-Aldrich, Saint-Louis, MO, USA), converted to cDNA using qScript cDNA Synthesis Kit (Quantabio, Beverly, MA, USA), and stored at −20 °C.

2.4.2. qPCR

Each qPCR reaction contained a volume of 25 µL and consisted of 12.5 µL 2× SYBR Green JumpStart Taq Ready Mix (Thermo-Fisher Scientific, Waltham, MA, USA), 1 µL each of the forward and reverse primers (10 µM final working concentration, Table 2), 0.5 µL of RNase and DNase free water (Sigma-Aldrich), and 10 µL of cDNA (concentration depended on the specific analysis). A C1000 Touch thermal cycler with CFX96 Real-Time System (Bio-Rad, Oslo, Norway) was used for qPCR, with the following cycle conditions: 94 °C for 5 min followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min. Melting curve analyses were performed after each run (60 to 92 °C at a rate of 1 °C/5 s) to ensure that the specificity of the primers and the qPCR products were visualized on a 2% agarose gel. Three parallel reactions were performed for each gene, and negative controls excluding cDNA (NTC) and cDNA reactions without reverse transcriptase (NRT) were included for all master mixes. The gene expression levels were calculated by the ΔΔCt method [65].

Table 2. Details of primers for reference genes and the target gene used for qPCR.

Gene	Primer Name	Sequence 5′-3′	Primer Length (bp)
gyrA	#27-B_GYRA_F3	GTTCATCGGGTATTGCGGTCGGTAT	25
	#28-B_GYRA_R3	TCCTGTGCGGTAAGCGTCTTCG	22
rpoD	#46-B_RPOD_F1	GGACGTGATGCGACACCTGAAGAAT	25
	#47-B_RPOD_R1	AGTGGCTGTGCAAGTGCAGTATCTT	25
Putative uncharacterized protein <Hia>	#70-B_HIA_F4	AGGTGTGGGTTCATTCGCTGTGG	23
	#68-B_HIA_R2	CCGATTGCTGCCGCTTGTTGTTC	23

2.4.3. Primer Design and Validation

Genes considered for this work were based on the results from an analysis of genomic regions and potential vaccine targets. The genes selected as reference genes were *rpoD* and *gyrA*. The target gene selected was the putative uncharacterized protein (<Hia>) (Table 2).

qPCR assays were designed using the software Primer Premier version 6.24 (Premier Biosoft, San Francisco, CA, USA). The five highest rated assays for each target sequence were then chosen for testing. The length of the amplicons was kept between 100 and 250 bp for optimal amplification efficiency. The specificity of the primers was confirmed by qPCR (20 ng of cDNA used in each qPCR reaction), and product size was observed by electrophoresis on 2% agarose gels. All of the qPCR assays produced single amplification products. The best assay for each target gene based on Cq value, non-template controls, melting curves, and the results of electrophoresis were then chosen for further work. The resulting primers used for qPCR are listed in Table 2.

2.4.4. Bacterial Exponential Growth Phase Analysis

P. atlantica was grown as described in Section 2.1. At different time points after inoculation, the bacteria were harvested, the RNA was isolated, and cDNA synthesis was performed as described in Section 2.4.1. The synthesized cDNA was diluted across a twofold dilution series to give a range from 10 ng/µL to 0.625 ng/µL of cDNA for both the target and reference genes in qPCR (100 ng to 6.25 ng in each qPCR reaction). The relative gene expression was calculated by the ΔΔCq method using the most suitable dilution from the range tested, and comparisons of gene expression were made to the lowest stable time point of the analysis.

2.4.5. Head Kidney Leucocyte (HKL) Exposure Analysis

Four lumpsuckers were quickly netted and killed by a sharp blow to the head. Leucocytes were isolated from the head kidney on discontinuous Percoll gradients as described previously [66] with the following modifications. The supplemented L-15 medium did not contain gentamicin sulphate since the cells were to be exposed to viable *P. atlantica*. Additionally, resuspension of the isolated leucocytes was performed in L-15 supplemented with 5% fetal calf serum (L-15/FCS). The leucocytes were counted in a CASY-TT Cell Counter TM (Innovatis AG), and the viability (95%) and aggregation factor (1.2) of the cells were determined. The concentration of the isolated leucocytes was then adjusted to 3.3×10^6 cells mL^{-1} in L-15/FCS, and 250 µL volumes were added to each well of the 24-well Nunc plates (approximately 8×10^5 cells per well) (Thermo-Fisher Scientific) and incubated overnight at 15 °C prior to exposure to *P. atlantica*.

A late exponential phase (18 h) culture of *P. atlantica* was prepared as described in Section 2.4.1, re-suspended in L-15/FCS, and adjusted to 1.5×10^8 bacteria mL^{-1}. Volumes of 250 µL were then added to each well (approximately 4×10^7 bacteria per well). Sterile L-15/FCS medium was supplied to the leucocytes and used for the non-challenged controls. The cells were then incubated at 15 °C, and samples were collected at 3, 6, 9, 12, and 24 h after exposure. Samples were collected from the wells and centrifuged to remove all media prior to storage at −80 °C, until the total RNA was isolated, DNase treated, and converted to cDNA as described above. For qPCR, 80 ng per reaction was used as the template for both the target and reference genes.

2.4.6. Statistical Analysis

The relative gene expression during the HKL exposure was calculated by the ΔΔCq method [65] and comparisons were made to the negative controls (bacteria without leucocytes). The results were analyzed using two-way ANOVA and Fisher's LSD for post hoc tests, and differences were considered significant when $p < 0.05$. All statistical analyses were carried out using SigmaPlot version 12 (Systat Software, San Jose, CA, USA).

3. Results

3.1. Novel Sequenced Genome and Accession Numbers

The length of the PacBio sequenced *P. atlantica* genome (NVI 9100) is 2,301,649 bp, with a GC content of 42.1%, and 2197 coding regions (CDS) were predicted. The length of the Illumina sequenced *P. atlantica* genome (UiBP1-2013) is 2,260,408 bp.

3.2. Phylogenomics and Taxonomic Classification

The *P. atlantica* strains were isolated from *Cyclopterus lumpus* in a previous study where preliminary phylogenetic analysis was performed based on 16S rRNA and *rpoB* gene sequencing [4]. To assess pairwise similarity percentages among *Pasteurella* species, nine different publicly availably *Pasteurella* species genomes (Table 1) and *P. atlantica* were analyzed using the OrthoANI tool (Figure 1).

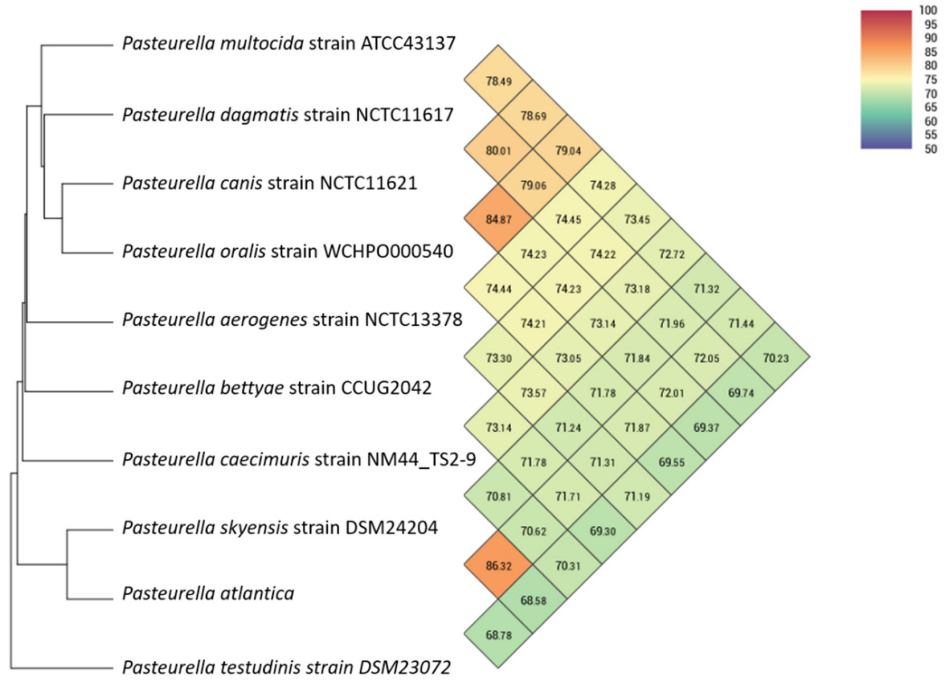

Figure 1. Heatmap generated with OrthoANI values calculated from the Orthologous Average Nucleotide Identity Tool (OAT) software.

When compared to our genome of interest, *P. skyensis* shows the highest OrthoANI values (86.32%) whereas the other species generated much lower values (70 to 72%).

Apart from *Pasteurella* genus, a genomic analysis of *P. atlantica* against the entire publicly available bacterial database, based on whole genome sequences, according to Similar Genome Finder, returned the *P. skyensis* strain DSM 24204 as the only similar genome at a genomic distance of 0.096 (Mash distance threshold: 0.1). The second most similar genome at a genomic distance of 0.145 was the *Ph. uteri* strain NCTC12872, and that was shown only when the threshold increased up to 0.5 (Figure 1). A bigger number of *Pasteurellaceae* family bacteria were found to be less similar, at genomic distances of >0.2, and for the sake of further analysis, representative genomes have been included in Table 1 as well. Substantially low-similarity levels compared to the bacterial database constitute an additional piece of evidence supporting *P. atlantica* as a novel species, knowing that a

pairwise Mash distance of at least ≤0.05, approximately corresponding to an ANI of ≥95%, is taken for granted among conspecific bacterial genomes [44].

Multiple codons through the entire genomes of Table 1 were used as genetic markers to finalize the taxonomy of *P. atlantica* (Figure 2).

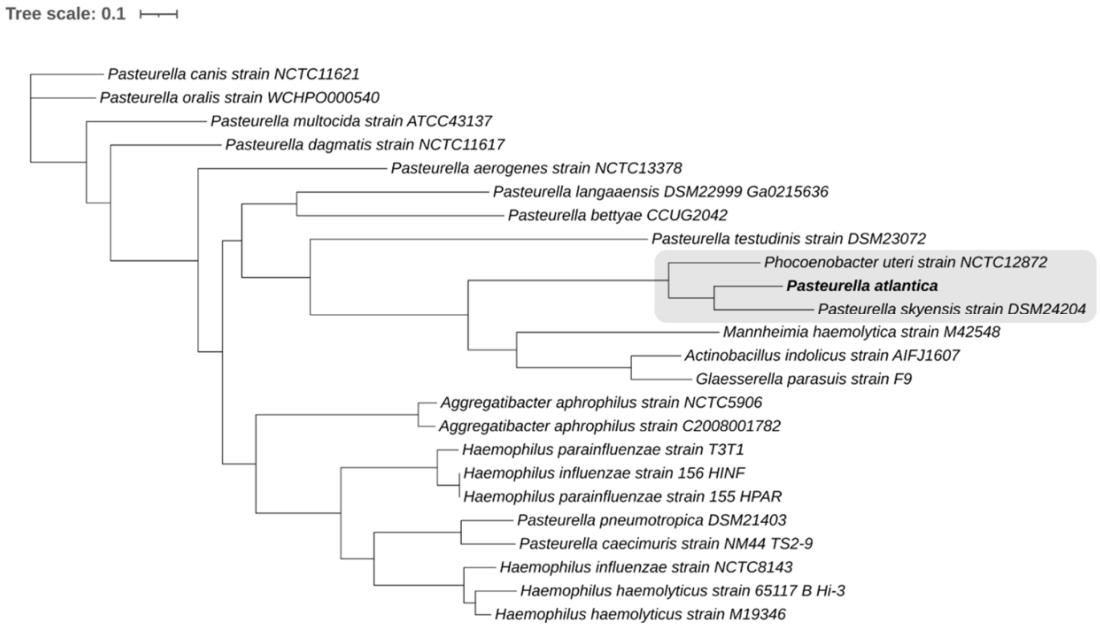

Figure 2. Phylogenetic tree of *P. atlantica* and Table 1 bacterial strains based on compariScheme 500 of randomly selected codons through their whole genome sequences. The monophyletic group in which the novel species belongs is highlighted, while the branch support in all nodes is >98% and has been based on 100 rounds of rapid bootstrapping.

According to the genome-wide analysis, *P. atlantica* clusters together with *P. skyensis* and *Ph. uteri*, forming a robust phylogenetic group supported by 100% confidence level (highlighted in Figure 2). Both monophyletic taxa are phylogenetically distinct since their nodes bear 100% support values.

3.3. Genomic Elements

The sequence of *P. atlantica* revealed mobile elements including genomic islands and prophages that should further be analyzed due to their possible role in pathogenicity [67–69].

3.3.1. Genomic Islands and Prophages

Bioinformatic analysis on the genome of *P. atlantica* illustrated eleven genomic islands (GIs) (Table 3) with various properties.

GC content of the GIs span between 29.4 and 36.6%. They encompass 207 coding sequences (CDS), approximately 10% of the *P. atlantica* total proteome, and many of them were attributed to virulence-related or broadly proteolytic activity functions. The most prominent putative virulence functions belong to CDS such as RTX-like toxin (GI_1), putative hemolysin toxin of *HlyC* family (GI_1), heme-binding protein (GI_4), bacterial peptidoglycan degrading enzyme of NIpC/P60 (GI_6), spermidine-putrescine binding (GI_8), acetyltransferase of gnat family (GI_9), ctx rstr-like repressor (GI_9), two-partner secretion (TPS) system protein (GI_10), and toxin-antitoxin system proteins (GI_11).

The genome of *P. atlantica* (NVI 9100) harbors five different prophages that were in silico predicted to be intact, hence inducible (Table 4).

Table 3. Genomic features of the eleven genomic islands that were found in the genome of *P. atlantica* (NVI 9100).

#	Sequence Location	Sequence Length (bp)	GC%	CDS
GI_1	GI_64353_82229	17,877	31.3	19
GI_2	GI_322423_340166	17,744	34.8	26
GI_3	GI_685873_700034	14,162	34.4	11
GI_4	GI_922164_930860	8697	36.6	12
GI_5	GI_966979_985107	18,129	34.7	26
GI_6	GI_1040130_1045358	5229	35.2	7
GI_7	GI_1058606_1061839	3234	33	9
GI_8	GI_1287395_1293893	6499	29.4	7
GI_9	GI_1369488_1396082	26,595	31.1	34
GI_10	GI_1468334_1477249	8916	29.7	12
GI_11	GI_1534357_1558731	24,375	35	44

Table 4. Genomic features of the five prophages that were found in the genome of *P. atlantica* (NVI 9100).

#	Sequence Location	Sequence Length (bp)	GC%	CDS	Most Closely Related Phage	Predicted Family	NCBI Accession Number
P_1	P_322716_342002	19,287	35	26	*Enterobacteria* phage P4	Unknown	NC_001609
P_2	P_972452_1005548	33,097	37.3	40	*Mannheimia* phage vB_MhS_535AP2	Siphoviridae	NC_028853
P_3	P_1030690_1066158	35,479	35.6	49	*Escherichia* phage D108	Siphoviridae	NC_013594
P_4	P_1527862_1576157	48,296	35.2	57	*Mannheimia* phage vB_MhM_587AP1	Myoviridae	NC_028898
P_5	P_2056529_2078637	22,109	35	28	*Hemophilus* phage SuMu	Myoviridae	NC_019455

P_1 and P_3 were found to be somewhat related to *Enterobacteria* phages, whereas P_2, P_4, and P_5 resemble prophages that have already been described in other *Pasteurellaceae* family bacteria [70,71]. According to BLAST sequence similarity, it was only possible to match 4 out of 26 CDS (7.7%) present in P_1 with any known phage genome implying that this is a quite novel prophage, uniquely present in the genome of *P. atlantica*. Similarly, P_3 seems to be a unique prophage as only 9 of its 49 CDS (18.4%) aligned with *Escherichia* phage D108. On the other hand, P_2 carried 9 of 40 CDS (22.5%) of *Mannheimia* phage vB_MhS_535AP2 while P_4 has homologs of 24 out of 57 CDS (42.1%) that were present in *Mannheimia* phage vB_MhM_587AP1. Last, P_5 has 23 out of 28 CDS (82.1%), resembling *Hemophilus* phage SuMu more, which was also anticipated due to the close phylogenetic relationships between *P. atlantica* and *G. parasuis* [40].

Interestingly, some of the predicted genomic islands and prophages do overlap in the genome (Figure 3). Specifically, the entire GI_2 is part of P_1, part of GI_5 is included in P_2, both GI_6 and GI_7 belong to P_3, while GI_11 is a part of P_5. Hence, there is a prominent connection between prophages and genomic islands that may be directly linked to the virulence properties of the bacterial host, with the former inciting the mobilization of the latter.

3.3.2. Virulence Factors

The proteome of *P. atlantica* consists of 2197 protein coding sequences (Supplementary Material S1), which were screened against an integrated matrix consisting of the VFDB, VICTORS, and PATRIC_VF databases to generate 565 unique amino acid sequence hits after discarding overlapping hits (Supplementary Material S1). This corresponds to ap-

proximately 26% of the 2197 predicted coding sequences. However, significant uncertainty needs to be removed from the analysis to achieve a better focus on the essential virulence factors present in the species. Subcellular localization of presumptive virulence factors showed that approximately 12% (70 out of 565) are presumably either extracellularly secreted (21) or with outer membrane surface exposure (49), setting these proteins as primary candidates for the pathogenicity of *P. atlantica*. The amino acid sequences of the predicted proteins accompanied by their most similar protein sequence GI number (SCL-BLAST), subcellular localization, adhesion, and transmembrane (TM) helices probabilities are reported in Supplementary Material S1. According to their amino acid sequences, 17 out of the 70 virulence factors with extracellular and outer membrane subcellular localization were the most critical for mediating bacterial invasion, such as adhesins with probabilities higher than 75%. Adhesins correspond to 0.8% of the total proteome (Figure 4), and since their expression may be a key determinant of the success of the infection process [72], they could play an imperative role in the pathogenicity of *P. atlantica* (Table 5).

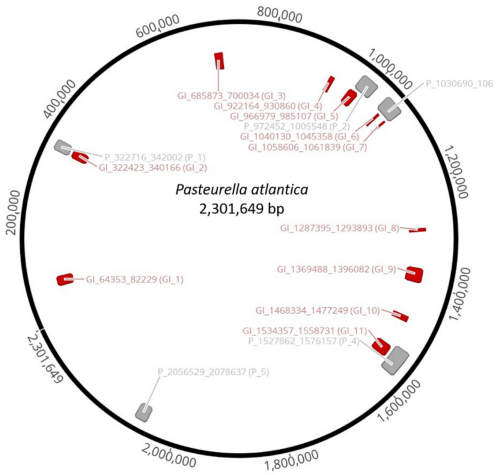

Figure 3. Genomic map of *P. atlantica* that highlights the eleven genomic islands (**red**) and five prophages (**grey**). Five out of eleven GIs are shown to overlap with four out of five prophages.

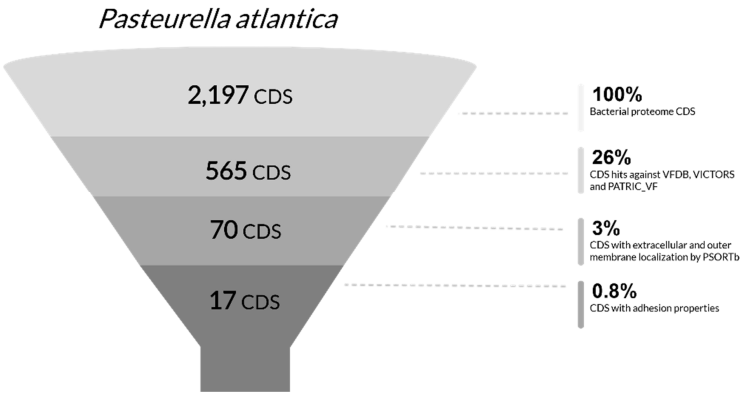

Figure 4. Filtration process of the CDS present in the genome of *P. atlantica* to determine the most likely virulence factors. According to the selection criteria applied during each step of the process, a total of 17 putative virulence factors were predicted as potentially important contributors to the pathogenicity of *P. atlantica*.

Table 5. Seven extracellular and ten outer membrane localized putative virulence factors of the genome of *P. atlantica* (NVI 9100). E: extracellular, OM: outer membrane, GI: Genomic island, P: Prophage, and ND: Not designated.

#	CDS	Localization (%)	Adhesin (%)	Signal Peptides	Protein Data Bank (PDB) Header (PDB Molecule)	Designated Genomic Area
1	Putative uncharacterized protein <Hia>	E (96)	92.5	n/a	Membrane protein/cell adhesion (Hia)	ND
2	Uncharacterized protein	E (96)	74.8	n/a	De novo protein (designed helical bundle)	GI_6 and P_3
3	Hemolysin-type calcium-binding region	E (96)	82	n/a	Cell adhesion (surface associated protein csha)	ND
4	Putative collagen triple helix repeat protein	E (96)	86.2	n/a	Membrane protein/cell adhesion (Hia)	GI_3
5	HbP1 protein	E (97)	81.5	n/a	Structural protein/contractile protein (collagen I alpha 1)	GI_2 and P_1
6	Uncharacterized protein	E (97)	76	Sec/SPI	Metal transport (hemophore)	ND
7	Pilus A	E (96)	82.9	n/a	Cell adhesion (fimbrial protein)	ND
8	Hep/Hag repeat protein	OM (95)	85.8	n/a	Cell adhesion (hep_hag family)	ND
9	Uncharacterized protein	OM (89)	82.6	n/a	Toxin (hemolysin)	ND
10	Protein PfhB1	OM (99)	75.4	n/a	Membrane protein (znud)	ND
11	Putative uncharacterized protein 4	OM (100)	74.7	n/a	Toxin (hemolysin)	ND
12	Putative septum site-determining protein MinC	OM (99)	82	n/a	Pili subunits (pili subunits)	ND
13	Putative uncharacterized protein 26	OM (95)	86.3	Sec/SPI	Pili subunits (pili subunits)	P_5
14	Phage-related protein tail component-like protein	OM (95)	74.6	n/a	Signaling protein receptor (interferon alpha beta receptor 1)	P_2
15	Type IV pilus biogenesis/stability protein PilW	OM (99)	80	n/a	Transferase (udp-n-acetylglucosamine–peptide n-)	ND
16	Auto transporter beta-domain protein	OM (95)	84.8	n/a	Hydrolase (esterase esta)	ND
17	Outer membrane antigenic lipoprotein B	OM (99)	79.7	n/a	Sugar binding protein (chitin elicitor-binding protein)	ND

The strongest virulence factor candidate is a novel uncharacterized protein, the function of which would have passed unnoticed without examining its protein structure. Therefore, PDB predictions based on protein fold recognition were included. All PDB predictions reported in Table 5 were above the 97% confidence level and encompassed major virulence components such as hemolysins, toxins, and pili subunits. Specific secretory signal peptides were detected in only 2 out of 17 adhesins, intended to be transported by Sec translocon followed by Signal Peptidase I cleavage (Sec/SPI) to be released to the host.

3.3.3. Vaccine Targets

All 70 CDS with extracellular and outer membrane localization were assessed as potential subunit vaccine candidates. However, apart from adhesion probability, additional factors such as transmembrane helices and orthologue analysis need to be considered for calculating their Vaxign-ML score, which was the metric applied to define their suitability as targets for vaccine development. All vaccine targets with Vaxign-ML scores higher than 99.5% are presented in Table 6, and they are therefore suggested as the most promising vaccine targets. All of the exact scores as well as further details about the remaining 60 extracellular and outer membrane CDS are included in Supplementary Material S1.

Table 6. Three extracellular and seven outer membrane localized proteins are the most promising candidates for vaccine development against *P. atlantica* (NVI 9100 and UiBP1-2013). E: extracellular, OM: outer membrane, GI: Genomic island, P: Prophage, and ND: Not designated.

#	CDS	Localization (%)	Adhesin (%)	Signal Peptides	PDB Header (PDB Molecule)
1	Putative uncharacterized protein <Hia>	E (96)	92.5	n/a	Membrane protein/cell adhesion (Hia)
2	Uncharacterized protein	E (96)	74.8	n/a	De novo protein (designed helical bundle)
3	Uncharacterized protein	OM (89)	82.6	n/a	Toxin (hemolysin)
4	Hep/Hag repeat protein	OM (95)	85.8	n/a	Cell adhesion (hep_hag family)
5	Protein PfhB1	OM (99)	75.4	n/a	Membrane protein (znud)
6	Putative uncharacterized protein 4	OM (100)	74.7	n/a	Toxin (hemolysin)
7	Serine protease sat autotransporter	E (84)	69.4	Sec/SPI	Hydrolase (hemoglobin protease)
8	PfhB1	OM (100)	69.5	n/a	Toxin (hemolysin)
9	Uncharacterized protein	OM (100)	63.3	Sec/SPI	Transport protein (translocation and assembly module tama)
10	Outer membrane protein assembly factor BamA	OM (100)	50.8	Sec/SPI	Membrane protein (outer membrane protein assembly factor BamA)

Since adhesion probability is not the only criterion for vaccine development, 6 out of the 10 CDS reported in Table 5 are potential virulence factors (Table 6). The list can further be confined by using VaxiJen as a tool to predict the protective antigenic properties of the vaccine targets. Only the putative uncharacterized protein and the Hep/Hag repeat protein had a score >80%, claiming that their antigenicity levels are high enough to trigger the host's immune response. However, the putative uncharacterized protein CDS not only was ranked first in both tables, scoring the highest Vaxign-ML (100%) and adhesin probability (92.5%), respectively, but also was characterized as a protective antigen, findings that render it the most significant candidate for virulence and potential vaccine development.

According to its amino acid sequence, BLASTP identified it as a YadA-like family protein, a collagen-binding adhesin originally characterized in *Yersinia enterocolitica* [73], that is also present in the genome of *P. skyensis*. However, although the query coverage was 100%, the identity rate was only 58.23%. Based on the predicted structure of the protein, Phyre2 [59] identified it as a membrane protein for cell adhesion similar to Hia (Tables 5 and 6), which is also a major adhesin expressed by *H. influenzae* [74]. Due to the prominent role that this uncharacterized membrane/cell adhesion protein seems to play in the virulence of *P. atlantica*, it was selected for further evaluation of its expression levels against lumpsucker head kidney leucocytes.

3.3.4. Functional Analysis of the Major Virulence Factor

Expression of <*hia*> in Bacterial Exponential Growth Phase

The predetermined time points for measurement were chosen based on growth curves of *P. atlantica* to coincide with the exponential growth phase of the bacteria. Furthermore, from the range of cDNA dilutions tested, 50 ng of cDNA per qPCR reaction yielded the clearest and most consistent results, and this dilution was used to calculate the <*hia*> expression. Out of the two reference genes tested, *gyrA* gave the most stable results compared to *rpoD* (Table 7) and was therefore used to calculate the relative expression of <*hia*> during cell growth and leucocyte exposure studies. This highlights the importance of

testing various reference genes for evaluation and stability for expression studies. In this study, although *rpoD* seemed to be a stable gene to use for analysis when primer assays were evaluated, experimental situations proved otherwise.

Table 7. Reference and target gene primers qPCR assay performance.

Gene	Amplicon Size (bp)	Assay Efficiency (%)	Correlation (R^2)
gyrA	200	102	0.998
rpoD	173	104	0.987
Putative uncharacterized protein <Hia>	192	96	0.984

The expression level of <*hia*> changed over time, when analyzing samples collected at different time points during the exponential growth phase. The expression levels were upregulated by 6.5×, 11.5×, and 15.7× at 16, 18, and 20 h, respectively, compared to the expression level at 14 h (Figure 5). The expression levels also mirror the exponential increase seen in bacterial numbers from previous work performed on growth curves for *P. atlantica* [28].

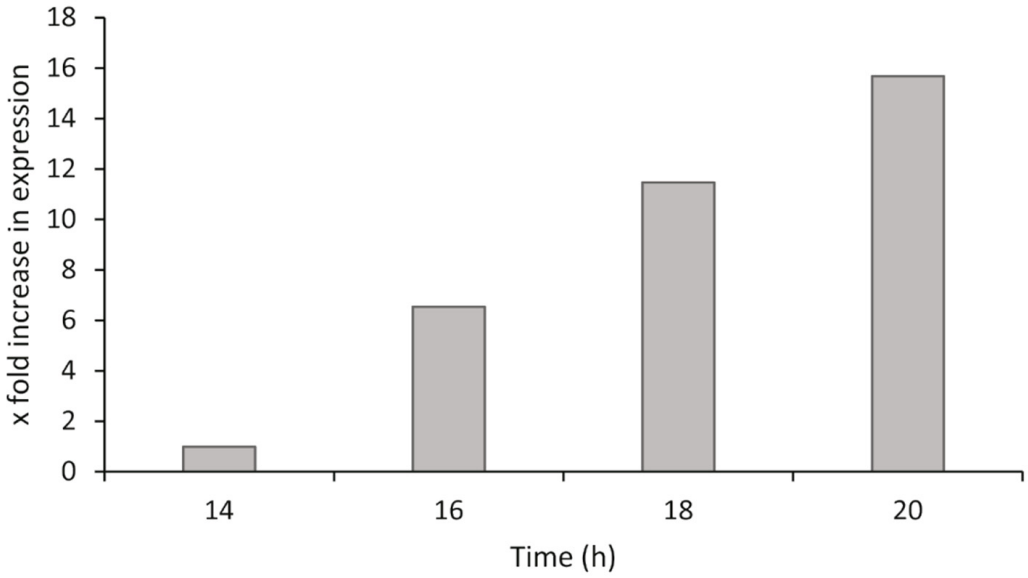

Figure 5. Expression levels of <*hia*> in *P. atlantica* (UiBP1-2013) during growth in liquid medium. The time period corresponds to the exponential growth phase of the bacteria.

Head Kidney Leucocyte (HKL) Exposure Analysis

The expression levels of <*hia*> increased with time, with the highest levels recorded at 9 and 12 h after leucocyte exposure (Figure 6), with significantly elevated expression levels compared with those in the early stages of the exposure (3 and 6 h after exposure). At 24 h after exposure compared to 3 and 6 h after exposure, the bacteria would have entered the lag phase of growth, and the expressions of both the reference and target genes are therefore not comparable with those from earlier time points. Furthermore, statistical analysis also showed that HKL from individual fish did not have an impact on the expression of <*hia*> ($p = 0.33$).

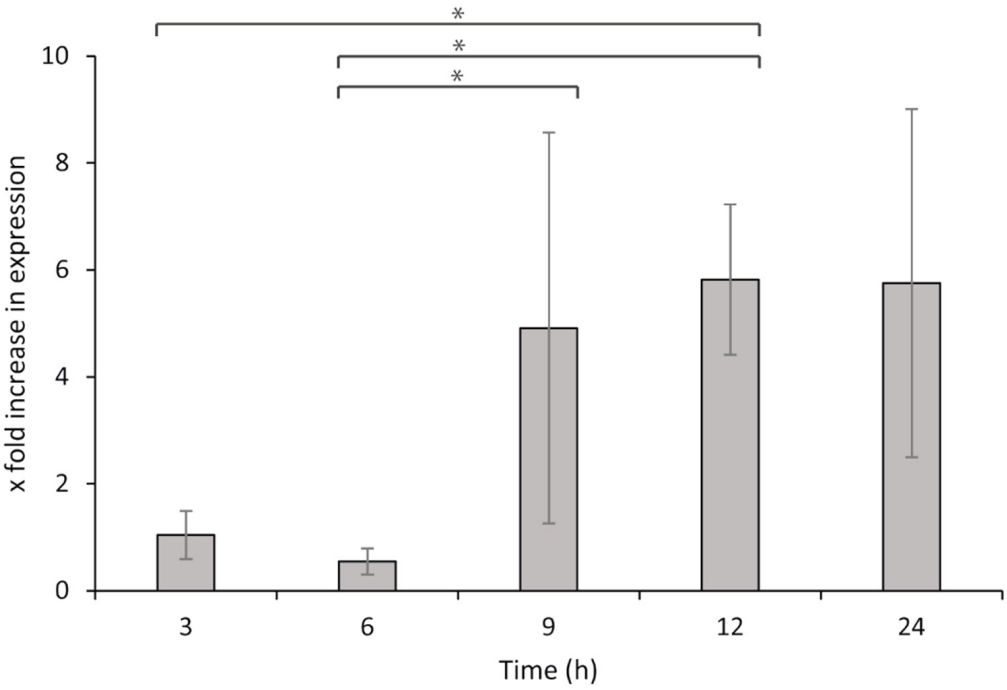

Figure 6. Fold increase in relative expression levels of <*hia*> in *P. atlantica* (UiBP1-2013) over time in the presence of lumpsucker head kidney leucocytes, compared to bacteria not exposed to HKL. Error bars represent standard deviation (n = 4). Asterisks represent significant differences in expression between the time points indicated by square brackets.

4. Discussion

The isolates of *P. atlantica* used in this work were isolated and reported as the causative agent of confirmed outbreaks of pasteurellosis in farmed lumpsuckers from Norway.

4.1. Phylogeny Reconstruction

Alarcón et al. [4] carried out preliminary phylogenetic analyses that included the isolates used in this study, using the 16S rRNA and *rpoB* genes, and it was suggested yet not concluded that the isolates might represent a novel species or subspecies. After examining the existing phylogeny of the *Pasteurellaceae* family, which is based on 16S rRNA genes, it can be deduced that using this molecular marker does not sufficiently resolve the true phylogenetic relationships, since it reflects rather incongruent phylogenetic relationships among its members. The available 16S rRNA based phylogenetic tree of the *Pasteurellaceae* family provides clear evidence that the polyphyletic nature prevails in the taxonomy of the family [75] and it has already been proven that there is room for improvement on molecular markers [76]. Despite ongoing research, the current phylogeny of the *Pasteurellaceae* family cannot be considered resolved according to the evolutionary principle of monophyletic taxa formation. Phylogenomic and molecular demarcation studies have been performed both on the major genera of the family such as *Pasteurella*, *Hemophilus*, and *Actinobacillus* [77] and on the reclassification of individual species such as the case of *Hemophilus parasuis* to *G. parasuis* [40].

Hence, the classification of *P. atlantica* in the current study was based on a dataset that includes representative species from both *Pasteurella* and other genera that belong to the *Pasteurellaceae* family to unravel a comprehensive taxonomy for *P. atlantica*. *P. multocida* is the most thoroughly studied member of *Pasteurellaceae* due to its high pathogenicity in a

broad range of livestock species [78,79]. The observed orthologous average nucleotide of 86.32% corroborates that *P. atlantica* and *P. skyensis* represent two different species. Whole genome-based phylogeny validated the novelty of the studied genome and its accurate taxonomic placement. Since *P. skyensis* and *Ph. uteri* are the only strains of marine origin out of the entire bacterial database, the habitat seems to be a strong differentiating factor for this taxon of *Pasteurellaceae*. Second, the host species of the bacteria in the marine cluster likely play a role for the speciation process. *P. atlantica* and *P. skyensis* are the closest relatives and both infect marine teleosts [35], whereas *Ph. uteri* isolated from a marine mammal is classified slightly more distant [36]. The major setback in this case is the lack of data in sequenced aquatic *Pasteurellaceae*, a fact that makes it quite precarious to formulate any solid conclusions regarding the trait of virulence. However, the highly emerging incidence and pathogenicity of *Pasteurella* in aquaculture has already brought [4,80] and will definitely bring more genomic data to the scientific community.

4.2. Virulence Genes

The scarcity of aquatic *Pasteurella* genomes, particularly from the aquaculture environment, highlights the significance of these *P. atlantica* genomes in exploring genomic elements, including putative virulence factors, antimicrobial resistance genes, and their potential implications for devising precaution strategies such as vaccine development in aquaculture. The abundance of mobile elements such as GIs (11) and prophages (5) in *P. atlantica* further enforces its high pathogenicity that has already been documented in previous studies [4,6,28]. GIs and prophages are very often found in pathogenic bacteria and have consistently been reported as major contributors of virulence factors to their bacterial hosts. Five out of the seventeen strongest candidate virulence factors of *P. atlantica* are in GIs and/or prophages, which in total account for roughly 10% of the genome. This finding is in line with the current literature, which supports the potential of bacterial mobilomes to harbor significant pathogenicity traits. A recent comparative genomic analysis of *Vibrio anguillarum*, a highly virulent aquaculture pathogen, revealed that 64 GIs that belonged to the accessory genome of the species were present in six out of nine of the most virulent strains [67]. In the same study, the role of prophages was also quite prominent, while it was recently proven in another study that marine *Vibrio* harbors a large number of prophage-encoded virulence factors [81]. In the pangenome analysis of *P. multocida*, GIs were also characterized as one of the most important mobile components not only as sources of virulence factors but also as facilitators of foreign genes acquisition. Prophages were again highlighted as core elements of the mobilome, and it was speculated that they may serve as precursors for the formation of GIs [69]. Additionally, facultative intracellular pathogens, such as *P. atlantica* [6,28,82], usually demonstrate quite variable genomes with a fourfold larger genomic content of mobile DNA such as transposable elements and prophages, when compared to obligate intracellular bacteria [83].

Mobile elements are characterized by a high frequency of transfer in the environment through the mechanism of horizontal gene transfer (HGT). HGT can be phage-mediated and can facilitate the dissemination of virulence-associated GIs within and between microbial communities [84], and the presence of a pathogenicity island can potentially define virulence in bacterial pathogens [85]. Apart from sophisticated algorithms [60,61], simpler methods that are based on the distinct GC content of horizontally transferred genes compared to that of the bacterial chromosome have been suggested for the prediction of GIs and prophages [86]. For instance, the pathogenicity islands in *Salmonella typhimurium* (SPIs) have lower GC content than the rest of the bacterial genome [87]. According to the in silico analysis of our study, the average GC content for GIs and prophages were 34.5% and 35.6%, respectively, in both cases lower than 42.1%, which is the average GC content of the *P. atlantica* genome.

Recent progress in in silico analysis tools allowed us to recognize and assess potential virulence factors by exploring the novel genome of *P. atlantica*. Such methodologies constitute fundamental principles of RV, which facilitate vaccine development, while turning

extensive, time consuming, and generalized lab work into target-oriented functional analysis and in vitro/in vivo trials. It was not until 2006 when the first standalone RV program designated as NERVE [88] was published, whereas great progress as well as comparative analysis have been achieved and performed in the RV field ever since [89]. The cornerstone of virulence factor prediction has been the determination of subcellular localization of bacterial proteins. Proteins that are extracellularly secreted or exposed on the bacterial surface have good chances to play a role in bacterial virulence.

4.3. Adhesins

Adhesins, for instance, which can facilitate the colonization of pathogens to mucosal or other biotic surfaces and which are essential for bacterial pathogenesis and survival may be prominent virulence factors and suitable candidates for vaccine development [72,90]. Screening of the *P. atlantica* genome for gene targets that can be further functionally evaluated as virulence factors generated the 17 strongest candidate genes that may have implications for bacterial virulence, based on subcellular localization and predicted adhesion properties.

Previously published bath, cohabitation, and intramuscular and intraperitoneal challenge trials that included the current *P. atlantica* isolate (UiBP1-2013) illustrated high and acute mortality rates, potentially indicating that extracellular proteins may have a substantial role in the infection dynamics [28], an interim conclusion that is supported by present findings. In our analysis, a putative uncharacterized protein ranked first as both a putative virulence factor and a vaccine candidate.

According to the in silico analysis, the role of this protein was directly linked to major adhesins YadA and Hia in *Yersinia* and *H. influenzae*, respectively, both of which are essential for successful infection of their hosts via human epithelia tissues [73,74,91]. Although their amino acid sequences were not identical, there was a clear connection based on query coverage and confidence level, which in both cases were 100%.

Trimeric autotransporters (TAAs) are a family of non-fimbrial, homotrimeric bacterial adhesins that are secreted by the Type 5c secretion system (T5cSS) and for which the main function is the attachment of pathogenic Gram-negative bacteria to hosts and abiotic surfaces. *Yersinia* adhesin A (YadA) is the most extensively studied member of this family, while others include *H. influenzae* adhesin (Hia) and *Neisseria* adhesin A (NadA).

The type 5c secretion system is of great importance in the development of multivalent recombinant and subunit vaccines, as it enables proteins to be exposed on the bacterial outer membrane, making them potential vaccine targets [92]. This could then also be used as the basis for further work to elucidate the structure of the Hia-like protein investigated in the current study.

YadA showcases the invasive nature of *Y. enterolitica* and *Y. pseudotuberculosis*, which colonize the intestine by adhering to the intestinal mucosa [93]. Immunologically, YadA has been shown to be a highly immunogenic antigen. In fact, Tahir and Skurnik (2001) [93] found that immunizing mice with live bacteria raised antibodies against only epitopes located in the N-termini, whereas using inactivated bacteria raised antibodies against other epitopes as well. In addition, a study [94] found that mice immunized using a recombinant YadA vaccine had higher survival compared to those vaccinated with inactivated *Y. pseudotuberculosis*.

Hia is an adhesin found in 25% of clinical non-typable strains of *H. influenzae* (NTHi). It mediates adherence to host cells and causes various diseases in humans, including respiratory tract infections and meningitis [95]. Hia differs slightly from other TAAs and is thought to represent a new sub-family. It too is highly immunogenic, however, and antibodies were strongly induced following natural infection observed in humans. Furthermore, opsonophagocytic antibodies were generated in serum in guinea pigs and mice [96]. However, due to its prevalence in so few strains and despite its high antigenicity, it cannot be considered a vaccine target on its own but in combination with High Molecular Weight adhesin 1 (HMW1), High Molecular Weight adhesin 2 (HMW2), and outer membrane

vesicles (OMVs). In this way, a vaccine containing the three adhesins covers 95% of NTHi and confers better protection [97].

The significantly higher expression levels of <*hia*> detected in the current study following in vitro exposure to lumpsucker leucocytes after 9 and 12 h together with the in silico results strengthen its candidacy as both a major virulence factor and a potential target for subunit protein antigen, mRNA, or DNA vaccine development.

The expression of <*hia*> in the absence of a host correlates with previously observed increases in bacterial growth rates [28] and indicates that the adhesin is typically expressed during bacterial growth. In the exposure experiment, bacteria were harvested from the growth medium at 18 h after inoculation when both bacterial growth and <*hia*> expression were at peak levels and combined with HKL in cell culture medium, which does not favor bacterial growth. The results from this experiment then demonstrate that the expression of <*hia*> increases significantly in the presence of the host, following an initial period of acclimation (up to 6 h, as seen in Figure 6). This implies that the presence of host leucocytes serves as a trigger for the bacteria to significantly increase the expression of adhesins (between 6 and 9 h after exposure) and shows that the increased expression is due to the presence of the host and not an increase in bacterial cell numbers. Furthermore, it indicates bacterial cell attachment to leucocytes as an initial step towards infection, as previously suggested by Ellul et al. (2019) [6].

4.4. Vaccine Development

Vaccines targeting adhesins can confer protection by two major mechanisms, one of which is by inducing neutralizing antibodies, which attach to adhesins and prevent attachment and subsequent infection. The other involves inducing opsonizing antibodies, which attach to adhesins and increase recognition of the pathogen by Fc receptors on phagocytes, leading to phagocytosis, or by activation of the complement system [98], as shown by Winter and Barenkamp [96] in their experiments with Hia.

Ellul et al. [6] recently assessed the efficacy of formalin-inactivated *P. atlantica* vaccine in lumpsucker, and although immune response and antibody production were prominent, the protection levels provided by the vaccine were relatively weak. Inactivated bacterin is the most common and traditional type of vaccine used in aquaculture; however, alternatives such as subunit recombinant, mRNA, or DNA vaccines may be the next step in solving the problem [99]. This is especially the case with the recent advances in human mRNA vaccines against SARS-CoV-2, which point to potential avenues for fish vaccines. These vaccines can be developed using various techniques, including harvesting and purifying the target directly from the pathogen, or using recombinant expression vectors, such as *E. coli* [99]. Pending further work, the former method could be a possible step forward in the case of *P. atlantica* as high expression rates were observed in bacteria in their exponential growth stage.

Some of the most successful recombinant protein subunit vaccines in the aquaculture industry include those against IPN (MSD Animal Health) and ISA in Chile (Centrovet). DNA vaccines have also been produced for use in Atlantic salmon against IHN in Canada and against SPD in Norway (Elanco). Subunit vaccines against the bacterial fish disease furunculosis in *Oncorhynchus mykiss* [100] have been tested. All targets for the vaccine development were identified in silico, employing the RV principles and found to be protective. Recombinant protein production has already been used in order to protect farmed fish against infections by *V. anguillarum*, *Edwardsiella tarda*, *Aeromonas hydrophila*, and *Francisella orientalis* [101] in all cases leading to the suggestion of potential vaccine candidates [102–104]. Following these lines, our study highlights the potential that in silico analysis may have in the future formulation of a novel vaccine against pasteurellosis.

Commercial vaccines are available only for a few infectious diseases in aquaculture, and given that the needs for fish and shellfish protein as well as the number of cultured aquatic species are constantly increasing, novel vaccine development will be essential to guarantee improved animal welfare and a safe, sustainable, and healthy product.

5. Conclusions

In this study, we report the first fully sequenced genomes of *P. atlantica*, an emerging pathogen in *Cyclopterus lumpus* in Norwegian and Scottish aquaculture. The wide phylogenetic distance from the closest bacterial relative, *P. skyensis*, and the very few available *Pasteurellaceae* members of aquatic origin highlight the need for intensification of sequencing efforts that should be focused on *Pasteurella* strains from the aquaculture environment. In silico genomic analysis revealed numerous target genes that may be responsible for the virulence of this species, and a major role was attributed to mobile elements such as prophages and GIs. Functional analyses showed that the expression levels of an uncharacterized protein increased significantly when *P. atlantica* was exposed to leucocytes from lumpsuckers, complementing the in silico analysis by designating a prospective virulence gene and by promising candidate for vaccine development. Further assessment of virulence in larger scale as well as formulation of experimental vaccines will shed more light on devising a precautionary strategy against a serious emerging disease in aquaculture.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms9061215/s1, Supplementary Material S1: Database with an extensive description of the sequences from the proteome-filtering process (virulence factors and adhesins)

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