Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Food Safety and Infection Biology

Philosophiae Doctor (PhD)
Thesis 2019:75

Comparative study of challenge models to evaluate protection after immunization with *Piscirickettsia salmonis*

Komparativ studie av smittemodeller for å evaluere grad av beskyttelse etter immunisering med *Piscirickettsia salmonis*

Karla Meza Parada

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Oslo (2019)

Thesis number 2019:75

ISSN 1894-6402

ISBN 978-82-575-1635-2

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“In my view, all that is necessary for faith is the belief
that by doing our best we shall succeed in our aims”
- Rosalind Franklin.

“If we knew what we were doing,
it wouldn’t be called research, would it?”
- Albert Einstein.

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ACKNOWLEDGMENT

I would like to acknowledge to the Norwegian research council and VESO for funding this project. To VESO for allowing me to perform fish trails in VESO Vikan facilities. I want to thanks to the Norwegian university of life science (NMBU) to let me perform the laboratory studies in the department of food safety and infection biology in the Veterinary faculty.

I, sincerely, would like to thanks:

First, to Arne Ruud and Marie Løvoll to believe in me and give me the possibility to do this PhD. To my supervisors Marie Løvoll, Henning Sørum, Ane M. Bjelland and Makoto Inami for guiding me in this long and hard process of learning about the research world and for sharing with me all your knowledge. To Duncan Colquhoun for kindly facilitating a Norwegian strain of *P. salmonis* and the specific antibody used in this study. To Kira Salonius for the reading proof of this thesis and to allow me to work on your team.

I would like to thanks to INBIOGEN laboratory staff from the Veterinary medicine faculty at University of Chile, for the training and the help before this journey, without your help at the beginning, this would have been much harder.

Thanks to all VESO Vikan staff for all the support (technical and personal) given during my two long stays in Namsos, you made it easier.

To Lindern crew for the amazing working environment, I hope you keep that essence wherever you go. Thanks to Øysten W., Elisabeth, Stine, Ingvild and Hege for the valuable discussions, technical support and the patience to help me with all my difficulties during this project.

To Alf S. Dalum and Christian Wallace for friendship and professional support during many talks, discussions and for sharing all your experience and knowledge with me along the way.

I do not have more than gratitude and affection to my entire bacteriology group: Gaute, Line, Silje, Erik, Simen, Leif, Tatiana, Cristopher, Håkon, Özgün, Aud Kari, Mari, Stanislav, Øystein K., Turhan, Ingrid, Sabrina, Ane and Henning for the amazing energy and support during the difficult and not that difficult days, without all of you this could not be possible. Sabrina and Mari, you made this last

summer of work less lonely and much funnier, and thanks for the support always. I want to thank, especially, to Aud Kari and Özgün for being my crisis management team in the last year of this journey, avoiding that I jumped from the third floor or I hit my head against the wall a couple (or many) times.

To the nicest PhD students Anne B. and Mette H. for the friendship and support, talks, coffees and laughs about our, sometimes or most of the times, frustrating process.

To my lovely and amazing friends: Kari, Alex, Kitty, Mauro, Felipe, Leidy and Sigmund for being my family in Norway and making me feel like at home so many times. To Cote, Vane, Pia, Dani, Carlos and Cristian B. for that unconditional support and good vibes from so far away. Thank you all for believe in me always! Love you all guys!

To the last, but not least, my family (I will do this in Spanish):
Tengo que agradecer a mis maravillosos padres, Carla y Mario, y mi hermano, Mario, por ese apoyo incondicional en todos los proyectos en los que me embarco y durante todos estos años. Gracias por nunca cortarme las alas. Sé que no fue fácil dejarme ir, pero nunca lo demostraron y lo único que recibí fueron palabras de apoyo y orgullo. Agradecerles también por las eternas conversaciones a la distancia llenas de consejos y paciencia, gracias por estar siempre presente. A mis abuelos, Chino y Ana, por estar siempre pendientes desde tan lejos, aprovecho de disculparme por no haber tenido una comunicación aún más fluida, espero me perdonen por eso. Al resto de la familia (consanguíneos y de la vida) muchas gracias por estar pendientes y por las buenas vibras que enviaron siempre y que recibí con cariño.

I will be grateful forever with all the people that was part of these years of my life and have been part of this process. I will never forget you all.

With love,
Karla Meza.
Oslo, 2019.

SUMMARY

The Chilean salmon industry is considered one of the most important aquaculture industries worldwide, only bypassed by Norway. In Chile, salmon represents the second-biggest export product after copper. Due to the fast development of the industry and the introduction of Atlantic salmon to the Pacific Ocean, the industry has been affected by infectious diseases caused by viruses, bacteria and parasites. Piscirickettsiosis is the infectious disease that has produced the highest economic losses during the last 30 years in Chilean aquaculture; around USD 700 million per year. The disease was described for the first time in 1989, in the Los Lagos region, a southern part of Chile. The salmonid species that are susceptible to piscirickettsiosis are coho salmon (*Oncorhynchus kisutch*), Atlantic salmon (*Salmo salar* L.), rainbow trout (*Oncorhynchus mykiss)* and chinook salmon (*Oncorhynchus tshawytscha*). Piscirickettsiosis is an aggressive systemic disease with mortalities that can fluctuate between 30-90% of the affected fish. In general, the outbreaks develop after transfer of the fish to the sea and close to harvest. The etiological agent of the disease is *Piscirickettsia salmonis*; a Gram-negative, non-motile, intracellular facultative bacterium. The most common pathological changes in fish infected with *P. salmonis* are lethargy, darkness of the skin, erratic swimming, and anorexia. Internally, the visceral tissues (liver, kidney and spleen) are most affected, but heart, brain, ovaries and skeletal muscle are also compromised. The control measurements used by the Chilean industry to reduce the casualties of the disease are among others the use of antibiotics (as treatment and prophylactic) and vaccination.

The frequent and widespread outbreaks of piscirickettsiosis account for the high use of antibiotics in Chilean salmon farms. In 2014, the use of antibiotics reached a total of 563.2 tonnes in comparison with the Norwegian aquaculture industry that used only 0.5 tonnes the same year. Vaccination is frequently used to prevent outbreaks of piscirickettsiosis. There are 57 vaccines registered for salmonid fish in Chile, of which 32 vaccines include a *P. salmonis* component. From the 32 vaccines with *P. salmonis* antigens, there are seven monovalent and 25 multivalent vaccines. The 32 vaccines with *P. salmonis* antigens comprise 28 inactivated vaccines, three subunit vaccines and one live-attenuated vaccine.

Experimental challenge trials represent an important tool for evaluation of the efficacy of health feeds, pharmaceutical treatments, prophylactic measurements and genetic resistance. *In vivo* challenge trials have been used for many years for the evaluation of vaccine efficacy. The challenge models commonly used are challenge by intraperitoneal injection of the test fish, cohabitation of i.p. injected shedders fish with test fish and by immersion of the test fish.

The main objective of this thesis was to increase the knowledge on experimental challenge models with *Piscirickettsia salmonis* and define the *in vivo* model of preference for evaluation of efficacy of vaccines intended for Atlantic salmon. In addition, the aim was to increase the understanding on piscirickettsiosis' pathogenesis and to characterize a *P. salmonis* isolate *in vitro*. To achieve this, three sub-goals were defined: 1) To compare the progression of piscirickettsiosis development after experimental challenge by intraperitoneal injection or cohabitation; 2) To compare the protection induced by experimental vaccines against *P. salmonis* after immunization and experimental challenge by intraperitoneal injection or cohabitation; and 3) To characterize the *P. salmonis* isolate used for *in vivo* experimental trials after cultivation in broth media.

As a result of this investigation, three scientific articles were written, each with the objective to answer the specific goals. Article I, "Development of piscirickettsiosis in Atlantic salmon (*Salmo salar L.*) smolts after intraperitoneal and cohabitant challenge using an EM90-like isolate: A comparative study", demonstrated that there were no significant differences in disease development after challenge by intraperitoneal injection compared with cohabitation. Furthermore, changing the bacterial cultivation from solid agar to liquid medium to produce the inoculum did not affect the disease development in a controlled environment. Article II, "Comparative study of experimental challenge by intraperitoneal injection and cohabitation of Atlantic salmon (*Salmo salar* L.) after vaccination against *Piscirickettsia salmonis* (EM90-like)", revealed that the protection induced by experimental vaccines is similar after challenge by intraperitoneal injection or cohabitation. Article III "Cultivation and characterization of a *Piscirickettsia salmonis* EM90-like isolate used for *in vivo* challenge of Atlantic salmon (*Salmo salar* L.)", provided new insight to the growth dynamics of *P. salmonis*. Furthermore, phylogenetic studies confirmed that the isolate used in the current work belongs to the EM90 genogroup and that there is a phylogenetic separation between the genogroups EM90 and LF89. Moreover, the study demonstrates that the change of cultivation media from solid agar to liquid broth did not affect the virulence of the isolate.

Further investigations should be done to obtain a better understanding of *P. salmonis* and its interaction with its host during infection. The use of experimental challenge models must be carefully evaluated to apply a model that most closely mimics real-life conditions in the field. The present work reveals that the protection induced by vaccination is similar whether the fish are challenged by i.p. injection or cohabitation. However, further work is needed to refine the model to better separate the effective vaccines from the ineffective ones.

SAMMENDRAG

Den chilenske laksenæringen regnes som en av de viktigste oppdrettsnæringene over hele verden, kun forbigått av Norge. Laks representerer Chiles nest største eksportprodukt etter kobber. På grunn av den raske utviklingen av oppdrettsnæringen og innføringen av atlantisk laks til Stillehavet, har industrien blitt påvirket av smittsomme sykdommer forårsaket av virus, bakterier og parasitter. Piscirickettsiose er laksesykdommen som har medført de største økonomiske tapene de siste 30 årene i chilensk akvakultur, og de tapte verdiene er beregnet til rundt 700 millioner US dollars per år. Piscirickettsiose ble beskrevet for første gang i 1989, i Los Lagos-regionen i den sørlige delen av Chile. Artene av laksefisk som er mottagelige for piscirickettsiose er coho-laks (*Oncorhynchus kisutch*), atlantisk laks (*Salmo salar* L.), regnbueørret (*Oncorhynchus mykiss*) og chinook-laks (*Oncorhynchus tshawytscha*). Piscirickettsiose er en aggressiv systemisk sykdom med dødelighet som varierer mellom 30-90% av den smittede populasjonen. Sykdomsutbrudd opptrer vanligvis etter overføring av fisken til sjø, samt i perioden før fisken er slakteklar. Sykdommens kausale agens er *Piscirickettsia salmonis*; en Gram-negativ, amotil, fakultativ intracellulær bakterie. De vanligste symptomene hos fisk som er smittet med *P. salmonis* er slapphet, pigmentforandringer i huden, unormal svømming og anoreksi. Patologiske forandringer sees først og fremst i viscerale organer (lever, nyre og milt), men hjerte, hjerne, eggstokker og skjelettmuskulatur kan også affiseres. Forebyggende tiltak mot sykdommen er bruk av antibiotika (både profylaktisk og som behandling) og vaksinasjon. Stor geografisk utbredelse, samt hyppige utbrudd har medført høy bruk av antibiotika. I 2014 nådde bruken av antibiotika totalt 563,2 tonn sammenlignet med den norske oppdrettsnæringen som bare brukte 0,5 tonn samme år. Vaksinasjon brukes ofte for å forhindre utbrudd av piscirickettsiose. Det er registrert 57 vaksiner for laksefisk i Chile, hvorav 32 vaksiner inkluderer en *P. salmonis*-komponent. Blant de 32 vaksinene med *P. salmonis*-antigener er det syv monovalente og 25 multivalente vaksiner. Blant de 32 vaksinene med *P. salmonis*-antigener inneholder 28 inaktiverte vaksiner, tre underenhetsvaksiner og en levende attenuert vaksine.

Eksperimentelle smitteforsøk representerer et viktig verktøy for å evaluere effekt av helsefôr, farmasøytiske legemidler, profylaktiske vaksiner og genetisk resistens. *In vivo* smitteforsøk har blitt brukt i mange år for å dokumentere effekt av vaksiner. Smittemodellene som ofte brukes er smitte ved i.p. injeksjon av forsøksfisk, kohabitasjon av i.p.-injiserte shedderfisk med forsøksfisk og badesmitte av forsøksfisk.

Hovedmålet med dette arbeidet var å øke kunnskapen om eksperimentelle smittemodeller med *P. salmonis* og å vurdere hvilken smittemodell som er best egnet til å avdekke effekt av vaksiner mot *P. salmonis* i atlantisk laks. I tillegg var målet for arbeidet å øke forståelsen for sykdomsutviklingen ved

infeksjon med *P. salmonis*, samt å karakterisere et *P. salmonis*-isolat *in vitro*. For å oppnå dette ble tre spesifikke mål definert: 1) Å sammenligne utviklingen av piscirickettsiose etter eksperimentell smitte ved intraperitoneal injeksjon eller kohabitasjon; 2) Å sammenligne beskyttelsen indusert av eksperimentelle vaksiner mot *P. salmonis* etter immunisering og eksperimentell smitte ved intraperitoneal injeksjon eller kohabitasjon og 3) Å karakterisere *P. salmonis*-isolatet brukt i *in vivo* smitteforsøk etter dyrking i flytende medium.

Med bakgrunn i resultatene ble tre vitenskapelige artikler utarbeidet. Artikkel I, "Development of piscirickettsiosis in Atlantic salmon (*Salmo salar* L.) smolts after intraperitoneal and cohabitant challenge using an EM90-like isolate: A comparative study", viste at det ikke var noen signifikante forskjeller i sykdomsutvikling etter smitte ved intraperitoneal injeksjon sammenlignet med kohabitasjon. Endring av dyrkingsforhold for bakterien fra fast agar til flytende medium påvirket ikke sykdomsutviklingen i et kontrollert smitteforsøk. Artikkel II, "Comparative study of experimental challenge by intraperitoneal injection and cohabitation of Atlantic salmon (*Salmo salar* L.) after vaccination against *Piscirickettsia salmonis* (EM90-like)", viste at beskyttelsen indusert av eksperimentelle vaksiner er tilnærmet lik etter smitte ved intraperitoneal injeksjon eller kohabitasjon. Artikkel III " Cultivation and characterization of a *Piscirickettsia salmonis* EM90-like isolate used for *in vivo* challenge of Atlantic salmon (*Salmo salar* L.)" ga ny innsikt i vekstdynamikken til *P. salmonis*. Videre bekreftet fylogenetiske studier at isolatet som ble brukt i dette arbeidet tilhører EM90-genogruppen, og at det er et fylogenetisk skille mellom genogruppene EM90 og LF89. Forsøkene viste videre at endring av dyrkingsmedium fra agar til flytende medium ikke påvirket virulensen til isolatet.

Ytterligere undersøkelser bør gjøres for å få en bedre forståelse av *P. salmonis* sin interaksjon med verten under et infeksjonsforløp. Bruken av eksperimentelle smittemodeller må evalueres nøye for å benytte en modell som best etterligner kommersielle oppdrettsforhold i felt. Dette arbeidet viser at beskyttelsen indusert ved vaksinasjon er lik om fisken deretter blir smittet ved i.p. injeksjon eller kohabitasjon. Det er imidlertid nødvendig med ytterligere arbeid for å optimalisere modellen for bedre å skille de effektive vaksinene fra de ineffektive.

RESUMEN

La industria del salmón Chilena es considerada como una de las más importantes a nivel mundial, posicionándose en segundo lugar de exportación de salmón después de Noruega. Al interior del país la industria es de gran importancia económica quedando, como producto de exportación, en segundo lugar después del cobre. Debido al rápido crecimiento, la industria ha tenido que enfrentar dificultades en diferentes aspectos como las relacionadas con enfermedades infecciosas producidas por virus, bacteria y parásitos. Piscirickettsiosis es la enfermedad infecciosa que mayores pérdidas económicas ha producido en los últimos treinta años para la acuicultura chilena con pérdidas que bordean los 700 millones de dólares al año. Se describió por primera vez en el año 1989, en la región de Los Lagos, al sur de Chile. Las especies susceptibles son salmón coho (*Oncorhynchus kisutch*), salmón del Atlántico (*Salmo salar* L.), trucha arcoíris (*Oncorhynchus mykiss)* y salmón chinook (*Oncorhynchus tshawytscha*). Piscirickettsiosis es una enfermedad sistémica agresiva con mortalidades que pueden fluctuar entre 30-90% de los individuos. Por lo general, los brotes se presentan luego de la transferencia de los peces al mar, más específicamente cuando están cercanos a la cosecha. El agente etiológico de esta enfermedad es *Piscirickettsia salmonis*, una bacteria Gram-negativa, no motil e intracelular facultativa. Los signos patológicos más comunes presentes en los peces afectados por *P. salmonis* son letargia, oscurecimiento de la piel, nado errático y anorexia. Internamente, los tejidos más afectados son los viscerales (hígado, riñón y bazo), pero también se ven afectados el corazón, cerebro, ovarios y musculo esquelético. Dentro de las medidas de control empleadas en el país para disminuir las causalidades de la enfermedad se encuentran el uso de antibióticos (como tratamiento y profiláctico) y la vacunación. Debido a que piscirickettsiosis es la enfermedad infecciosa que produce el mayor porcentaje de mortalidad, se le ha responsabilizado por el uso excesivo de antibióticos en la industria salmonera Chilena, llegando a utilizar la cantidad más alta en el año 2014 con un total de 563.2 toneladas en comparación con Noruega que solo utilizo 0.5 toneladas ese mismo año. En Chile, existen 57 vacunas registradas para el uso en peces, de las cuales 32 son contra *P. salmonis*, de estas, 25 son multivalente y siete son monovalente. De las vacunas específicas contra la enfermedad 28 son inactivadas, tres son sub-unitaria y una viva atenuada.

Los desafíos experimentales con peces son una herramienta importante no solo para la evaluación de la eficacia de muchos productos como alimentos, fármacos como tratamientos para enfermedades, medidas profilácticas y estudios genéticos. Para la evaluación de la eficacia de las vacunas se han utilizado por muchos años los ensayos de desafío *in vivo*. Los modelos para los ensayos de desafío comúnmente utilizados son el de inyección intraperitoneal de los peces, de cohabitación con peces inyectados intraperitonealmente y el de inmersión.

El objetivo principal de este estudio fue incrementar el conocimiento sobre modelos de desafío con *Piscirickettsia salmonis* y definir el modelo *in vivo* de preferencia para la evaluación de la eficacia de vacunas desarrolladas para salmón del Atlántico. Junto con ello, el objetivo era aumentar la comprensión sobre la patogenia de piscirickettsiosis y caracterizar un aislado de *P. salmonis in vitro*. Para ello tres objetivos específicos fueron definidos: 1) comparar la progresión de la enfermedad después del desafío experimental de peces por inyección intraperitoneal o cohabitación; 2) comparar la protección inducida por vacunas experimentales después de la inmunización de los peces y desafío experimental de los mismos por inyección intraperitoneal o cohabitación; 3) caracterizar el aislado de *P. salmonis* usado para ensayos de desafío *in vivo* después del cultivo en medio líquido.

Como resultado de esta investigación se desarrollaron tres artículos científicos, cada uno con el fin de responder cada uno de los objetivos específicos. Artículo I, "Development of piscirickettsiosis in Atlantic salmon (*Salmo salar* L.) smolts after intraperitoneal and cohabitant challenge using an EM90-like isolate: A comparative study.", demuestra que el cambio en la forma de cultivo de la bacteria no cambia el desarrollo de la enfermedad bajo ambientes controlados. Al mismo tiempo, se puede observar la ausencia de diferencias importantes entre ambos modelos de desafío. Articulo II, "Comparative study of experimental challenge by intraperitoneal injection and cohabitation of Atlantic salmon (*Salmo salar* L.) after vaccination against *Piscirickettsia salmonis* (EM90-like).", donde luego de comparar los dos modelos de desafío, esta vez después de la inmunización de los peces, se confirmaría las similitudes presentes en ambos modelos. Junto con ello se evaluaron algunos inmunogenes que dan como resultados algunas directrices relacionadas con la respuesta inmunológica de los peces frente a la vacunación y al desafío de los individuos contra *P. salmonis*. Además de contribuir con mayores antecedentes relacionados con la patogénesis de piscirickettsiosis. Finalmente, articulo III, "Cultivation and characterization of a *Piscirickettsia salmonis* EM90-like isolate used for *in vivo* challenge in Atlantic salmon (*Salmo salar* L.)", proporciona nuevas ideas relacionadas con la dinámica de crecimiento de *P. salmonis*. Además, el estudio filogenético confirmó que el aislado utilizado en este estudio pertenece al genogrupo EM90 y la separación entre los genogrupos EM90 y LF89. También, se demostró que el cambio de medio de cultivo de agar a liquido no afecto la virulencia del aislado.

Futuras investigaciones deberían desarrollarse para lograr un mejor entendimiento de *P. salmonis* y su interacción con el hospedero durante la infección. El uso de modelos de desafío experimental debería ser cuidadosamente evaluado para poder aplicar el modelo que simula más realísticamente las condiciones en terreno. Este trabajo revela que la protección generada por la vacunación es similar entre peces desafiados con el modelo intraperitoneal o por cohabitación. Sin embargo, es necesario

desarrollar más investigaciones con el fin de perfeccionar el modelo que logre separar de una mejor forma las vacunas eficaces de aquellas que no lo son.

LIST OF ABBREVIATIONS

Abs	Antibiotics
dpc	Days post challenge
IHC	Immunohistochemistry
in vitro	"within the glass"
in vivo	"within the living"
i.p.	Intraperitoneal
RT-qPCR	Quantitative reverse transcription PCR
qPCR	Quantitative polymerase chain reaction
SAG	Servicio agrícola y ganadero (Agricultural and livestock service)
SERNAPESCA	Servicio nacional de pesca y acuicultura (Fishing and aquaculture national service)
SUBPESCA	Subsecretaria de pesca y acuicultura (Fishing and aquaculture sub-secretary)
wpc	Weeks post challenge

LIST OF ARTICLES

Article I:

Development of piscirickettsiosis in Atlantic salmon (*Salmo salar* L.) smolts after intraperitoneal and cohabitant challenge using an EM90-like isolate: A comparative study.

Karla Meza, Makoto Inami, Alf S. Dalum, Ane M. Bjelland, Henning Sørum, Marie Løvoll.

J Fish Dis. 2019;00:1–11. https://doi.org/10.1111/jfd.13004

Article II:

Comparative study of experimental challenge by intraperitoneal injection and cohabitation of Atlantic salmon (*Salmo salar* L.) after vaccination against *Piscirickettsia salmonis* (EM90-like)

Karla Meza, Makoto Inami, Alf S. Dalum, Hege Lund, Ane M. Bjelland, Henning Sørum, Marie Løvoll.

Accepted in Journal of Fish Diseases.

Article III:

Cultivation and characterization of a *Piscirickettsia salmonis* EM90-like isolate used for *in vivo* challenge in Atlantic salmon (*Salmo salar* L.)

Karla Meza, Marie Løvoll, Leif Lotherington, Cristian Bravo, Simen F. Nørstebø, Jessica Dörner, Victor Martinez, Henning Sørum, Ane M. Bjelland.

Submitted to Veterinary Microbiology

INTRODUCTION

Chilean salmon industry

Cultivation of fish species has been undertaken for centuries around the world. Freshwater species were reared in ponds and over time, the cultivation of salmonid species gradually developed to include both a freshwater and a seawater phase as is natural of an anadromous species. The breakthrough for farming of Atlantic salmon, as we know it today, started in Norway in the 1970s by fishermen and coastal businesses. Since then, the salmon industry has developed rapidly because of technological improvements and governmental support promoting the economic development of the industry[1, 2]. The salmon production around the world is dominated by four countries: Norway, Chile, Scotland and Canada[3]. The coastlines and suitable water temperatures are key factors for the location of the salmon farms around the world (Figure 1).

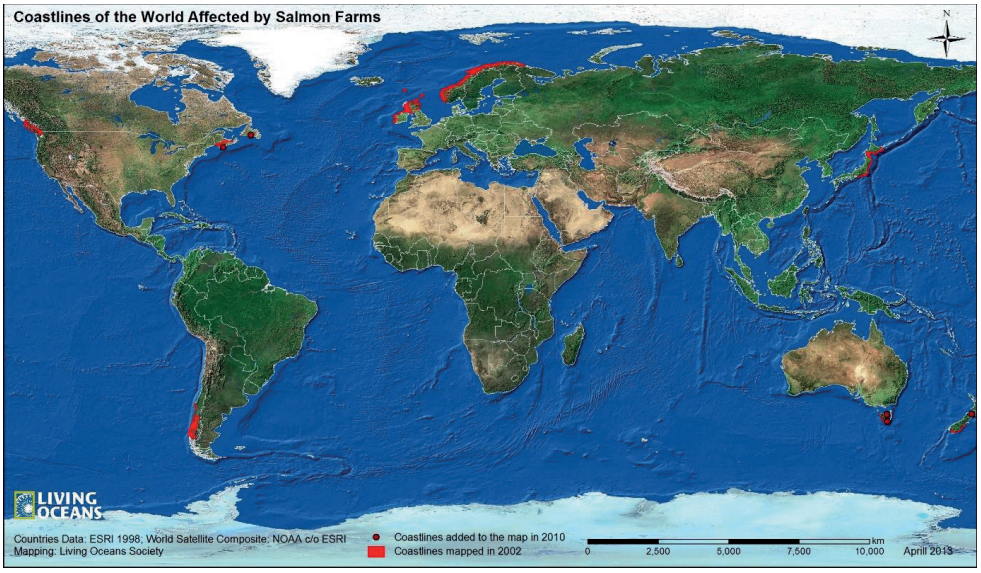

Figure 1. Worldwide distribution of salmon farms. Source: Living Oceans, 2013.

In Chile, the salmon industry had its inception in 1979[4], when Chile was considered a suitable location based on its geography and seasonal characteristics and initial investment was made by companies producing other farmed animals[5]. By 1992, Chile was the second largest producer of

farmed salmonids after Norway[4]. Chile maintained its position until 2007 when the industry was affected by the viral disease infectious salmon anemia (ISA). The production rapidly decreased from 379 000 tonnes in 2007 to around 211 000 tonnes in 2009[6]. More recently, the Chilean salmon industry has returned to be the second largest aquaculture industry worldwide with a production of 605 800 tonnes annually of Atlantic salmon (*Salmo salar* L.) in 2016[7] and an export value of US $2.6 billion[8]. The Chilean salmon industry is responsible for the second largest national export product after copper. Currently, in Chile there are a total of 15 companies producing salmon, of those, five are international companies and 10 are national[9]. The farms are located in the southern part of the country, concentrated in the Los Lagos and Magallanes regions[5, 10]. The importance of the Los Lagos region to the aquaculture and fisheries reveals the growing importance of the Chilean salmon aquaculture to raise the national profile in the global economy[5]. The Chilean production of salmonids comprises three species: coho salmon (*Oncorhynchus kisutch*), Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss)*. In 2018, 70% of the salmonid species produced by marine farms were Atlantic salmon. Rainbow trout and coho salmon accounted for 19% and 11% of the production, respectively[11] (Figure 2).

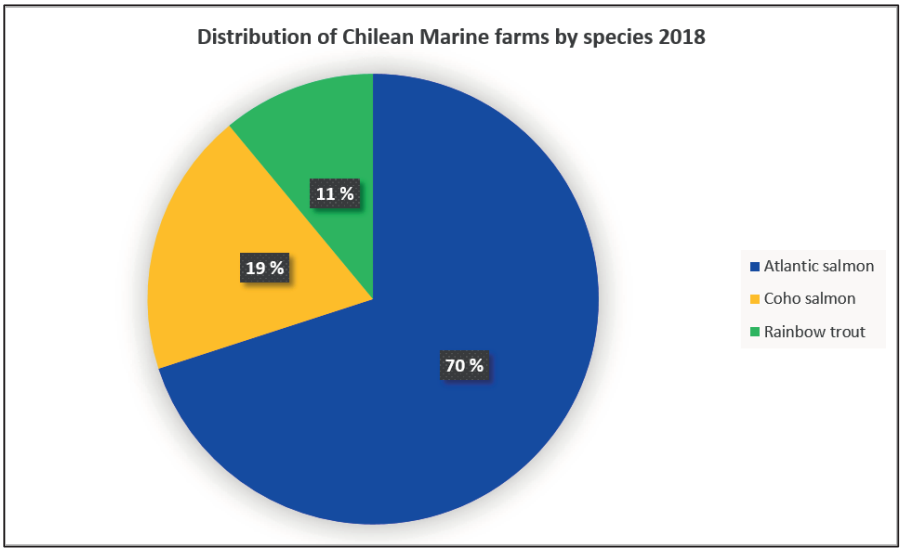

Figure 2. Distribution of Chilean marine farms by specie 2018. Source: SERNAPESCA

Figure 3. Chilean salmon farm and its cages. Source: The fish Site (https://thefishsite.com).

Aquaculture has been the fastest growing food-producing sector worldwide for years[12] and the Chilean industry is considered the fastest-growing salmon producer in the world[6]. The rapid growth in aquaculture brings along well-known challenges when it comes to keeping animals within a confined space. The fish are susceptible to transmittable, infectious diseases and the increased stocking density elevates the stress levels in fish and make them more vulnerable to diseases[12]. Infectious diseases caused by viral, bacterial and eukaryote pathogens have been a major yield-limiting problem in the production[13, 14]. The diseases that are considered as high-impact for salmonids in the Chilean industry are listed in Table 1 and the mortality by cause is shown in Figure 4[11].

Table 1. List of high-impact diseases of Chilean salmon industry based on classification developed by the Fishing and aquaculture sub-secretary (SUBPESCA) from the Economy and tourism ministry of Chile[15].

Disease	Etiological agent
Infectious salmon anemia (ISA)	*Orthomyxovirus* ISA virus (ISAV)
Infectious pancreatic necrosis (IPN)	Infectous pancreatic necrosis virus (IPNV)
Piscirickettsiosis	*Piscirickettsia salmonis*
Caligidosis	*Caligus rogercresseyi*
Streptococcosis	*Streptococcus phocae*
Flavobacteriosis	*Flavobacterium psychrophilum*
Atypical furunculosis	*Atypical Aeromonas salmonicida*
Vibriosis	*Vibrio ordalii; Listonella anguillarum*
Amoebic gill disease	*Neoparamoeba perurans*
Smolt hemorrhagic syndrome	Not identified
Heart and skeletal muscle inflammation (HSMI)	*Piscine reovirus* (PRV)
Tenacibaculosis	*Tenacibaculum* sp.

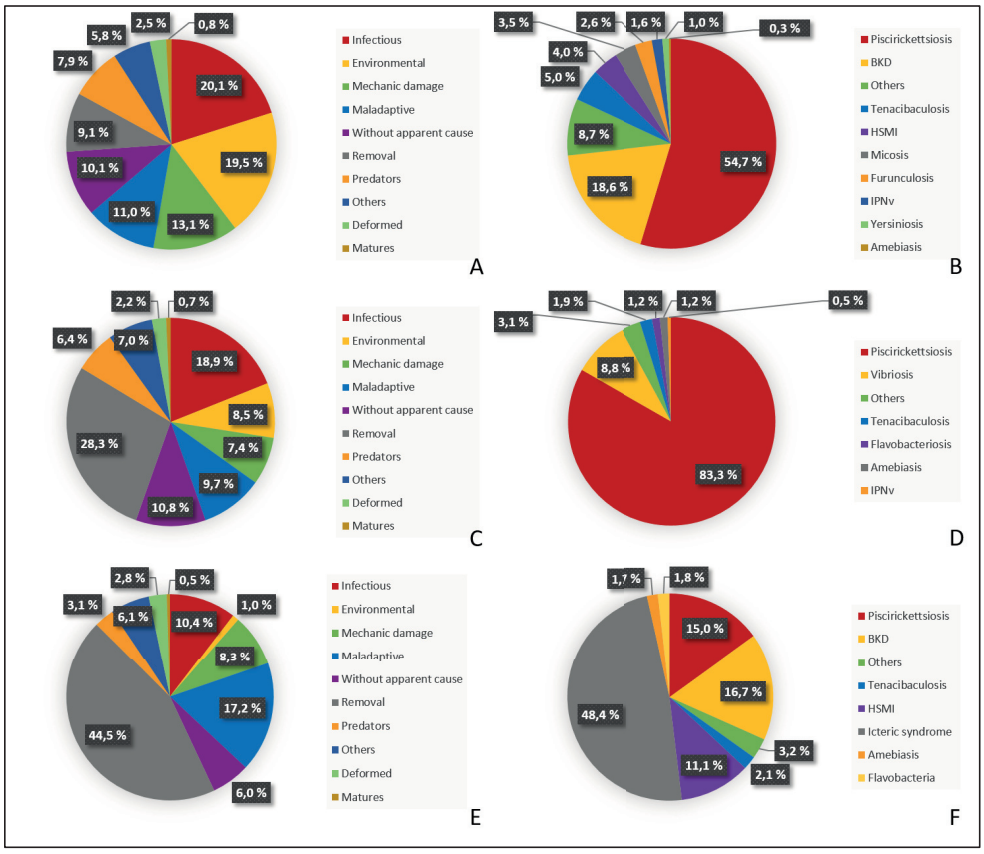

Figure 4. Production mortality by cause. In Atlantic salmon, by primary cause (A) and by disease (B). In rainbow trout (*Oncorhynchus mykiss*), by primary cause (C) and by disease (D). In coho salmon (*Oncorhynchus kisutch*), by primary cause (E) and by disease (F). Source: SERNAPESCA.

The most common measures against disease are prophylactic vaccines, and treatment with antibiotics. The Chilean salmon industry uses a high amount of antibiotics, reaching a maximum amount in 2014 with 563.2 tonnes[16]. The amount was reduced to 322.7 tonnes in 2018[17]. In 2018, the most commonly used antibiotics were oxytetracycline in freshwater and florfenicol in seawater, and the treatments are primarily targeting diseases in Atlantic salmon[17]. The amount of antibiotics used in Chile leaves the industry in a distant position in relation to the Norwegian salmon industry where only 0.641 tonnes was used in 2017[18]. The frequent use of antibiotics in Chile has an impact on the farmed salmon, but also the environment[19]. The constant use of antibiotics in the sea produced alterations in

the bacterial flora in the aquatic environment, and the repeated use will eventually increase the probability of inducing antibiotic resistance[20].

Piscirickettsiosis

Epidemiology

Piscirickettsiosis is one of the most challenging diseases to the sustainability of the Chilean salmon industry[21-25]. This disease is the main cause of infection-related mortality in the Chilean aquaculture[26-28], and has been estimated to account for up to 83.3% of the mortalities depending on the affected species[11]. The causative agent is the bacterium *Piscirickettsia salmonis*[22, 23, 29-31], and the disease affects mainly salmonid species, including Atlantic salmon (*Salmo salar* L.), rainbow trout (*Oncorhynchus mykiss)*, coho salmon (*Oncorhynchus kisutch*) and chinook salmon (*Oncorhynchus tshawytscha*)[29, 32, 33]. Piscirickettsiosis can be present mainly in brackish water and seawater, however the disease has also been described in freshwater[21]. Piscirickettsiosis in Chile is present in the Los Lagos and Aysén regions[34]. The number of reported outbreaks is higher during the warmer season when the water temperature is between 8 to 18°C in Los Lagos and reaching up to 15°C in Aysén[35]. Coho salmon is considered particularly susceptible to the disease[21, 22, 36], producing high mortality rates between 30% and 90%[24, 29, 32, 37, 38]. Infections with *P. salmonis* are not restricted to salmonid fish, and the bacterium has been described to cause a disease similar to piscirickettsiosis in non-salmonid hosts, such as white seabass (*Atractoscion nobilis*), yellow perch (*Perca flavescens*) and muskellunge (*Esox masquinongy*)[21, 39, 40]. Piscirickettsiosis was reported for the first time in 1989 in Calbuco, Los Lagos, Chile in coho salmon[41, 42]. The disease is responsible for enormous economic losses in the Chilean salmon industry[7, 43], with an estimation US $700 million in 2017[23, 44].

Piscirickettsiosis has also been reported in Ireland[45], Norway[46], Canada[47], Scotland[48] and recently in Turkey[44]. Outside of Chile, piscirickettsiosis outbreaks are sporadic and the mortality can be as low as 0.06%[24]. Worldwide piscirickettsiosis has been recognized as an emerging problem due to the increment of outbreak reports over the last years[7, 21, 24, 31, 42]. This could be due to differences in virulence related to the geographical origin of the *P. salmonis* isolates[21, 44] and could also be related with the non-native nature of salmonids in Chile[49].

Environmental impact

Mortality related to piscirickettsiosis has been noted in salmon as early as 10-14 days after introduction to infected seawater areas in Chile[21, 50], but outbreaks typically occur 6-12 weeks after

healthy fish are introduced into seawater[22, 24, 29, 32, 43, 51]. This could suggest the endemic nature of the bacterium, endemic bacteria usually only cause sporadic disease but may induce an epizootic in the confined cages of aquaculture[24]. Disease outbreaks are less frequently observed during the freshwater stage of the salmonid life cycle[39]. Stress has been described to play an important role in the development of piscirickettsiosis, and this could be induced by multiple factors such as smolt transfers, water temperature changes and severe storms, among others[24]. The higher incidence of piscirickettsiosis outbreaks is independent of the geographic location[52]. During the last years the climate changes have affected many areas and environments including rise in seawater temperature, which may contribute to different patterns of pathogen transmission[53]. As mentioned previously, in Chile the temperature of the seawater in summer could rise up to 18°C and in winter can go below 5°C, but not reaching freezing point[35]. This variation in the seawater temperature could be the reason for the massive problem that piscirickettsiosis creates in the Chilean industry. On the contrary, in the Norwegian seawater the temperature goes from 14.7°C in the summer to 4.2°C in the winter, a range that could avoid development of the disease[54].

Pathological signs

Piscirickettsiosis is a septicemic disease affecting multiple organs of salmonids[39, 51, 55]. Clinical signs of piscirickettsiosis in fish are lethargy, anorexia, skin lesions, respiratory distress, pale gills and surface swimming[22, 29, 32, 39]. At the same time, some fish could present just minor signs of disease[21, 24, 56]. The skin lesions include darkness, perianal and periocular haemorrhages, petechia in the abdomen and shallow hemorrhagic ulcers (Figure 5B). Additionally, bilateral exophthalmia and ulcerative stomatitis have been described[22]. Organs commonly affected are liver, spleen, intestine and hematopoietic tissue of the kidney[38]. The most characteristic internal lesions observed in heavily infected fish are off-white to yellow sub-capsular nodules throughout the liver, ascites, peritonitis, general pallor, diffuse swelling and presence of multifocal pale areas in the kidney and spleen (Figure 5A)[29, 38]. Petechia and ecchymosis on the serosa surfaces of the pyloric caeca, swim bladder and caudal intestine have also been reported in Atlantic salmon[22, 57]. In coho salmon, the renal lesions have been interpreted as chronic damage characterized by fibrosis[22]. Pale organs have been described in natural cases of piscirickettsiosis and a low hematocrit value is commonly found. The hematocrit values could fall to 2-20% compared to the normal 40-45%, but it is not clear whether anemia is a characteristic of the disease[24, 29, 32, 38]. The most typical microscopic lesions are found in the liver, kidney, spleen and intestine but pathological changes in the brain, heart, ovary and gill can also be observed as severe multifocal necrosis and inflammation, and mild injury in the cardiac, pancreatic and ovarian tissues[32,

[38, 43, 57]. Granulomas of macrophages and neutrophils as perivascular infiltration are also typical findings[43]. Differences in the severity of the lesions may be due to variations in the host species such as age, mode of infection, chronicity of the infections, and water temperature[38].

The disease has been reproduced fulfilling the Koch´s postulates by experimental infection of the fish with *P. salmonis* demonstrating a dose-response with mortality reaching nearly 100% in some groups of coho and Atlantic salmon injected with the bacterium[38, 51, 58-60]. Fish experimentally infected with *P. salmonis* are described to present clinical symptoms and typical lesions of piscirickettsiosis[50, 61].

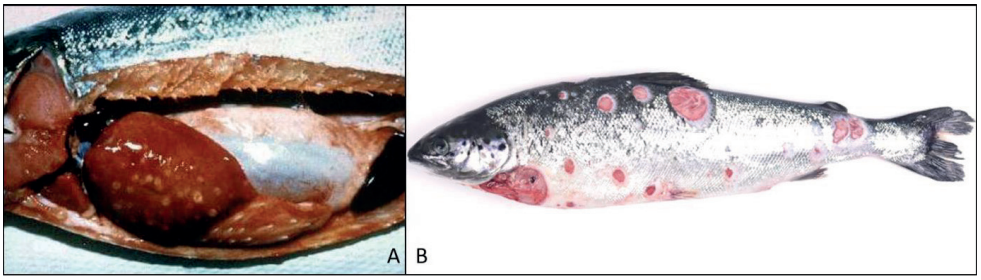

Figure 5. A, Coho salmon (*Oncorhynchus kisutch*) infected with *Piscirickettsia salmonis* (Source: Fryer and Mauel, 1997). B, Atlantic salmon (*Salmon salar* L.) affected by piscirickettsiosis, cutaneous presentation (Source: Patología en acuicultura, Marco Godoy).

Diagnostics

Piscirickettsiosis is preliminarily diagnosed by clinical signs[21, 62]. Other methods include solid culture of *P. salmonis* coupled with Gram and Giemsa stain techniques[24, 63]. Isolation of the bacteria by cultivation is considered one of the best methods for detection, however, bacterial isolation in the field is complicated due to the difficulty to obtain a pure *P. salmonis* culture and for the requirement of special media for cultivation[24]. Molecular diagnostic techniques, such as conventional PCR, real-time PCR, and indirect fluorescence antibody test are more accurate and sensitive than culture techniques[28, 29, 64]. The tissues of choice for isolation of *P. salmonis* include kidney, liver and blood during active infection[57]. Smears or impressions of the kidney, liver, spleen or infected cell cultures on glass or plastic substrates can be fixed and stained with Gram, Giemsa or methylene blue solutions for direct observation of *P. salmonis* within host cells[57].

Treatment and prevention

The most common treatment against piscirickettsiosis is use of antibiotics distributed as medicated feed. The drug of choice is florfenicol, however oxytetracycline is also commonly used[65, 66]. Disease prevention strategies include reduced stress, improved husbandry practices, screening of brood stock and vaccination[43], which all depend on good management practices[21]. In addition to antimicrobial drugs and vaccines[23], selective breeding for resistance against infectious diseases represents a realistic and more sustainable strategy to control disease outbreaks in aquaculture species. Recent evidence demonstrates the presence of significant variation for resistance against *P. salmonis* in Atlantic salmon and coho salmon population and it has been demonstrated a significant heritability for resistance against *P. salmonis* in Atlantic and coho salmon[50, 67]. This low-susceptibility of some Atlantic salmon families has been suggested to be related with a reduced intracellular iron content, which may suggest that iron deprivation could work as an innate immune defense mechanism against *P. salmonis*[50, 68, 69].

Piscirickettsiosis has evolved over time and the control of the disease has been proven to be difficult[70]. New outbreaks are typically increasingly insidious and refractory to treatments, and often show increased bacterial virulence as well as increased clinical and pathological severity[21, 36, 71]. To reduce the amount of antibiotics that has been used during the last years, the industry must continue focus on best sustainability practice in farming, like regulating reduced stocking densities and developing prophylactic measurements against piscirickettsiosis, such as vaccines.

Piscirickettsia salmonis

Bacterial properties

Piscirickettsia salmonis is a facultative intracellular, aerobic and non-motile Gram-negative bacterium. The pleomorphic bacterial cells are mainly coccoid ranging in size from 0.5 to 1.8 um in diameter[22, 33, 37, 41, 72, 73]. This is a marine bacterium that mainly induces clinical disease in seawater, though it has been reported in freshwater as well[43]. *P. salmonis* was the first rickettsia-like organism recognized as a fish pathogen, affecting several cultured salmonid species[22, 24].

P. salmonis replicates by binary fission within membrane-bound cytoplasmic vacuoles in cells of susceptible fish hosts or fish cell lines inducing a characteristic cytopathological effect[21, 62, 64]. Despite its rickettsia-like properties, the 16S rRNA sequence of the organism shows that it is a member of the gammaproteobacteria[21, 74], which includes the genera *Francisella*, *Coxiella* and *Legionella*[24, 42, 64].

In a recent description developed by Nourdin-Galindo, et al (2017)[75], *P. salmonis* isolates were re-classified in two genogroups; LF89 and EM90. The genogroups differ in phenotype, geographic

location, antibiotic resistance, host specificity and clinical manifestation[52, 61, 75]. Virulence factors analyzed at genome level revealed that both genogroups carry similar genes encoding for endotoxins, enzymes and surface components, and functions for adherence, iron uptake and stress response. However, some of the virulence factors were also considered genogroup specific[75].

The EM90 genogroup is widely disseminated and responsible for a major proportion of the piscirickettsiosis cases. This genogroup is more prevalent in the Aysén region of Chile, while the LF89 genogroup is reported to be predominant in the Los Lagos region[52]. Both genogroups have been identified in fish from a single outbreak of piscirickettsiosis in Chile[30, 37, 52]. The isolates from salmon in Canada, Norway and Ireland appear to be serologically and genetically related to the original Chilean isolate of *P. salmonis* (LF89), but different to the Chilean isolate EM90[30]. The geographical distribution has demonstrated differences in virulence, and the Chilean isolates are in general more virulent than the Norwegian isolates[21, 24, 33].

Phenotypic characterization and growth conditions

The colonies of *P. salmonis* are slightly convex, grey-white, shiny, and centrally opaque with translucent, slightly undulating margins. No distinctive colony or cell morphology differences have been observed between genogroups[37]. As for all bacteria, *P. salmonis* has nutrient requirements for cultivation. Iron is considered essential for *P. salmonis* growth and the bacterium has developed strategies to capture iron from endogenous (interaction with proteins that contain iron) and exogenous (siderophores) sources[69, 76, 77]. The amino acid cysteine has been suggested to be essential for the cultivation of *P. salmonis*[63]. In contrast, fetal bovine serum (FBS) and glucose has been demonstrated not to be essential for *P. salmonis* growth[72]. Peptone concentration has been documented to be more significant for *P. salmonis* growth compared to the concentration of yeast extract[72]. Culture media for *P. salmonis* are highly nutritive and non-selective, therefore they can easily become contaminated by other bacteria[28].

By being a facultative intracellular bacterium, cell-free *in vitro* cultivation of *P. salmonis* is challenging. *In vitro* cultivation of *P. salmonis* was initially performed using eukaryotic cell lines susceptible to infection, such as chinook salmon embryo (CHSE 214) and insect tissue cells[22, 72, 74]. Susceptible fish cell lines for *P. salmonis* also includes EPC, CSE-119, RTG-2, FHM and CHH[64, 78]. Cell culturing has been the gold standard for the isolation of *P. salmonis*[63]. In those cell lines, the bacterium produces a cytopathological effect. Experiments have suggested that *P. salmonis* has the potential to continue to be viable in invertebrates and non-fish poikilotherms due to the possibility of replication in insect and frog-derived cell lines[21].

In 2007, the cultivation of *P. salmonis* in an artificial cell-free medium was reported for the first time[79]. Studies reveal that *P. salmonis* remains pathogenic *in vitro* after growing in cell-free media[72]. The two first cell-free agar media developed for cultivation of *P. salmonis* were based on cysteine heart agar supplemented with 5% bovine blood (CHAB) and an enriched sheep blood agar with cysteine addition (BFCG)[63, 79, 80]. After the introduction of these first cultivation media, several cell-free media have been developed through the last years. Cell-free cultivation is less resource-intensive compared to the time-consuming and costly maintenance of cell lines, and the method also eliminates contamination from host cell debris. In addition, the ability to isolate and culture *P. salmonis* on artificial media simplifies the identification of piscirickettsiosis in remote fish-culturing facilities and the early detection of the agent enhances treatment and prevention strategies[21, 63]. However, the growth of *P. salmonis* on agar media is not suitable for determining the number of bacteria present in an *in vitro* culture, because bacterial growth is slow and the phenotypic properties of *P. salmonis* make it difficult to count on plates. Growth on solid media usually takes from four to eight days for visible colonies to appear[62]. From 2009, the cultivation of the bacterium in cell-free liquid media became possible[62, 81]. Marine broth medium supplemented with L-cysteine, named AUSTRAL-SRS broth, is one of many liquid media that facilitated the growth of *P. salmonis* strains[62]. The incubation period for the cultivation of *P. salmonis* in broth has been described to be between 6 to 13 days[62, 72, 81].

The optimal temperature for cultivation of *P. salmonis* is reported to be 15-18°C. The bacterial growth rate is inhibited at temperatures below 10°C and above 20°C, and growth does not occur above 25°C[21, 24, 37, 52, 72]. The generation time of the bacterium is approximately 5-7 hours in cell-free media[41]. After growing *P. salmonis* with gentle shaking there has been described a lag phase of approximately 18 hours, followed by a logarithmic growth of 126 hours and a stationary phase of 96 hours[62].

During bacterial cultivation, it has been reported an increment in pH that could be a consequence of the degradation of amino acids present in the peptone and yeast extract components acids in the growth media, as well as a consequence of assimilation of organic acids[72].

There is an interest to achieve pure high cell density growth of *P. salmonis* in a liquid medium free from eukaryotic contaminants[72] for possible vaccine development.

Transmission and host specificity

Vertical transmission of *P. salmonis* has been suggested due to its presence in the milt and celomic fluid of adult brood fish and in fry[43, 82]. Nevertheless, the low incidence of the disease in freshwater suggests that vertical transmission may not be common for *P. salmonis*[21]. The bacterium has been demonstrated to be transmitted horizontally, mainly in seawater, between fish[29, 51]. It has been

documented that the bacterium remains viable in seawater however without a capacity to grow[70]. *In vitro* experiments have demonstrated that *P. salmonis* in salt water maintains its infectivity for 10 to 15 days[51]. Due to the length of survival time in salt water, horizontal transmission may occur without a vector[24, 43]. Recently it was confirmed that stress could be responsible for the formation of biofilm in this bacterium[41, 42, 83]. Biofilm formation could be the reason that *P. salmonis* may survive and remain latent for long periods in the ocean without a host.

A vector or reservoir related with *P. salmonis* has not been demonstrated[22, 29], however a marine reservoir for *P. salmonis* has been suggested[14, 39]. At the same time, previous studies suggest that ectoparasites could have an important role in the transmission of *P. salmonis*[21]. Furthermore, the crowded hatchery or aquaculture conditions may influence in the dissemination of the disease[39].

Initially, *P. salmonis* was believed to be a pathogen only affecting salmonids but some reports have suggested a broader host range that includes European seabass (*Dicentrarchus labrax*) in Greece and white seabass (*Atractoscion nobilis*) in southern California, USA[42]. Together with this, genetic material from a *P. salmonis*-like organism has been found in native fish, such as *Eleginops maclovinus*, *Odontesthes regia*, *Sebastes capensis, Callionymus lyra, Oreochromis nilótico, Panaque suttoni, Parapristipoma trilineatum, Epinephelus melanostigma, Oreochromis mossambicus, Sarotherodon melanotheron* and *Salilota australis*[69, 84]. In addition, the EM90 genogroup was previously suggested to be specie-specific to Atlantic salmon[33], but this genogroup has also been isolated from Atlantic salmon, coho salmon and rainbow trout.

The incubation period of the disease is dependent on the bacterial isolate and infection method, and environmental and host factors[61].

Pathogenesis and virulence factors

The pathogenesis of the bacterium is unclear. There is no doubt that *P. salmonis* is an intracellular pathogen, however, it has not been determined in detail if *P. salmonis* spreads from cell to cell via the extracellular space or uses mechanisms that spreads intracellularly between cells[43].

The portal of entry of infection is not fully clarified, but studies suggest that the pathogen enters into the host through the oral route, gills or skin[21, 22, 43]. After *P. salmonis* has entered the host, the bacterium firmly attaches to macrophages (target) surfaces, after this the host immune system may be manipulated by *P. salmonis* to permit enhanced microbial growth and survival[85]. *P. salmonis* is then carried and disseminated through the circulatory system within leukocytes and reaches the main organs by infecting the endothelial cells of blood vessels[38].

The virulence mechanisms of *P. salmonis* can be related to the stimulation of the innate immune response and inhibition of the adaptive humoral and cell-mediated immune response to evade the host reaction[86]. Even though the virulence mechanisms have not been established, some virulence factors have been described. It has been suggested that *P. salmonis* has the potential to develop a flagellum controlled by a transcriptional cascade[42]. It has also been described the presence of genes that modulate a pili-like structure in *P. salmonis*[87]. Studies developed by Chilean and Norwegian researchers described the presence of outer membrane vesicles (OMV) in *P. salmonis* and the possibility that OMV stimulate the immune system of the fish[88, 89]. Previous studies suggested that iron could be a crucial element in the growth and virulence, this due to the existence of a set of genes involved in the iron metabolism[69, 90, 91] as in most of living organisms[92].

The *P. salmonis* bacteria have been identified in cytoplasmic vacuoles in hepatocytes and macrophages associated with liver, kidney, spleen and peripheral blood demonstrating that the bacterium can infect a variety of cell types in salmonid hosts. Consequently, the bacterium has been considered a highly adaptable microorganism. Nevertheless, the main target cells are the macrophages in which this bacterium can survive and replicate extensively[21, 32, 38, 88, 93]. It has not been fully documented where the bacterium resides, if it is in the endosome/phagosome or if it is released from there to the cytosol[43]. *P. salmonis*-containing vacuoles are demonstrated to not fuse with lysosomes, this could indicate that there is an interference in the endosomal maturation process to ensure the bacterial survival through the evasion of the phagocyte-lysosome fusion, and enabling *P. salmonis* to avoid the fish's primary immune defense[21, 88, 94]. *P. salmonis* affects the immune system of the host, activating the innate immune response in the head kidney, the muscle and the liver. This potentially induces inflammatory responses in the head kidney and an interferon-mediated response in the liver[21]. *P. salmonis* may inhibit cellular apoptosis by down-regulating apoptosis-related genes, whereas it may stimulate cell proliferation by up-regulating cell-proliferation-related genes[21, 23, 95]. During the beginning of the infection *P. salmonis* may replicate in large quantities within macrophages and decrease the immune cells' apoptosis frequency, this could facilitate the survival of the bacterium when the disease development has started[77]. In addition, it has been described that *P. salmonis* could induce apoptosis in macrophages *in vitro* as a possible *in vivo* strategy to evade the host immune system[41], and to colonize and disseminate within host tissues[44].

It has been described that *P. salmonis* secretes extracellular products, and at least one of the components has cytotoxic effects *in vitro* and probably mediates some tissue damage *in vivo* in salmonid fish[21]. The antioxidant system of the host may be affected by *P. salmonis*, eventually causing death and necrosis, as observed in several tissues of moribund fish infected by this bacterium[21].

Antibiotic resistance

The bacterium has demonstrated susceptibility to antibiotics *in vitro*, but treatments in the field are not always successful[96]. The use of antibiotics both prophylactically and during early outbreaks of piscirickettsiosis may inhibit the growth of *P. salmonis*, but such treatments have been largely unsuccessful in stopping disease outbreaks[70, 97]. Some reports exist about the development of antibiotic resistance in *P. salmonis*. A study conducted by Mauel and Miller (2002)[24] found that *P. salmonis* was susceptible *in vitro* to streptomycin, gentamicin, tetracycline, chloramphenicol, erythromycin, oxytetracycline, flumequine, Imequil, oxolinic acid, sarafloxacin, clarithromycin and resistant to penicillin, lincomycin, furazolidone, and sulfonamide-trimethoprim. Another study developed by Saavedra, et al (2017)[52] reported that the majority of investigated LF89-like isolates demonstrated resistance to quinolones, florfenicol and oxytetracycline compared to the EM90-like isolates that were all susceptible to the tested antibiotics in that study. In 2016, Evensen (2016)[43], described increased resistance to penicillin, streptomycin, oxolinic acid, and oxytetracycline in *P. salmonis* isolates. On the other hand, the same year, Otterlei, et al (2016)[37], described that all tested isolates were susceptible to oxytetracycline. As mentioned previously, the most used antibiotics for treatment of piscirickettsiosis is florfenicol. This antibiotic, is relatively environmentally innocuous but may induce resistance development and the genetic determinants for this resistance can be shared between fish and human pathogens[98]. One of the concerns should be the possibility of increased antibiotic resistance of *P. salmonis* and the reduction in the efficacy of treatments. Sediments under salmon farms in Chile were analyzed and an increment of other bacteria resistant to florfenicol were found[99].

The focus of the Chilean industry is the reduction in the use of antibiotics. This could contribute to reduce the possibility in obtaining a highly resistant pathogen and in reducing the imprint of the salmon farms on the environment.

Fish vaccines

Fish immune system

In fish as in most vertebrates, the innate and adaptive immune responses are considered essential components to fight pathogens. The poikilothermic nature of fish leads to some limitations in the adaptive immune system, such as limited repertoire of antibodies, slow kinetics of antibody responses and poor affinity maturation[69, 100].

The innate immune response is on the other hand well-developed in fish. It represents the first line of defense against pathogen invasion and include physical barriers as well as humoral and cellular responses[85]. Macrophages primarily act as antigen-presenting cells, but these cells are also responsible for most phagocytic activity in addition to regulating the immune system cascade triggered by the secretion of pro-inflammatory cytokines[85]. The adaptive immune response has also a humoral and a cellular arm including specific antigen receptors that drives a secondary, faster and stronger, immune response[101]. In the cellular components of the immune system, there are B and T cells. One of the functions of B cells is the production of antibodies to neutralize pathogens and mark them to be removed by the immune system. On the other hand, T cells act as coordinators of the T-helper cells involved in the immune response to kill infected cells[101]. B cells secrete immunoglobulins (Ig) or antibodies. Three types of Ig have been described in teleost fish including IgM, IgD and IgT, the last one designated as IgZ in zebrafish[101, 102]. IgM has been recognized as an important antibody in the teleost immune system, being the most ancient and the only isotype conserved in all jawed vertebrates[7]. The IgM produced by plasma cells and immature plasma cells located in the head kidney is the most abundant type of immunoglobulin[44]. The exact function of IgT is not clear, but IgT appears to be important in the gut, skin and nasal mucosa[44] suggesting an important role in the mucosal immunity[100]. It has been described that non-mucosal IgT responds to viral infections[101]. Little is known in relation to IgD, but this type of antibody could be related to the mucosal immunity and together with IgM, IgD seems to be essential for all teleost species[101]. In salmonids, cytotoxic (CD8) and helper (CD4) T cells are present[103]. These cells are mainly responsible for the immune response against viral infections (intracellular organisms), as demonstrated by *in vitro* study where leucocytes expressing CD8 and T cell receptor produced high levels of cytotoxicity in virus infected cells[102, 104].

Cytokines are small proteins that are involved in both the innate and the adaptive immune responses by mediating cell signaling. The cytokines regulate the immune function by interaction with specific receptors on the surface of the cell. A group of cytokines includes the interleukins (ILs)[101]. IL-12 is one of the key components for efficient performance of phagocytes in teleost fish, similar to the roles played by IL homologs in mammals[85].

The development of long-lasting humoral immune response in fish after immunization has been described associated with some pathogens[100]. However, vaccination against piscirickettsiosis may lead to protection only during the first months after transfer to sea yet still represents an important tool to reduce the number of disease outbreaks and subsequent use of antibiotics[12].

Vaccines

Vaccination has a central role in attenuation of known and emerging diseases in fish[13]. Although the development of fish vaccines began in the 1930s, commercialization of fish vaccines did not occur before the second half of the 1970s[105]. The first licensed fish vaccine came in 1976 and was against enteric redmouth disease caused by *Yersinia ruckeri*[106, 107]. Since 1988, Norwegian salmonids have been successfully vaccinated against cold-water vibriosis[108]. Vaccines against furunculosis, a disease caused by *Aeromonas salmonicida* ssp *salmonicida* were introduced in Norway in the late 1980s with variable degree of protection. It was just until 1992-3 when the first oil-based vaccine to control furunculosis was commercialized[106, 109, 110]. In Chile, the use of fish vaccines started in the early 1980s but it was not until 1995 that the use of fish vaccines became a common practice[111]. Since the control of furunculosis by oil-based vaccines, new vaccines were developed to control bacterial diseases[112]. Viral diseases are, on the other hand, typically more difficult to prevent by vaccination[110]. Viral infections are intracellular, which makes the virus less exposed to the immune system due to their capacity for immune evasion by avoiding the immune detection and inhibiting the host defense[103]. The same characteristics are displayed by intracellular bacteria, which make the development of vaccines against intracellular bacterial infections a challenging task[113]. There are historically at least three different types of vaccines; live-attenuated, inactivated and sub-unit vaccines. The live-attenuated vaccine are those vaccines where the pathogen has been weakened, but still keeps its capacity of multiplication within the host, and thus being able to develop a strong cell-mediated immune response[106, 114]. In an inactivated vaccine, the pathogen is killed by chemical or heat treatment, however the antigen is still able to develop an immune response. The sub-unit vaccines include one or more selected parts of a microorganism that is/are able to stimulate a specific immune response[106]. For the control of many bacterial diseases the use of inactivated pathogens has been successful[115], the vaccines based on inactivated pathogens are considered environmentally safe because the inactivation reduces the possibility of dissemination of diseases as a result of the vaccination routines. It has been described that the inactivation of the pathogen could be the cause of the short protection provided by vaccines based on the inactivated pathogen[12]; this due to the possibility that the chemical inactivation may reduce the quality of immune stimulants in the surface of the bacteria[116]. Furthermore, it has been reported that the inactivated vaccines are less efficient against intracellular pathogens[106], since the inactivated vaccines mainly induce humoral immunity and this vaccines should be considered as exogenous antigens[117]. In 2005, the first DNA vaccine for fish was registered in Canada, targeting the infectious hemorrhagic necrosis virus (IHNv)[118]. In 2017, a DNA vaccine against salmonid pancreas disease virus (SPDV) was approved in the EU and Norway[119]. Fish vaccines are typically administered by injection through the intraperitoneal (i.p.) or intramuscular

(i.m.) routes, by immersion or through the oral route[12, 120, 121]. Injectable vaccines are known to elicit a good, specific immune response, but intraperitoneal administration has historically been associated with some side effects such as tissue inflammation, adhesion and necrosis [12]. Intramuscular injection, on the other hand, induces less adverse events, and is an efficient way to target antigen presenting cells located in the skin and muscle of the fish. Intramuscular vaccination seems to be the optimal route of administration for DNA vaccines[120]. Administration of vaccines by injection requires extra handling of each fish, which may have a negative impact with regards to stress and the following growth. Immersion vaccines are typically used in smaller fish in freshwater and aim to protect against diseases that typically appear during the earliest life stages. Oral vaccines often induce a weaker protection and are typically used as a booster or primer vaccination[12].

In Chile there are 57 mono- and multivalent vaccines registered for fish in 2019[12]. Among these 57 vaccines, 32 vaccines include a component to immunize against piscirickettsiosis[44, 122]. From those 32 vaccines against piscirickettsiosis, seven are monovalent and 25 are multivalent[122]. The high number of available vaccines developed against piscirickettsiosis may imply both that the efficacy obtained is not very high and that the evaluation of the efficacy of those vaccines is not very discriminatory[21, 37, 44]. The majority of piscirickettsiosis vaccines are inactivated and composed of *P. salmonis* pre-treated with heat or formalin[7]. Live-attenuated vaccines have succeeded in activating both the innate and the adaptive immune system, and, in many ways, mimic a natural infection upon immunization[7]. There is no available literature that documents the effect of the live-attenuated vaccine against *P. salmonis*[43], but the use of *Arthrobacter davidanieli* as a live vaccine against *P. salmonis* and *Renibacterium salmoninarum* has been described to be able to induce a specific immune reaction[123]. For the time being there is only one vaccine available based on live-attenuated antigens against piscirickettsiosis[122].

Vaccines for aquaculture may also include recombinant antigens and bacterins, however, there is no recombinant vaccine against piscirickettsiosis on the Chilean market today. Most of the vaccines with *P. salmonis* bacterins have demonstrated to have a reasonable effect in preventing the initial piscirickettsiosis outbreak when the fish are transferred from freshwater to seawater. However, the vaccines do not have the same efficacy in preventing more aggressive outbreaks that occur later in the production phase close to harvest[20, 23, 44]. This suggests that early infections of piscirickettsiosis do not produce specific antibodies to prevent later outbreaks.

The role of both specific and non-specific cell mediated immunity in protection of fish against intracellular infections is receiving increased attention. As a result of *P. salmonis* being an intracellular pathogen, stimulation of cell-mediated immunity including enhanced phagocytosis and intracellular killing, is most likely critical for success in development of effective vaccines in order to confer significant protection against the disease[30]. Marshall, et al (2007)[36] assumed that the most exposed

bacterial antigens might also be the most immunoreactive epitopes and those rendering immune protection. This makes reasonable sense to evaluate the potential immunity against piscirickettsiosis by purifying the bacteria and/or by searching for a single antigen[36]. A successful outcome from vaccination against piscirickettsiosis depends on an activation not only of the innate immune system also of the antibodies and cellular mediated immune system to provide a sufficient protection[7]. It has been reported that when the primary immunization is done by the parenteral route (i.p.) followed by a booster by oral delivery the circulating antibody levels have shown to be elevated[43]. However, the circulating antibodies may not be able to protect against the infection and/or piscirickettsiosis development[43]. Understanding the immune response against *P. salmonis* may come from examining the biology of other intracellular pathogens[44] for example, *P. salmonis* has been demonstrated to be able to inhibit phagosome-lysosome fusion[85].

Vaccine regulations

The approval and use of vaccines and pharmaceuticals are strictly regulated by the relevant authorities. Vaccines to be used in Norwegian aquaculture must be approved by the Norwegian Medicines Agency (NoMA) and for the European market must be approved by the European Medicines Agency (EMA)[124]. For those approvals, the vaccines must pass pre-clinical laboratory studies to demonstrate efficacy, potency and safety during the registration process for market authorization. The tests are performed by experimental immunization and challenge of fish in a controlled environment. After the market authorization has been granted, each vaccine batch must be tested before release to the market. In addition to the laboratory studies, some authorities require the conduct of clinical field studies to document large scale efficacy and safety under commercial farming conditions [124]. Evaluation of a vaccine's efficacy and potency is usually performed by measuring the mortality and calculating the relative percentage of survival (RPS)[112]. The RPS is a quantitative and dynamic measurement that express the ratio between the percentage (%) of mortality in the vaccinated fish over the controls [125]. The most common scale utilized is with a RPS of ≥ 60% mortality in the control group and a vaccinated group with a mortality ≤24%[125]. The European regulation for fish vaccines is based on a monograph from 1996 and the last update and revision was in 2011[126].

In Chile, the regulation of fish vaccines is controlled by the Chilean agricultural and livestock service (SAG). The vaccines currently on the market are approved according to the prevailing provisional registration requirements for immunological products for salmonids. The protocol states that the RPS_{60} after immunization and challenge with *P. salmonis* should be ≥70% for vaccines administered by injectable and oral administration and ≥ 60% for vaccines administered by immersion[127]. In 2018, SAG

established a protocol for full registration of salmonid vaccines that applies for all products with provisional market authorization. The process aims to be completed by 2021[128] and is likely to reduce the number of vaccines available on the market. In the new Chilean guideline, for full registration it is emphasized that to demonstrate the efficacy of fish vaccines the pharmaceutical company must apply the challenge model that closely mimics infection under real-life conditions and in a more reliable manner mimic the mortality pattern of the disease. The possible challenge models to use for registration are by immersion, cohabitation and intraperitoneal injection[128].

Experimental challenge models

Experimental challenge of fish

The efficacy of vaccines and pharmaceuticals intended for aquaculture are tested using experimental challenge of fish. Experimental challenge models represent a key tool for development of all vaccines and pharmaceuticals, and the quality of the models is essential for a valid result that reflects the actual efficacy of the products. *In vivo* methods of challenge are typically used for the evaluation of the efficacy of new vaccines under development. Challenge models are also used to document the consistency of vaccine batches of registered vaccines that are produced for the aquaculture industry.

The development of robust and reproducible challenge models is a time-consuming task and several factors must be taken into consideration. The trials are typically conducted in well-established wet lab facilities that are run according to high quality standards to ensure the validity of the trials. The facilities must be able to closely monitor parameters such as water temperature, light, salinity and flow/water exchange. The quality of the test fish with regard to genetic background, individual variation, size and physiological status must also be taken into consideration. The test fish must be pathogen-free, which calls for screening of brood fish, incubated eggs and the fish population after start-feeding by qPCR to document the absence of known pathogens. For some trials designed to evaluate the efficacy of vaccines, there is an additional requirement to document the absence of antibodies in the test fish prior to immunization.

The infectious material used to challenge the fish must be representative of pathogens isolated in field outbreaks of the disease. Whether the pathogen is a virus, a bacterium or a parasite, the pathogen must be re-isolated and cultivated in a pure culture to ensure a causal relationship between the etiological agent and disease development (Koch's postulates). *In vitro* cultivation of infectious material can be a challenging task as pathogens often are known to lose virulence over time. Optimization of parameters such as cell culture, broth, temperature, agitation is crucial to ensure cultivation of pathogens that are still representative of the strains isolated from the field.

The challenge models typically applied comprise challenge by injection, cohabitation or immersion. The challenge models aim to mimic real-life conditions in the field as closely as possible. The validity of the models must be studied for each pathogen separately since the dynamics of the infections vary significantly. Internal trials performed at VESO Vikan, for example, revealed that fish immunized with vaccines against *Moritella viscosa* infection demonstrate different degrees of protection against disease dependent on the challenge model applied. Challenge by immersion will give a valid presentation of the protection induced by the vaccines, while challenge by i.p. injection will give a false impression of high protection and this has direct relation with the bacterial route of infection. It is therefore important to thoroughly consider which challenge model that should be applied to reveal the protection provided by different vaccines. For experimental challenge with *P. salmonis*, it was recently demonstrated (unpublished results, VESO Chile) that salmon families bred for different genetic resistance against *P. salmonis* classified similarly resistant independent of being challenged by i.p. injection or cohabitation. The current thesis aims to document whether similar conclusions can be drawn after immunization of fish against *P. salmonis* followed by i.p. or cohabitation challenge.

Challenge models with *P. salmonis* are well-known to be difficult to establish due to the intracellular characteristics of the bacterium in addition to the high mortality rate. The virulence of the *P. salmonis* strains varies significantly, and only some strains are capable of horizontal transmission from i.p. injected shedder fish to naïve cohabitant fish[74]. The infectious pressure can be controlled by adjusting the injected dose or the percentage of shedders introduced. However, the challenge pressure must be above a certain threshold level for horizontal transmission to occur. The incubation period for the disease depends on the bacterial isolate, the challenge dose administered or the number of shedders introduced, as well as environmental factors such as temperature, flow/water exchange and salinity. The physiological status of the fish (parr or smolts) and age/weight must also be taken into consideration when designing a *P. salmonis* challenge trial.

The extensive use of fish as experimental animals has been discussed in terms of ethics and animal welfare by scientists for a long time[129]. The 3Rs principles was established in 2010 for pharmaceutical use[130]. The 3Rs focus on replacement, reduction and refinement. Replacement means to avoid or prefer non-animal methods in research. Reduction means to induce the use of fewer animals achieving comparable levels of information or to obtain more information with the same number of fish. Refinement means use of methods for alleviation or reduction the level of pain, suffering or distress, prioritizing the fish welfare[130, 131].

Challenge by injection

Challenge by injection is the most cost-effective model due to shorter disease development and thus duration of the experiment compared to the cohabitation challenge models. In addition, the use of correct challenge dose is easily feasible because the pathogen is injected directly into the fish. The main issue with this model is the artificial route of infection[132, 133]. There are two different challenge models based on injection of the pathogen: intraperitoneal (i.p.) and intramuscular (i.m.). In the i.p. injection challenge, the pathogen is injected directly into the peritoneal cavity of the fish. It has been described that the first affected tissues in fish challenged by i.p. injection are those closer to the injection site[134]. In addition, the substances administered intraperitoneally may undergo hepatic metabolism before reaching the systemic circulation[135]. Thus, this challenge model might not, in some cases, be the best approach for the evaluation of diseases or vaccines.

In case of the i.m. challenge, the pathogen is injected into the muscle tissue of the fish. This route of administration utilizes the high number of antigen presenting cells present in the muscle. For some pathogens, such as the piscine myocarditis virus (PMCV), i.m. administration of the inoculum may represent a good way of challenging the fish[136]. The method of i.m. injection requires some practice to be performed in a consistent manner in a large group of fish. Injection of individual fish requires handling that is known to be very stressful.

Challenge by cohabitation

The cohabitation challenge model is based on a certain number of shedder fish (trojans) being introduced to infect naïve healthy fish held in the same tank. The shedders are typically naïve fish from the same fish population as the test fish that are i.p. injected with infectious material. Dependent of the incubation time of the specific pathogen, the shedder fish will start shedding the virulent pathogen to the water, which will infect the naïve cohabitants through horizontal transmission by direct contact or through the water.

The advantage of this type of challenge model is that it closely mimics the natural infection and transmission of disease in a population[133]. Even though the port of infection of *P. salmonis* has not been proven, the fish may be challenged orally or via the gill surface, and have been shown to display a systemic pattern of infection[51]. Thus, cohabitation challenge models accurately represent natural exposure and may provide predictable results for mortality[21]. Furthermore, the amount of infectious material that must be produced for the challenge is limited because typically just the 20% of the test fish will be injected. This is particularly important for large scale trials that include a high number of

fish, or for pathogens that are difficult to cultivate *in vitro*, which leads to limited need for supply of inoculum[137]. Another advantage of challenge by cohabitation is reduced handling of the fish in comparison with the injection model. Although there are several advantages of using the cohabitation model, it is typically more time-consuming than challenge by injection or immersion. The prolonged period for transmission of the pathogen from shedder to cohabitant fish results in higher costs due to rental of tanks and use of water. It is therefore essential to determine whether challenge by i.p. injection will reveal similar results when compared to challenge by cohabitation or immersion with regards to the efficacy of a vaccine or pharmaceutical.

Challenge by immersion

The immersion challenge model is based on submerging the fish in a pathogen broth culture for a certain period of time. The fish are typically kept in one tank, the water level is lowered, and the broth containing cultivated pathogen added[138]. After a set period of time, the water flow is restored, and the water level raised back to normal. Challenge by immersion is typically applied for pathogens that are known to infect the outer barriers of the fish, such as mucus and skin. The model ensures that the handling of the fish is reduced to a minimum, which is crucial to leave the outer barriers intact for a representative infection and corresponding immunity to develop. Challenge by immersion requires large volumes of the pathogen to be cultivated. Large scale *in vitro* cultivation of pathogens may be difficult to achieve in an efficient time, and long culture period increase the risk of losing virulence. Furthermore, the duration of a typical immersion challenge trial is longer than challenge by injection. However, the route of infection more closely resembles natural infection, and challenge by immersion is a preferred method to document the efficacy of many products.

AIMS OF STUDY

The main objective of this thesis was to increase the knowledge about experimental challenge models with *Piscirickettsia salmonis* and to define the *in vivo* model of preference for evaluation of efficacy of vaccines intended for Atlantic salmon. In addition, the aim was to increase the understanding on piscirickettsiosis' pathogenesis and to characterize a *P. salmonis* isolate *in vitro*.

To achieve the main objective three sub-goals were defined:

1. To compare the progression of development of piscirickettsiosis after experimental challenge by intraperitoneal injection or cohabitation.

2. To compare the protection induced by experimental vaccines after immunization and experimental challenge by intraperitoneal injection or cohabitation with *P. salmonis*.

3. To characterize the *P. salmonis* isolate used for experimental trials *in vivo* after cultivation in broth media.

SUMMARY OF ARTICLES

Article I

Development of piscirickettsiosis in Atlantic salmon (*Salmo salar* L.) smolts after intraperitoneal and cohabitant challenge using an EM90-like isolate: a comparative study

Karla Meza, Makoto Inami, Alf S. Dalum, Ane M. Bjelland, Henning Sørum, Marie Løvoll.
Journal of Fish Diseases. doi: 10.1111/jfd.13004

Piscirickettsiosis, caused by the intracellular Gram-negative bacterium *Piscirickettsia salmonis* is at present the most devastating disease in the Chilean salmon industry. The aim of this study was to analyze disease development after challenge with a *P. salmonis* strain (EM90-like) under a controlled environment by comparing intraperitoneal challenge with cohabitation challenge. The *P. salmonis* EM90-like isolate was cultured in a liquid medium for the challenge of 400 Atlantic salmon (*Salmo salar* L.) smolts. Cumulative mortality was registered, necropsy was performed and bacterial distribution in the tissues and histopathological changes were analyzed. The results revealed a similar progression of the disease for the two different challenge models. Pathological and histopathological changes became more visible during the development of the clinical phase of the disease. Bacterial DNA was identified in all the analyzed tissues indicating a systemic infection. Bacterial tropism to visceral organs was demonstrated by real-time quantitative PCR and immunohistochemistry. Better knowledge of disease development during *P. salmonis* infection may contribute to further development of challenge models that mimic the field situation during piscirickettsiosis outbreaks. The models can be used to develop and test future preventive measures against the disease.

Article II

Comparative study of experimental challenge by intraperitoneal injection and cohabitation of Atlantic salmon (*Salmo salar* L.) after vaccination against *Piscirickettsia salmonis*.

Karla Meza, Makoto Inami, Alf S. Dalum, Hege Lund, Ane M. Bjelland, Henning Sørum, Marie Løvoll.

Accepted in Journal of Fish Diseases.

The Chilean aquaculture has been challenged for many years by the bacterial disease, piscirickettsiosis. A common prophylactic measurement to try to reduce the impact from this disease is vaccination, but the development of vaccines that induce full protection of the entire fish population has so far not been successful. Experimental challenge models are typically used to document the efficacy of vaccines. The aim of this study was to evaluate the performance of experimental vaccines after challenge by the two most widely used challenge routes; intraperitoneal injection and cohabitation. A total of 1120 Atlantic salmon were vaccinated with non-commercial experimental vaccines with increasing amounts of an inactivated *Piscirickettsia salmonis* EM90-like isolate. Differences in mortality, macroscopic and microscopic pathological changes, bacterial load and immune gene expression were compared after challenge by different routes. The results revealed a similar progression of the disease after challenge by both routes and no gross differences reflecting the efficacy of the vaccines could be identified. The analysis of the immune genes could provide some guidelines related to the pathogenicity of *P. salmonis* and suggesting the possible suppression of the cellular immunity by CD8 T-cells and with this stimulate the bacterial survival and replication. Comparative studies of experimental challenge models are valuable with regards to identify the best model to mimic real-life conditions and performance of vaccines.

Article III

Cultivation and characterization of a *Piscirickettsia salmonis* EM90-like isolate used for *in vivo* challenge in Atlantic salmon (*Salmo salar* L.)

Karla Meza, Marie Løvoll, Leif Lotherington, Cristian Bravo, Simen F. Nørstebø, Jessica Dörner, Victor Martinez, Henning Sørum, Ane M. Bjelland.

Submitted to Veterinary Microbiology

Piscirickettsia salmonis is one of the most widespread fish pathogens in the Chilean aquaculture and the causal agent of piscirickettsiosis, a disease that is responsible for big economical losses in the salmon industry. *P. salmonis* is a facultative intracellular bacterium classified in two different genogroups, LF89 and EM90. A majority of the published studies of *P. salmonis* are related with the reference strain LF89. The aim of this study was to characterize phylogenetically and phenotypically a *P. salmonis* EM90-like isolate cultured in FN2 liquid medium. The isolate has previously been used in experimental challenge trials of Atlantic salmon (*Salmo salar* L.). The growth dynamics of the bacteria in different media was studied, and the enzyme activity and the protein expression profile of the bacterium was described after cultivation in a new liquid medium. Optimization of cultivation is essential to ensure that the bacterial culture remains pure, maintains bacterial yield and virulence. Some differences were observed in the protein expression profile after cultivation of *P. salmonis* on CHAB agar plates compared with FN2 liquid medium. Furthermore, differences in protein expression were identified when comparing fresh bacteria with bacteria inactivated with formalin. A morphological difference in the color of the colonies was observed when comparing the *P. salmonis* EM90-like isolate cultured on CHAB agar plates with the bacteria cultured in FN2 medium. Results from *in vivo* challenge trials with fish indicated that the virulence of the bacteria was conserved independent of cultivation method.

DISCUSSION

In the current project, the objectives were to compare the challenge models used for evaluation of the efficacy of vaccines intended for Atlantic salmon against piscirickettsiosis. The focus was the addition of improvements and the intention to define which model is preponderant to study new vaccines against *P. salmonis*. Furthermore, more knowledge in relation to the pathogenesis of piscirickettsiosis was intended. First, the challenge models by i.p. and cohabitation were studied in unvaccinated fish challenged with a *P. salmonis* EM90-like isolate (**article I**). Afterwards, the same challenge models were studied after immunization of fish against *P. salmonis* (**article II**). Finally, the bacterial isolate was characterized after *in vitro* cultivation in liquid medium (**article III**). The results described in the three articles led to new knowledge that may be taken into consideration in further research. Those findings will be discussed in this section.

Development of piscirickettsiosis in challenged fish

To identify factors that could modify and influence the development of piscirickettsiosis and its mortality during challenge trials are of interested. The objective of this is to tune the mortality curve and to be able to obtain a higher separation between the different efficacies of tested products. In this case, to be able to separate the mortality curves obtain from vaccines that develop higher protection levels against piscirickettsiosis from those vaccines that produce lower protection. For *P. salmonis*, this has been difficult due to its inner characteristics.

Modification of the environmental conditions could affect piscirickettsiosis' pre-clinical period

The challenge model's setup has an impact on the results from the challenge trials. The results obtained from both experimental challenges conducted in this study (**article I** and **II**) suggest that the salinity of the water could be a factor in the disease development. In **article I** where the salinity was 32‰ it was demonstrated that the mortality for unvaccinated fish challenged by i.p. injection was observed to occur from 14 days post challenge (dpc) and by cohabitation from 28 dpc. This, compared with the challenge in **article II** where the salinity was 25‰, the i.p. injected fish started to die at 11 dpc and the cohabitation at 24 dpc. This could suggest that the salinity of the water has some impact on the incubation period of the disease. This may imply that *P. salmonis* holds a high preference for

estuarine water, which is likely since salmonids are mostly located in fjords where the salinity of the water is lower than in seawater, and is in that production phase where most outbreaks occur.

Another variable to consider is the water temperature. Based on unpublished results (VESO Vikan), it was found that when the temperature was reduced the onset of mortality was delayed. Results from a trial to document the impact of temperature showed that fish kept at 15°C started to die at 17 dpc (i.p. injected shedders) and 40 dpc (cohabitants). Fish kept at 12°C started to die at 24 dpc (i.p. injected shedders) and 64 dpc (cohabitants). The reduction of temperature by 3°C resulted in seven days delay in mortality for the i.p. injected shedders, and a 24 days delay for the cohabitants (Figure 6). After the onset of mortality, the progression of the disease development and mortality rates were similar and independent of temperature.

The results may suggest or reaffirm that piscirickettsiosis is an environmentally dependent disease, and the virulence of the bacterium may be affected by the geographical locations both with regards to temperature and salinity. The adjustment of these parameters are able to tune, in some cases, the outcome of challenge trial. That is the case of challenges with *Moritella viscosa*, where the reduction in the temperature reduces the mortality. However, this is not applicable for *P. salmonis* where a decrease in the temperature just increases the pre-clinical period, but there are not changes in the percentage of mortality. This has not been reflected in field outbreaks, due to in field epidemiological studies in Chile like the study performed by Jakob, Stryhn, Yu, Medina, Rees, Sanchez and St-Hilaire (2014)[139], have also revealed that the temperature of the water could affect the mortality of the fish. There was an increase in mortality among smolts transferred to seawater in the spring-summer season (higher temperature), in contrast to the smolts transferred in the fall-winter season (lower temperature). It has been documented that Atlantic salmon is more susceptible to, for example, the salmonid pancreas disease virus during the Norwegian spring/summer when the water temperatures are increasing[140]. Also, it has been described that Atlantic salmon obtain higher counting of amoebas at higher temperatures during amoebic gill disease[141]. Other reason of changes in virulence of *P. salmonis* by the temperature of the water could have relation with the optimal temperature for bacterial grow. The water temperature in Chile during the warmer season is close to the temperature defined as optimal for *in vitro* cultivation of *P. salmonis*. Another possibility could be related with the increment of the metabolic rate that increased the activation of immune response in the fish at higher temperatures and in addition to the bacterial intracellular characteristics, could result in an increase in the development of piscirickettsiosis. This is because *P. salmonis* targets mainly the cells involved in the immune response. Moreover, the susceptibility of smolts transferred during the warmer seasons may perhaps be related to the stress induced by increasing temperatures.

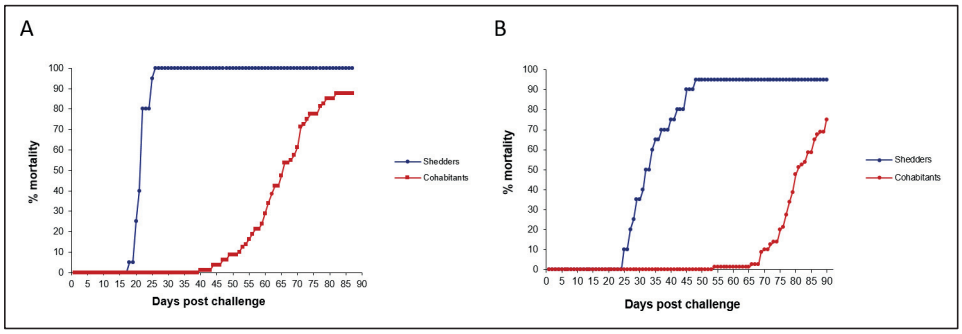

Figure 6. Mortality curves from parr challenged with *P. salmonis* at different temperatures. A, 20% shedder fish and 80% cohabitants at 15°C. B, 20% shedder fish and 80% cohabitants at 12°C.

Different percentage of shedders modified the piscirickettsiosis pre-clinical period, but not the percentage of mortality

For cohabitation challenge, the percentage of shedders may to some extent influence the level of mortality. In the trials described in **article I** and **II** it was demonstrated that the percentage of shedders did not influence the percentage of fish mortality. In **article I,** 50% shedders were used in contrast to **article II** where only 20% shedders were added. The challenge dose was identical for both trials and the mortality reached 100%. The main difference found when comparing the challenge trials was in relation to the time when the mortality started. For the challenge with 20% shedders the cohabitant fish started to die 24 dpc, contrarily in the challenge with 50% shedders the cohabitants started to die 28 dpc. This was in line with previous trials conducted at VESO Vikan (unpublished data) in which different percentages of shedders were compared for challenge with *P. salmonis*. One tank contained 50% of i.p. injected shedders while the other tank contained 30% of i.p. injected shedders, the injected dose was identical and the fish were kept in freshwater at 16°C. In both tanks, cohabitant fish started to die just one day apart, being later in the tank with 50% shedders. The mortality of the fish challenged by cohabitation reached almost 100%. Different challenges presented equal characteristics independent of the number of shedders added into the tanks (**article I**, **II** and Figure 7). Small differences present in those challenges could be related with other factors as those mention previously (water temperature and salinity).

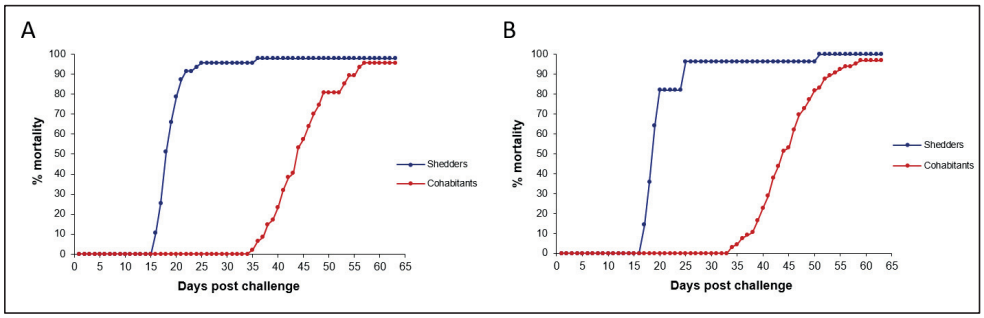

Figure 7. Mortality curves after challenge of parr with *P. salmonis* with different percentages of shedders. A, 50% shedders and 50% cohabitants. B, 30% shedders and 70% cohabitants.

Culture method and inoculum concentration are important for challenge trials

The method used for bacterial cultivation may influence the outcome of a challenge trial and should therefore be taken into consideration when designing an experiment. In the challenge trials executed in this study (**article I** and **II**) the bacterium was cultured in FN2 broth (recipe in **article III**). The main difference between the two culture media was in relation to the incubation period demonstrated in **article III**, where bacteria cultivated on CHAB plates needed at least six to ten days to be ready for harvest while bacteria cultivated in FN2 broth only needed four days of incubation at 18°C. The use of broth as a culture medium for preparation of *P. salmonis* inoculums requires a sterile environment (cabinet) due to the high risk of contamination. Media for cultivation of *P. salmonis* are typically highly nutritive[28]. For the agar media, it is also necessary to work in a clean environment, however, unlike with the liquid media it is possible to identify contamination by visual observation of the agar plate.

Calculation of the bacterial concentration in inoculums is complicated by the growth characteristics of *P. salmonis*, which makes it difficult to count colony forming units (CFU). This was demonstrated in **article III** where *P. salmonis* was plated, but single colonies were not possible to count. Prior to the two challenge trials performed in this study (**article I** and **II**) a theoretical challenge dose was calculated based on the measurements obtained by Berger (2014)[97]. The challenge dose resulted in 100% mortality after challenge by i.p. injection as well as cohabitation. It has been described that the level of mortality of the fish in challenge trials correlates with the concentration of the inoculum when the bacteria is grown in CHAB plates[79]. Previous challenge model development performed at VESO Vikan revealed that a certain degree of correlation between dose/percentage shedders and level of mortality could be obtained when fish are challenged by i.p. injection. At the dose needed for the shedder fish to transmit disease to the cohabitant fish, however, the mortality rate in the cohabitant

fish will be similar independent of initial challenge dose. These results are independent of cultivation media and are likely related to the growth characteristics and virulence displayed by *P. salmonis* as an intracellular bacterium.

Culture method may not affect piscirickettsiosis development, but biomass could

To verify that *P. salmonis* cultured in FN2 broth was able to induce mortality in Atlantic salmon, a small challenge trial (Figure 8) was conducted to evaluate the potential differences in mortality induced by *P. salmonis* cultured in CHAB plates compared with FN2 broth. In this challenge, three tanks were used; in each tank 10 i.p. injected (shedders) and 10 cohabitants were included. One tank was challenged with *P. salmonis* EM90-like isolate culture in FN2 broth (tank A), other with the same bacterial isolate culture on CHAB plates (tank B), and the third tank was challenged with a *P. salmonis* LF89-like isolate culture in FN2 broth (tank C). The fish challenged by i.p. injected in tank A started to die at 11 dpc, in contrast with tank B where the fish started to die at 13 dpc (**article III** and Figure 8). For the cohabitants, no mortality was obtain in fish challenged in tank A, but there was mortality in the cohabitants of tank B (41 dpc). For the tank C no mortality was observed. There was a difference in two days in the mortality, being the i.p. injected fish in tank A earlier than in tank B. The absence of mortality in the cohabitant fish in tank A could be related with the reduced biomass included in the trial. The results have been confirmed in additional trials at VESO Vikan (unpublished data). This demonstrated that to obtain mortality in the challenge with *P. salmonis*, not only the characteristics of the isolate are important, but also the biomass. If the biomass is too low, mortality in cohabitant fish may not occur. Furthermore, the absence of mortality in the fish infected with the LF89 strain, it may have been due to lack of virulence or biomass.

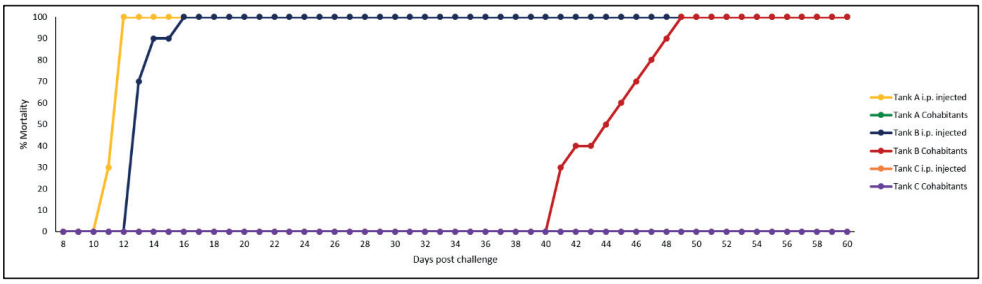

Figure 8. Mortality curves after a challenge of smolts with LF89- and EM90-like isolates of *P. salmonis* cultivated in FN2 broth or CHAB agar plates. Tank A, challenged with EM90-like isolate cultured in FN2 broth; Tank B, challenged with EM90-like isolate cultured on CHAB agar plates; Tank C, LF89-like isolate cultured in FN2 broth. 50% shedder fish were i.p. injected with 0.1 mL of an inoculum of 1.0×10^5 cfu mL^{-1} *P. salmonis* LF89- or EM90-like and introduced to the naïve cohabitant fish.

Stress caused by handling impacts the fish's susceptibility to piscirickettsiosis; this could have an impact on the outcome of experimental challenge trials

Mortality curves of the trial described in **article II** are shown in Figure 9A-D. All tanks were similar with regards to groups as well as water quality and temperature. In all tanks, the fish were exposed to handling during removal of morbid and dead fish once a day. In tank C and D, fish were exposed to additional handling during sampling once a week. Four tanks were kept in parallel, two tanks for sampling (Figure 9C and D) where the fish were sampled once a week and two tanks for mortality recording (Figure 9A and B). In the graph representing the tanks utilized solely for mortality recording it is possible to observe better separation of mortality curves, which reflects the dose-relation of the vaccines used in the study. In contrast, in the tanks where the samplings were performed, the mortality in the fish population challenged by cohabitation started earlier compared to the fish that were not exposed to handling. Furthermore, the dose-relation of the vaccines is not as clear. This could be explained by what has been described previously related to the stress that can result in an increment in the production of corticosteroids in the fish and this response is related to the increased susceptibility of the individuals to diseases[142]. In addition, the stress has been related with the exacerbation of infectious diseases like piscirickettsiosis[21, 139].

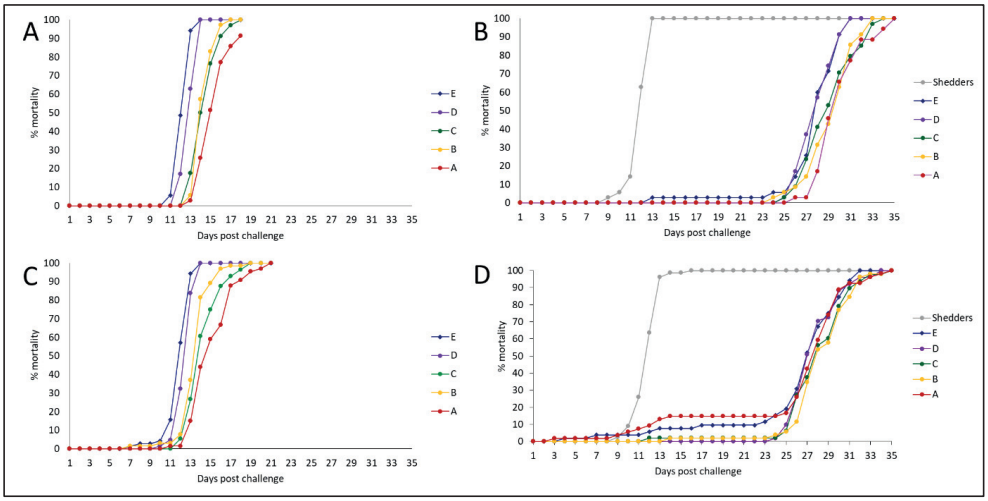

Figure 9. A, mortality curves of fish from a tank challenged by i.p. injection without handling; B, mortality curves from a tank with fish challenged by cohabitation without handling; C, mortality curves of fish from a tank challenged by i.p. injection with handling (sampling once a week); D, mortality curves of fish from a tank challenged by cohabitation with handling (samplings once a week). E = control group (saline); D = 0.5× antigen concentration; C = vaccine 2× antigen concentration; B = vaccine 3× antigen concentration; A = vaccine 5× antigen concentration.

Challenge by intraperitoneal injection or cohabitation

No major differences between i.p. injection and cohabitation challenge models with *P. salmonis* were detected

There has been a discussion related to which challenge model should be used, or not, in the evaluation of pharmaceutical products and prophylactic measurements in relation to piscirickettsiosis. **Article I** and **II** were developed with the objective to obtain an answer to this question by comparing the most used challenge models against *P. salmonis*. First, unvaccinated fish (**article I**) were challenged by i.p. injection or cohabitation. The duration of the i.p. injection study was as expected clearly shorter compared to the cohabitation study. Otherwise, no major differences between the challenge models were detected. Secondly, fish immunized (**article II**) with five different antigen proportions were tested. Again, no major differences were detected between the two challenge models. Based on these results, the suggestion is that the challenge model to be used depends on the aim of the study. If the aim of the study is the evaluation of the efficacy of vaccines, the i.p. injection challenge model could be the most appropriate related to cost-effectiveness and animal welfare issues. If the objective is to

study piscirickettsiosis as a disease, the challenge model of preference may be by cohabitation since this model mimics in a more natural way the disease development.

Furthermore, it could be of interested to compare Figure 9C and Figure 9D, where even though the fish were under stressful conditions (handling once a week) in the tank challenged by i.p. injection the vaccines with highest antigen levels seem to induce better protection against the disease. On the other hand, in the tank challenged by cohabitation where the fish were under the same conditions there was no differentiation between vaccines. This could strengthen the idea of using the i.p. challenge model to test vaccine efficacy. The indicated, was reflected by a better separation of the mortality curves (Figure 9A and 9C) demonstrating that the vaccine with higher concentration of *P. salmonis* antigens was able to delay the mortality in two days compared with the control vaccine. The groups started to die from those with less antigen concentration to the one with highest *P. salmonis* antigens concentration.

Differential evaluation of challenge models by pathology for registration of immunogenic products for fish should be established

For many years, the conduct of challenge trials with fish has been accomplished by the same parameters for different diseases without having any consideration about the impact of the pathology in the target population. Every disease in every species is different and diseases in fish are not an exception. There are some diseases with acute presentation and with high mortality rates like piscirickettsiosis[38, 143]. Other diseases have moderate or no mortality and could be classified as chronic diseases like some presentation of winter ulcers (caused by *Moritella viscosa*), bacterial kidney disease (caused by *Renibacterium salmoninarum*) and francisellosis (caused by *Francisella* sp. with a mortality not higher than 20%)[138, 144-147]. *In vivo* trials are part of the requirements for evaluation of the prophylactic measurements for fish diseases. However, the same parameters are used in evaluation of prophylactic measures for fish diseases, even though diseases can be very different in presentation and pathogenicity. The regulations for the registration of pharmaceutical products for veterinary medicine in Europe (EMA/CVMP/IWP/314550/2010, European Pharmacopoeia (Ph Eur) monograph 0062: vaccines for veterinary use, 92/18/EEC, 90676/EEC, CMDv/BPG/002)[148] were last updated in 2011 and they were developed based on a monograph from 1996[125, 126]. Based on improved scientific methods and animal welfare aspects, a revision of the regulations for vaccine testing could be useful. A similar process would be recommended for products intended for the Chilean market, where it is important to implement the new permanent vaccine registration regulations established by SAG.

The regulations would probably benefit from an update that takes into consideration the differences between diseases. It is known which fish diseases are considered chronic, acute or with moderate impact to the fish population and on the production. Each disease classification should have its own parameters for evaluation. For some of the diseases, the mortality as RPS value could be the most reliable indicator of vaccine efficacy, but for others it is necessary to find alternative outcome parameters. Especially, for those pathologies where the pathogenicity of the etiological agent is not fully understood like with piscirickettsiosis. Some of the alternatives that were suggested in this study (**article I** and **II**) such as histology, antibody titers, immune genes expression, and bacterial detection by qPCR. With the exception of histology, more studies are necessary to define if the other analysis are able to evaluate in a predictable manner the vaccine efficacy against piscirickettsiosis. Previous studies have aimed to develop less invasive methods for the documentation of protection against *P. salmonis* non-mortality based. This can be done for instance through antibody measurement, detection of bacterial DNA in serum and the use of mass spectrometry technologies[149-151]. Likewise, in this study (**article I** and **II**) some analyses were conducted with the objective to obtain more knowledge about the immune response of the fish and pathogenesis of *P. salmonis*, expecting to obtain some alternative outcome parameters other than RPS. An alternative to the RPS scale has been studied by Wilda, et al (2012)[150], where it was established a correlation between the serum IgM levels and the RPS after challenge with *P. salmonis*. It was, however, not possible to establish a cut-off value that ensured a high level of specific antibodies that could be applied in the vaccine development. Probably, a vaccine with good efficacy could facilitate the detection of good protection markers. Those markers could be a less invasive alternative to evaluate vaccine efficacy. Unfortunately, the efficacy of the research vaccine utilized in **article II** was not optimal. The lacking of fish protection was demonstrated by reaching 100% mortality and by measuring IgM titers. Even though there was an increment in the levels of antibodies present in plasma, the amount was not considerable to produce a strong immune response; therefore, no protection markers were identified. Improvements in the research vaccine can be apply such as the increment in the antigen concentration, which could result in a better immune response based on the IgM kinetics observed in **article II**.

In addition, the possibility that histology or IHC could be one of the candidates was suggested. In **article I** and **II**, the histology provided some guidelines related to *P. salmonis* infection and the IHC confirmed the presence of the bacterial cells in the lesions observed. It was demonstrated that histology and IHC are not the most sensitive analytical tools for the evaluation of the vaccine efficacy against piscirickettsiosis. Histopathological studies are, however, excellent tools for diagnosis of piscirickettsiosis. Histopathological analysis may also be utilized for vaccine safety evaluation due to the possibility to visualize the presence or absence of tissue damage induced by the test vaccines.

The European medicine agency (EMA), mentions in the "Guideline of the design of studies to evaluate the safety and efficacy of fish vaccines" that the mortality as an evaluation parameter should always be questioned, and that humane endpoints are always preferable[126]. This supports the necessity to find an alternative scale to measure vaccine efficacy.

From an ethical aspect, there is a strong drive to replace mortality as outcome parameter with other, less invasive methods. Indicators of disease that can be measured at an early stage of pathological changes are highly valuable candidates to replace the current mortality requirements. The changes may be implemented over time, but discussions are needed to make sure that the regulations at all times are in accordance with current scientific background.

The Chilean salmon industry was for several years an industry with poor investment in research. However, during the last years there has been a change in the industry, and better understanding of the fact that to be able to solve any difficulty the scientific view and collaboration between the academia and private sector are important.

In vitro studies on *P. salmonis*

Better knowledge of *P. salmonis* could improve the chance to prevent the disease

For characterization of bacteria, different *in vitro* methods can be utilized like microscopic observation, morphological description of the bacterial culture, Giemsa and Gram-staining and molecular techniques. Molecular techniques include among others enzymatic and biochemical profiling by commercial kits as well as protein profiling methods such as Western blotting, and finally genetic and phylogenetic characterization.

In **article I** and **II**, an *in vivo* characterization of the disease and pathogenesis after experimental challenge was performed, with the objective to understand how the bacterium behaves in the different challenge models and identify other measures than mortality for vaccine efficacy. For *in vivo* studies, the efficiency in the bacterial cultivation is important, for this any improvements in the inoculum preparation are relevant. Even tough, in the *in vivo* studies developed in **article I** and **II**, was demonstrated that the cultivation of *P. salmonis* in the new broth (FN2) was more efficient than in the old method, in **article III**, a validation of this was developed. It was compared the bacterial growth in CHAB agar plates to the bacterial growth in FN2 agar plates demonstrating that the bacterial growth was faster in the FN2 agar plates. Furthermore, in challenge trial, the inoculum preparation should be prepare when the bacteria is in the logarithmic phase of growth. To confirm that the *P. salmonis*

isolate was in the correct phase when the inoculums were prepared (**article I** and **II**), a growth curve was developed in **article III**. In that growth curve, was described that the logarithmic phase was reach at 40 hours and lasted for 56 hours. This demonstrated that the bacteria was harvested in the correct growth phase. Subsequently, in **article III**, phylogenetic and phenotypic characterization of the EM90-like isolate was done and a protein profile of the bacterium was described under different growth conditions, as well as after inactivation with formaldehyde. After the phylogenetic study, it was possible to confirm that the isolate used in this study belongs to the EM90 genogroup and differ to the LF89.

In **article II**, experimental vaccines were developed and utilized in the challenge trial. The results were not clear in terms of protection, possibly because the vaccines were not able to induce a sufficiently strong immune stimulation to elicit a reasonable amount of specific antibodies against *P. salmonis*. Also, was not able to stimulate the immune response based on T-cell. To try to identify the differences between an inactivated bacterium and a fresh culture of *P. salmonis* a Western blot protein profile was described in **article III**. The differences seen could reflect the reduction of the immunogenicity of the pathogen as a result of the treatment with formaldehyde[152], but this has not been proved. It is worth noting that several vaccines against extracellular pathogens have been developed based on inactivation with formaldehyde and are capable of inducing a successful long-lasting protection. The results from the Western blot revealed some bands that were only present in non-inactivated bacteria cultivated in FN2 broth. For characterization of these bands, a proteomic analysis would be necessary, with the objective to define the function of these proteins, which could have a role in the immune response against *P. salmonis*.

Detailed knowledge about the bacterium is important due to the fact that the more you know your enemy; the easier it is to fight him. In this case, the more we know *P. salmonis* it should be easier to find a long-term solution to prevent piscirickettsiosis and its consequences to the Chilean aquaculture.

Methodological considerations

Importance of considering the bacterial strain for challenge trials

The bacterial species, strain and isolate that should be used in a challenge is linked with the aim of the study. The results from a challenge trial may be affected by the isolate of choice as a consequence of different virulence[139]. These differences have been demonstrated in a study described by Rozas-Serri, et al (2018)[23] in which it was shown that two *P. salmonis* isolates induced significantly different levels of mortality. The study showed that i.p. injected and cohabitant fish infected with an EM90-like

isolate died significantly faster than fish infected with an LF89-like isolate. The findings were consistent with results from a trial performed at VESO Vikan (unpublished data) where an LF89-like isolate (FIUCHILE-89L01, isolated from Atlantic salmon in a commercial farm in Puerto Montt, Chile) did not induce any mortality (Figure 8). Similar results were obtained in a challenge with the Norwegian isolate NVI-5692 (obtained from the Norwegian Veterinary Institute) generating moderate mortality (Figure 10). The reason why the Norwegian isolate was not chosen for the present study is related to the geographical differences in the virulence of the bacteria described previously[153]. Based on this, it was more relevant to work with the EM90-like isolate which is described as the most widespread strain in the Chilean salmon industry[52].

The bacterial isolate used for experimental challenge should be representative for field outbreaks of the disease. Bacteria often target one fish species in particular, which is typically the case for *P. salmonis*. The current work was conducted using Atlantic salmon since this fish species accounted for 74% of the salmon harvest in Chile in 2018.

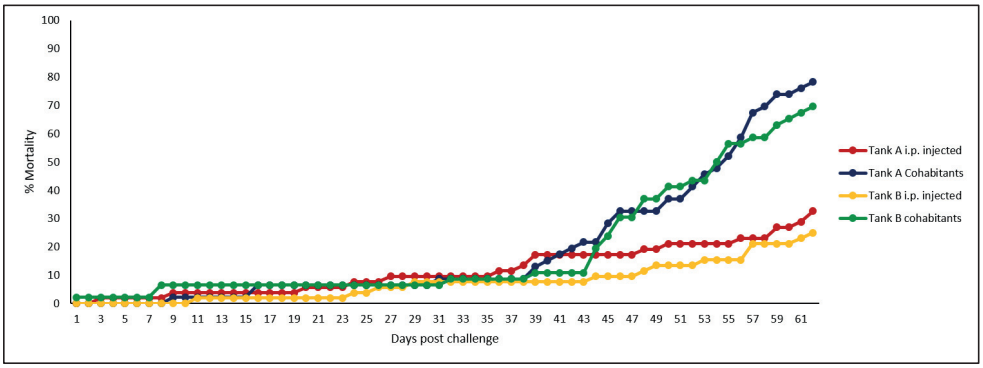

Figure 10. Mortality curve of unvaccinated Atlantic salmon (*Salmo salar* L.) challenged by cohabitation and i.p. injection with the Norwegian isolate, NVI-5692 (kindly provided by the Norwegian Veterinary Institute). Smolts were kept in seawater at 15°C. Atlantic salmon smolts challenged: 50% by i.p. injection (shedders) and 50% by cohabitation.

Expression of immune genes, antibody kinetics and the immune response are important variables to have under consideration in the development of a vaccine study

When the immune gene expression is studied by RT-qPCR it is essential to adjust the method by the use of house-keeping genes. Normalization to a reference house-keeping gene is done to reduce the errors related to variations in samples and technical variables as extraction, RNA quality among others[154]. How many house-keeping genes that should be considered for studies related with gene expression must be evaluated for each individual study. Relative quantification of expression of immune genes by qPCR is a well-established method. The stability of house-keeping gene expression has been evaluated and described in several studies[155-157]. The elongation factor 1 alfa beta (EF1αβ) was selected for the analysis performed in **article II** based on a previously reported trial performed by Løvoll, Austbø, Jørgensen, Rimstad and Frost (2011)[158].

The selection of the immune genes of study should be defined depending on which genes are likely to contribute to obtaining the major amount of information. In **article II**, one of the aims of the study was to obtain more information related to the immune response developed by fish vaccinated against *P. salmonis*. Previous descriptions indicate that the expression of genes associated with the adaptive immune response showed significant correlation with genes related to cell mediated immunity in fish infected with *P. salmonis*[23]. The genes of choice for **article II** were thus selected based on this.

In addition, in **article II**, specific antibodies kinetic was measured and some insights about vaccination/immune response interaction were obtained like the expression of *CD8α* immune gene, associated with cytotoxicity in infected cells, was down-regulated through the challenge trial for i.p. injection and cohabitation models, and the *CD4-1*, associated with T-helper cell, was up regulated. In case of the *CD8α*, in the i.p. injected fish a dose-response relation with the vaccines was observed at 2 wpc. Where a higher expression of *CD8α*, was observed in the vaccine with higher concentration of antigen. However, the half-life of specific antibodies against *P. salmonis* and their efficacy in protection are still unknown. It has been described that for some intracellular pathogens the development of specific antibodies take longer than expected, on the other hand, could be that the duration of the specific antibodies is short[103]. The efficacy of vaccines against piscirickettsiosis has been controvert due to the absence of long lasting protection, even though in some cases boosters are applied. Perhaps, the vaccination protocol has been administering incorrectly in terms of periods of administration, to define this could be necessary to do more analysis related with the half-life and kinetic of the specific antibodies against *P. salmonis*.

Specific *P. salmonis* primers and probes for bacterial DNA detection facilitate bacterial quantification

Primers, probes and antibodies for *P. salmonis* detection were developed several years ago using limited genomic information[28]. During the years, more sequence information was obtained, and more specific assays could be designed.

For detection of bacterial DNA, the specificity of the primers was tested to avoid cross-reaction with other bacteria that may be present. Due to some unspecific background, the use of a probe was decided. The probe used for the analysis described in **article I** and **II** was a result of a modification of the probe originally designed by Karatas, Mikalsen, Steinum, Taksdal, Bordevik and Colquhoun (2008)[159]. The primers used in **article I** and **II** were obtained from the same study and kept as the originals. Primers and probes were difficult to design because the whole genome of the EM90 genogroup utilized in this project was not available at the time of designing. Accordingly, identification of conserved and unique areas of the genome was challenging. In **article I**, the aim was to identify the presence of bacterial DNA in different tissues. The amount of DNA in each sample was not standardized, and accordingly no calculations of bacterial load could be made. Consequently, it was not possible to identify the highest bacterial load, which could have contributed to obtain more information related to the organ of preference or could have helped to clarify the port of entrance of *P. salmonis* to the host. Nevertheless, it was possible to corroborate the preference of *P. salmonis* for visceral tissue. In **article II**, the quantity of DNA in the samples was standardized, generating the possibility to identify liver as the visceral tissue that is most affected by *P. salmonis*.

From the results obtained in **article I** and **II**, it is possible to suggest that for further analysis, the tissue of study should be the liver.

Phenotypic and phylogenetic description of the EM90-like isolate of *P. salmonis* used in the studies described in **article I** and **II** was presented in **article III.** The isolate was confirmed to belong to the group of EM90-like isolates. New information about the bacterial genome such as conserved sequences opens for the possibility to design specific primers that may be used without an additional probe, thereby reducing the cost of the qPCR analysis. A fully sequenced *P. salmonis* genome may also contribute to identify, in the future, new antigens that may be evaluated with regard to immunogenic properties for vaccine development.

Necropsy: strong diagnostic tool, but difficult to standardize

Necropsy is the examination of the dead animal with the objective to corroborate the cause of the death of the animal. The examination is performed based on the observation of pathological changes. The pathological changes recorded in **article I** and **II** were described previously in the literature as part of the piscirickettsiosis development[24, 29, 38, 43, 50, 57, 61, 73].

The setup of the necropsy was a standard procedure established previously by VESO Vikan. The necropsy involved registration of pathological changes consistent with piscirickettsiosis. The registration was performed as presence or absence of a defined set of different pathological changes as ascites (A), pale focal nodes in the liver (PFNL), swollen kidney (SK), splenomegaly (SP), hemorrhages in the digestive tract (HDT), distended ventricle with gelatinous content (DV/GC) and general hemorrhages (GH). The main challenge with this kind of registration relates with the subjective classification of mild or more severe changes. Although the criteria are well-defined, it is difficult to maintain an objective evaluation. Necropsy is a valuable tool for diagnostic and for the study of pathogenesis. The amount and quality of the information obtained could be improved by implementing a more detailed scoring system.

The fish described in **article I** and **II** were starved prior to handling for challenge and samplings. Starvation of fish for a certain number of day degrees before handling or treatment is a well-established procedure[160, 161]. The procedure of starvation was implemented mainly to reduce the intestinal content in the fish, promoting a cleaner environment avoiding contamination of the samples. Additionally, the method is used to reduce the trauma of the remaining fish that were not sampled. The method of starvation of fish is frequently discussed, and several publications claim that there is no negative impact of short-term starvation with regards to health and stress levels.

CONCLUSION AND FUTURE PERSPECTIVES

Experimental challenge models are time consuming to develop and must be fully validated before use. A well-established challenge model aims to be robust and reproducible and must be optimized for each individual pathogen. The infectious material must be carefully selected and representative for field outbreaks of the disease.

Changes in the media for bacterial cultivation could improve the challenge trial by reducing the time of inoculum preparation. In the current study, a new FN2 broth media was used and validated in two challenge trials. The results with regards to mortality were similar compared with the old cultivation method on solid agar.

The challenge model of choice must be carefully selected based on the objective of the study. For evaluation of the efficacy of vaccines against piscirickettsiosis, this study showed that there are no major differences between the results obtained after challenge by i.p. injection or cohabitation.

Without considerable differences, it may be recommended to use the i.p. injection challenge model to evaluate vaccine efficacy. This model offers a short duration of the trial, which is valuable with regards to animal welfare since the fish are exposed to the pathogen for a shorter period of time. Otherwise, if the objective of the study is evaluation of disease pathogenicity, the cohabitation challenge model may be preferred.

The analysis that today are employed to evaluate fish vaccines may not apply for all the various diseases because each disease has a different pathogenicity. This difference could affect how the fish reacts to vaccines and how the vaccines should be evaluated to prove their efficacy, potency and safety. New outcome parameter for evaluation of vaccine efficacy should be implement for animal welfare reasons. Regulation for vaccines registration, in Chile, should be established, defined and regularly updated by the authorities.

Additional studies should be performed to better understand the pathogenicity of *P. salmonis* and the effect on the fish. It should keep studying the fish immune response and specific antibody kinetic against *P. salmonis* and their half-life. Aforementioned, could add more knowledge in the development of prophylactic measurements against piscirickettsiosis. Likewise, all documentation in terms of efficacy and all the information that could improve the fish protection should be taken under consideration.

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APPENDIX (ARTICLES I-III)

ARTICLE I

Received: 1 February 2019 | Revised: 11 March 2019 | Accepted: 14 March 2019

DOI: 10.1111/jfd.13004

ORIGINAL ARTICLE

Journal of Fish Diseases WILEY

Development of piscirickettsiosis in Atlantic salmon (*Salmo salar* L.) smolts after intraperitoneal and cohabitant challenge using an EM90-like isolate: A comparative study

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Funding information
VESO; Norwegian Research Council, Grant/Award Number: 258783

Abstract

Piscirickettsiosis, caused by the intracellular Gram-negative bacteria *Piscirickettsia salmonis*, is at present the most devastating disease in the Chilean salmon industry. The aim of this study was to analyse disease development after challenge with a *P. salmonis* strain (EM90-like) under a controlled environment by comparing intraperitoneal challenge with cohabitation challenge. The *P. salmonis* EM90-like isolate was cultured in a liquid medium for the challenge of 400 Atlantic salmon (*Salmo salar*) smolts. Cumulative mortality was registered, necropsy was performed, and bacterial distribution in the tissues and histopathological changes were analysed. The results revealed a similar progression of the disease for the two different challenge models. Pathological and histopathological changes became more visible during the development of the clinical phase of the disease. Bacterial DNA was identified in all the analysed tissues indicating a systemic infection. Bacterial tropism to visceral organs was demonstrated by real-time quantitative PCR and immunohistochemistry. Better knowledge of disease development during *P. salmonis* infection may contribute to further development of challenge models that mimic the field situation during piscirickettsiosis outbreaks. The models can be used to develop and test future preventive measures against the disease.

KEYWORDS

Atlantic salmon, challenge model, cohabitation, intraperitoneal, *Piscirickettsia salmonis*, Piscirickettsiosis

1 | INTRODUCTION

Piscirickettsiosis is the most challenging disease present in the Chilean salmon industry generating economical annual losses of around USD 700 millions (Maisey, Montero, & Christodoulides, 2017). The disease was reported for the first time in 1989 in Calbuco, Los Lagos, Chile, followed by isolation and characterization of the causative bacterium *Piscirickettsia salmonis* (Fryer, Lannan, Garcés, Larenas, & Smith, 1990; Garcés et al., 1991). Piscirickettsiosis is a septicaemia that affects mainly salmonid species resulting in high mortality rates (Otterlei et al., 2016). The last year, the most affected specie was Atlantic salmon (*Salmo salar*) followed by rainbow trout (*Oncorhynchus mykiss*) ([SERNAPESCA], 2018). At the beginning, Coho salmon (*Oncorhynchus kisutsch*) was the most affected salmonid species (Almendras & Fuentealba, 1997), but after a reduction in the production, the cases of piscirickettsiosis decreased.

Various isolates of *P. salmonis* have been characterized and are grouped based on genotyping as LF89-like (Fryer, Lannan, Giovannoni, & Wood, 1992) or EM90-like (Mauel, Giovannoni, & Fryer, 1999). The EM90-like genotype has been demonstrated to be widely distributed when piscirickettsiosis is diagnosed in field cases in Chilean salmon farms and is responsible for a high number of piscirickettsiosis outbreaks (Saavedra et al., 2017). Piscirickettsiosis has also been described in Canada (Brocklebank, Evelyn, Speare, & Armstrong, 1993), Ireland (Rodger & Drinan, 1993) and Norway (Olsen, Melby, Speilberg, Evensen, & Håstein, 1997). However, the incidences and losses from the disease are lower in these countries than the ones reported in Chile (Birkbeck, Griffen, Reid, Laidler, & Wadsworth, 2004). *Piscirickettsia salmonis* is an aerobic Gram-negative, facultative intracellular and non-motile bacterium (Bravo & Martinez, 2016). During disease development, the most affected organs are liver, spleen, intestine and the hematopoietic tissue in the head kidney (Almendras & Fuentealba, 1997; Evensen, 2016). Histopathological lesions have been classified in the categories of necrosis and inflammation (Almendras & Fuentealba, 1997; Mauel & Miller, 2002). The presence of the bacteria has been described in cells similar to macrophages (Rozas & Enríquez, 2014) in the kidney, liver and heart (Rozas-Serri et al., 2017). The bacterial cells have also been detected in hepatocytes and blood (Mauel & Miller, 2002).

Prophylactic treatment against piscirickettsiosis is widely used in the Chilean industry. Around 70 million vaccinated Atlantic salmon smolts were transferred to sea in 2016 (Navarro, 2018). In 2017, 32 different vaccines against this disease were available in Chile ([SAG], 2017). Nevertheless, vaccination is currently unable to stop outbreaks and the industry highly depends on antibiotics to treat the disease. In 2016, the Chilean salmon industry reported the use of 382.5 tons of antibiotics in total, of which piscirickettsiosis treatment accounted for 89.3% of the total use ([SERNAPESCA], 2017). Thus, there is a strong need for the development of more efficient vaccines against piscirickettsiosis. More knowledge about the different genotypes of the bacterium and their interaction with the host is crucial to develop protective vaccines.

Controlled challenge models represent an essential tool to study disease development in fish. The experimental models are designed to mimic authentic infections in a controlled environment and are typically performed by intraperitoneal (i.p.) injection of the pathogen, by cohabitation of naïve fish with i.p. injected shedder fish or by immersion. Intraperitoneal injection of the pathogen typically results in faster disease development and therefore provides results in a cost-efficient manner. Even when cohabitation and immersion challenges may seem to be more representative for comparison with field infection conditions compared to i.p. challenge in terms of route of infection, the challenge models have not been compared to assess their similarity and/or differences.

The aim of the study was to describe the disease development after infection with a *P. salmonis* EM90-like isolate by comparing an i.p. challenge model with cohabitation challenge. The pathological findings were evaluated in relation to bacterial distribution. The sequential development of pathological changes in the fish challenged with *P. salmonis* throughout the study was described with regard to challenge method and tissue tropism.

2 | MATERIALS AND METHODS

2.1 | Fish

Experimental fish were produced at the VESO Vikan hatchery. The fish were smoltified at the hatchery by manipulating the photoperiod using artificial light. The fish were exposed to 6 weeks of alternation between 12 hr light and 12 hr darkness followed by a 6 weeks continuous light photoperiod. A total number of 400 unvaccinated Atlantic salmon with an average weight of 102.3 g were used. The fish population was confirmed negative for antibodies against *Aliivibrio salmonicida*, *Vibrio anguillarum* O1 and O2a, *Vibrio ordalii*, *Aeromonas salmonicida* and *Moritella viscosa*. The absence of *P. salmonis*, infectious pancreas necrosis virus (IPNV), infectious salmon anaemia virus (ISAV), salmon pancreas disease virus (SPDV), piscine orthoreovirus (PRV) and piscine myocarditis virus (PMCV) was confirmed by qPCR (PatoGen Analyse AS) prior to trial start. Non-vaccine was used for this study.

2.2 | Bacterial cultivation

A *Piscirickettsia salmonis* EM90-like isolate (VESO Vikan) originally isolated from an outbreak in 2004 in Calbuco, Los Lagos, Chile, was used. This isolate was previously confirmed by BLAST® alignment after sequencing. This *P. salmonis* isolate was aerobically cultured from an ampule stored at −80°C in 10 ml of FN2 liquid medium (unpublished media, original recipe provided by FAVET-INBIOGEN, Universidad de Chile) with agitation (150 rpm) at 18°C for four days. Spectrophotometric analysis (Jenway 6300) was used to measure the density of the liquid culture. Control of contamination was evaluated by visual examination of the liquid culture under a phase contrast microscope, and in addition, 100 µl of culture was plated on cysteine heart agar (CHAB) supplemented with ovine blood (5%) (Norwegian Veterinary Institute) and on blood agar with 2% NaCl (Kystlab-PreBio, Namsos, Norway). The CHAB plate was incubated aerobically at 18°C and the blood agar was incubated aerobically at 22°C, both for ten days. No bacterial colonies other than *P. salmonis* were observed on CHAB plates. No bacterial colonies were observed on blood agar plates.

2.3 | Challenge procedure and cumulative mortality

The challenge trial was approved by the Norwegian Animal Welfare Authority (ID 9409) and conducted at VESO Vikan research facility (Namsos, Norway). Fish were acclimatized in sea water with a salinity of 32‰ at 15°C for 14 days prior to trial start and starved for 48 hr prior to challenge. At day 0, the fish were distributed into duplicated tanks (tank A and tank B). The tanks had a capacity of 420 L, and the stocking density was not higher than 40 kg/m^3 for the trial. Each tank included 100 i.p. injected fish (shedders) and

100 cohabitant fish (naïve). The shedders were anesthetized with 0.001% benzocaine chloride (Sykehusapoteket Oslo, Ullevål) and marked by shortening the adipose fin. The bacterial inoculum for i.p. injection of shedder fish was adjusted to OD_{600nm} = 1.0 and then further diluted by four tenfold dilutions giving a theoretical dose of 1.0×10^5 cfu/ml. Each shedder fish was injected with 0.1 ml of the bacterial inoculum on day 0. The concentration used was based on a previous publication where OD_{600nm} = 1.0 of the bacterial culture was estimated to be 1.0×10^{10} cfu/ml (Berger, 2014). The fish were kept in sea water with a salinity of 32‰ at 15°C and 24 hr light. The fish were fed with a commercial diet (Skretting AS) twice a day from day 1 post-challenge (dpc). The fish were monitored daily, and dead fish were registered. Fish in the terminal stage with clear signs of disease (erratic swim, lethargic, ulcer in the skin, pale gills, among other) were euthanized with an overdose of benzocaine chloride and registered as dead. Bacteriological examination of dead fish was performed daily by inoculating head kidney samples on blood agar with 2% NaCl and incubation at 22°C for 48–72 hr.

2.4 | Fish sampling

The fish were selected randomly by netting from tanks A and B and euthanized with an overdose of 0.002% benzocaine chloride. Ten control fish were sampled prior to challenge on day 0. Samples were collected weekly (7, 13, 20, 27 and 34 dpc) from six i.p. injected fish and six cohabitants per tank.

2.4.1 | Blood samples

Blood samples were obtained immediately after euthanizing the fish and before the necropsy was performed. Blood was drawn from the dorsal vein (*Vena caudalis*) and/or dorsal artery (*Aorta caudalis*) by puncturing the fish lateroventrally behind the anal fin. Vacutainer® blood collection tubes containing ethylenediaminetetra-acetic acid (EDTA) anticoagulant were used (BD, New Jersey, USA). After sampling, the tubes were centrifuged at 797 *g* at 4°C for 10 min before the plasma was transferred to eppendorf tubes and stored at −80°C.

2.4.2 | Necropsy

Necropsy was performed immediately after blood sampling. No necropsy was performed in control (untreated) fish. The pathological changes were classified as ascites (A), pale focal nodes in the liver (PFNL), swollen kidney (SK), splenomegaly (SP), haemorrhages in the digestive tract (HDT), distended ventricle and/or with gelatinous content (DV/GC) and general haemorrhages (GH). The pathological changes were registered as percentage of the occurrence among the 12 sampled fish per group.

2.4.3 | Tissue samples

Tissue samples were dissected from spleen, skeletal muscle, liver and head kidney. The samples for qPCR were submerged in RNA*later*® (Sigma-Aldrich) and stored at 4°C overnight, followed by −20°C until DNA extraction. Samples for immunohistochemistry and histology were fixated in 4% formaldehyde for 24 h followed by transfer to 70% ethanol and storage at 4°C.

2.5 | DNA extraction and real-time quantitative PCR

A total of 470 samples were analysed by real-time quantitative PCR (qPCR). Out of these, 40 tissue samples and 10 plasma samples were from control fish, and 336 tissue samples (72 from i.p. injected fish and 180 from cohabitant fish) and 84 plasma samples (24 from i.p. injected and 60 from cohabitant fish) were from challenged fish. Tissue samples were weighed (up to 10 mg of spleen and 25 mg for other tissues) in OHAUS® Analytical plus (Sigma-Aldrich) scale before they were homogenized using sterile steel beads and TissueLyser II (Qiagen) for 15 s at 25 Hz and incubated with protein kinase (Qiagen) at 56°C overnight. The DNA extraction was performed using QIAcube and QIAGEN DNeasy® Blood & tissue kit (Qiagen) according to the manufacturer's protocol. The quality of the DNA was confirmed using NanodropTM ND-1000 spectrophotometer (Thermo Scientific). Primer and probe sequences targeting *P. salmonis* were obtained from Karatas et al. (2008). Primer sequences were 5′-AGG GAG ACT GCC GGT GAT A-3′ for forward primer and 5′-ACT ACG AGG CGC TTT CTC A-3′ for the reverse primer. The probe sequence was modified to increase the specificity using the software Primer Express v 2.0 (Thermo fisher Scientific). The resulting probe sequence was 5′-TCG CTC CAC ATC GC-3′.

For the detection of bacterial DNA, qPCR was performed. Each reaction contained 10 µl TaqMan qPCR Supermix, 300 nM of specific forward and reverse primer, 100 nM of a specific probe and 2 µl DNA template in a total volume of 20 µl. The qPCR thermal profile started with 10 min denaturation at 95°C followed by the cyclic protocol applying a melting point at 95°C for 15 s, and annealing and elongation at 58°C for 45 s, for 40 cycles. Samples were analysed in triplicate. Results with C_t values above 35 were considered negative.

2.6 | Histology and Immunohistochemistry

All sampled fish (n = 70) were evaluated subjected to histological and immunohistochemical analysis. Formalin-fixed samples from liver, head kidney, spleen and skeletal muscle were processed for histological analysis and immunohistochemistry according to standard procedure (Suvarna, Layton, & Bancroft, 2018). For haematoxylin and eosin staining (HE), paraffin-embedded tissues were sectioned at 2 µm thickness. HE sections were evaluated in a blinded manner by two independent scientists with a scoring system from 0 to 3. The criteria for scoring the samples are shown in Table 1. After scoring the samples, the average of both values was calculated. For immunohistochemistry (IHC), tissues were sectioned at 4 µm thickness and mounted on poly-L-lysine-coated slides (KF FROST, VWR International BV, Amsterdam, The Netherlands). Protocol for immunohistochemistry was adapted

TABLE 1 Score system for haematoxylin–eosin slides

Score	Liver	Spleen	Head kidney	Skeletal muscle
0	No visible changes	No visible changes	No visible changes	No visible changes
1 Mild changes	Focal changes dominated by inflammation	Mild congestion	Oedema hematopoietic tissue	Oedema causing interfibre space increase
2 Moderate changes	Focal changes dominated by necrosis	Loss of tissue architecture due to oedema	Inflammation and oedema hematopoietic tissue and thickening Bowman's capsula	Fibre degeneration
3 Extensive changes	Bridging or diffuse necrosis surrounded by inflammation	Oedema and congestion, loss of tissue architecture, perivascular aggregation of immune cells and necrosis	Necrosis of hematopoietic tissue with oedema and an increase in inflammatory cells, glomerulone-phritis and enlargement of Bowman's space	Oedema causing interfibre space increase and fibre degeneration

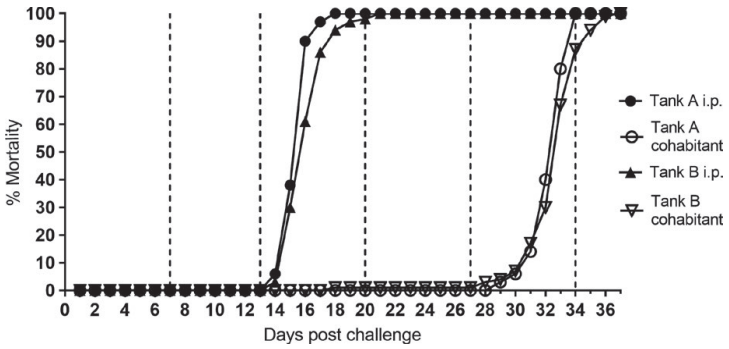

FIGURE 1 Cumulative mortality after experimental challenge with *Piscirickettsia salmonis* (EM90-like). Parallel groups of i.p. injected shedders and naïve cohabitant fish were included in both tanks A and B. Discontinuous vertical lines indicate time of sampling

from Dalum et al. (2016) including a primary antibody against *P. salmonis* (kindly provided by Duncan Colquhoun, NVI). The slides were incubated at 58°C overnight prior deparaffinization in xylene. Heat-induced epitope retrieval was performed at 121°C for 10 min in 0.01 M citrate buffer, pH 6, followed by washing in 0.01 M phosphate-buffered saline (PBS, pH 7.3). The sections were incubated for 40 min at 37°C in pre-heated PBS with 0.05% phenyl-hydrazine (Aldrich chemistry, MO, USA) followed by washing with PBS. Slides were blocked with a solution of 0.05 M tris-buffered saline (TBS, pH 7.6) with 0.2% goat normal serum and 5% bovine serum albumin (BSA) for 20 min. The primary polyclonal rabbit antibody was diluted 1:40,000 in TBS with 1% BSA. The antibody was labelled with polymer-HRP anti-rabbit (Dako EnVision + System-HRP) and developed with 3-amino-9-ethyl carbazol (AEC) producing a red staining when reacting with tissue-bound primary antibody. Counterstaining was performed with haematoxylin followed by washing in distilled water. A descriptive observation was performed.

3 | RESULTS

3.1 | Mortality and challenge data

Similar mortality curves were obtained from tank A and tank B (Figure 1). In the i.p. injected group, dead smolts were recorded from 14 dpc in both tanks and reached 100% mortality at 18 dpc in tank

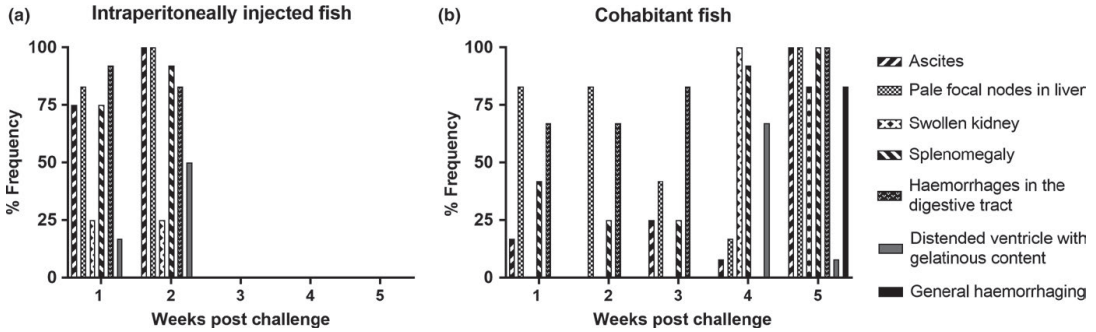

FIGURE 2 Pathological findings in sampled fish after (a) challenge by intraperitoneal injection and (b) challenge by cohabitation. wpc: weeks post-challenge. % Frequency = number of sampled fish that showed pathological changes divided by the total number of sampled fish per time point

A and 21 dpc in tank B. In the cohabitant (naïve) group, dead smolts were detected from 29 dpc in tank A and 28 dpc in tank B. In this group, mortality reached 100% on 34 and 37 dpc in tanks A and B, respectively. A minor tank effect was observed between tanks A and B during the challenge experiment (Figure 1). The mortality curve of the i.p. challenged fish was slightly steeper compared with the more prolonged mortality curve of the cohabitant challenge fish. A long incubation period was detected in both challenge models, 14 days from challenge to onset of disease outbreak was observed in i.p. injection challenge model and 28 days for cohabitation model. The duration of the disease until all smolts were dead was in average 5.5 and 7.0 days in the i.p. and cohabitation challenge, respectively (Figure 1).

3.2 | Necropsy

The type and frequency of pathological findings are shown in Figure 2. All the sampled fish were alive at the time of sampling. Pathological changes such as ascites, pale focal nodes in the liver, splenomegaly and haemorrhages in the digestive tract were observed from 1 week post-challenge (wpc) in both challenge models. Swollen kidney and distended ventricle with gelatinous content were only observed at 1 and 2 wpc in i.p. challenged fish and at 4 and 5 wpc in cohabitant fish. Pathological signs were more present in i.p injected fish at 2 wpc compared with cohabitant fish at 4 wpc, except from swollen kidney and distended ventricle with gelatinous content that were identified in 100% (12/12) and 92% (11/12) of cohabitant fish compared with 25% (3/12) and 50% (6/12) of the i.p. injected group, respectively.

Pathological changes were observed from 1 wpc in the i.p. injected fish. Ascites was observed in 75% (9/12) of the sampled fish, pale focal nodes in the liver in 83% (10/12), swollen kidney in 25% (3/12), splenomegaly in 75% (9/12), haemorrhages in the digestive tract in 92% (11/12) and distended ventricle with gelatinous content in 17% (2/12) of the sampled fish 1 wpc. Ascites and pale focal nodes in liver were observed in 100% (12/12) of the sampled fish in 2 wpc, splenomegaly in 92% (11/12), haemorrhages in the digestive tract in 83% (10/12), swollen kidney in 25% (3/12) and distended ventricle with gelatinous content in 50% of the sampled fish. General haemorrhages were not observed after i.p. challenge.

Fish challenged by cohabitation developed pathological changes differently compared with the i.p. challenged fish. Ascites was observed in less than 25% (3/12) of the fish from 1 wpc to 4 wpc. No ascites was observed 2 wpc and reached 100% (12/12) during 5 wpc. Pale focal nodes in the liver were observed in more than 75% (9/12) of the sampled fish at 1 and 2 wpc. Pale focal nodes in the liver were only observed in 42% (5/12) of the fish at 3 wpc and 17% (2/12) of the sampled fish at 4 wpc. Pale focal nodes in the liver were observed in 100% (12/12) of the fish at 4 wpc. Swollen kidney was not observed at 1, 2 and 3 wpc but was observed in 100% (12/12) and 75% (9/12) of the sampled fish at 4 and 5 wpc, respectively. Splenomegaly was observed in less than 50% (6/12) of the fish at 3 wpc, increase at 4 wpc (92% (11/12)) and reach 100% (12/12) at 5 wpc. Haemorrhages in the digestive tract were observed at 1, 2 and 3 wpc with increase by time until 4 wpc where no haemorrhages were observed but then reach 100% (12/12) at 5 wpc. Distended ventricle with gelatinous content was not observed until 4 wpc (67% (8/12), and there was a reduction in the frequency reaching an 8% (1/12) of the sampled fish at 5 wpc. General haemorrhages in the fish were observed at 5 wpc in 83% (10/12) of the sampled fish.

3.3 | Histology: Haematoxylin and eosin staining

The histopathological changes were evaluated according to an established scoring system from 0 (no changes) to 3 (extensive changes) (Table 1). The scoring of sampled fish throughout the challenge experiment is presented in Figure 3. Scores 0–2 were observed during disease development in the i.p. injected fish. In the cohabitant fish, however, score 3 was described at 5 wpc. In the i.p. injected fish, a slight increment in histopathological changes was observed

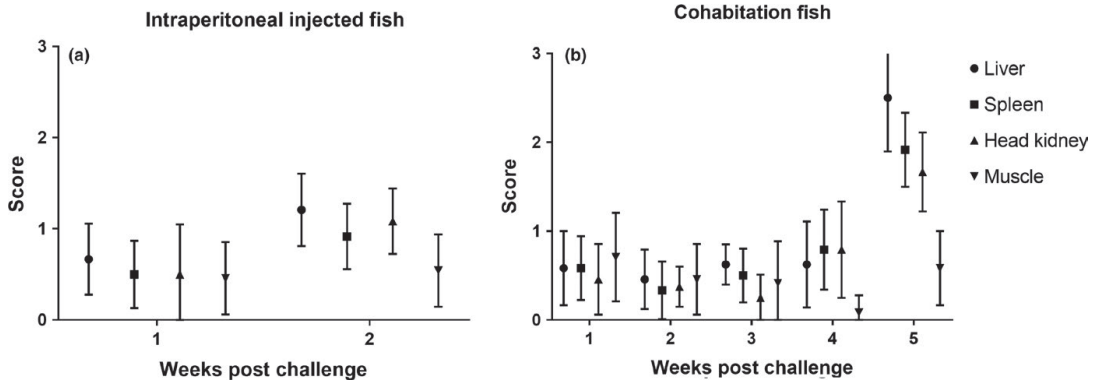

FIGURE 3 Distribution of the average of the histopathological scoring after i.p. and cohabitation challenge. The vertical bars show the average standard deviation of the scoring. Score 0 represents no changes in the tissue. Scores 1 and 2 represent mild and moderate changes in the tissue, respectively. Score 3 represents extensive changes in the architecture of the tissue

between 1 and 2 wpc. For the cohabitant group, a slight increment in histopathological changes was observed between 3 and 4 wpc, while an increase in the scoring was observed between 4 and 5 wpc. Histopathological changes representative for each scoring value are shown in Figure 4.

3.4 | Immunohistochemistry

Immunohistochemistry (IHC) revealed the presence of bacteria in different host tissues, demonstrating that the histopathological findings may be due to the bacterial infection (Figure 5). The bacteria were identified in all types of analysed tissues, with an increasing number of positive reactions in the last period of the challenge, especially at 5 wpc in cohabitants compared with 2 wpc in the i.p. injected fish. Results of the IHC observation are summarized in Table 2. No bacteria were detected at 1 wpc in either challenge model. At 2 wpc, the bacteria were detected in all tissue in i.p. challenge but no bacteria were observed in the cohabitant fish. At 3 wpc, the bacteria were detected in spleen and head kidney. The bacteria were observed in all tissues at 4 and 5 wpc.

The IHC demonstrated the presence of the bacteria free in the tissue and inside cells resembling macrophage as a red coloured reaction. *P. salmonis* was found to be focally distributed in liver (Figure 5m) tissue in contrast to spleen (Figure 5o), head kidney (Figure 5n) and skeletal muscle (Figure 5l) tissues where the bacterial distribution was more spread and homogenous. No bacteria were detected in tissues with a histopathological score 0 (Figure 5a–c). A sparse amount of bacteria (score 1, Figure 5e–h) was mainly observed in liver tissue (Figure 5e). A clear increase in the red staining is demonstrated in Figure 5i–l representing score 2. The most extensive occurrence of positive reactions of *P. salmonis* was observed in the liver of cohabitant fish at 5 wpc shown in Figure 5m where focal necrotic areas were visualized.

3.5 | Distribution of bacterial DNA by qPCR

The presence of *P. salmonis* DNA was confirmed by qPCR. The detection limit of *P. salmonis* was set to a Ct value of 35; all samples with a Ct value lower or equal to 35.0 were considered positive. The 10 control fish were negative for the presence of *P. salmonis* DNA.

In the first wpc, *P. salmonis* DNA was detected in spleen tissue of 58% (7/12) of the i.p. challenged fish. All tested tissues were positive in 91.7% (11/12) of the sampled fish in the i.p. challenged group 2 wpc. Bacterial DNA was detected 2 wpc in the spleen and skeletal muscle of 8.3% (1/12), and in the head kidney of 50% (6/12) of the cohabitant fish. The presence of *P. salmonis* was detected in head kidney, liver and muscle samples 3 wpc in 16.6% (2/12), 8.3% (1/12) and 8.3% (1/12) of the sampled fish, respectively. All tissues were positive in 50% (6/12) and 100% (12/12) of the sampled fish at 4 and 5 wpc, respectively. The results are summarized in Table 3.

4 | DISCUSSION

For the present study, the bacteria were cultured in liquid medium at 18°C for four days before challenge. Authors of previous reports (Almendras, Fuentealba, Markham, & Speare, 2000; Berger, 2014; Díaz et al., 2017; Garcés et al., 1991; Rozas-Serri et al., 2017; Smith et al., 2015) have cultivated the bacteria for challenge on solid media or cell culture. To our knowledge, this is the first report on *P. salmonis* cultivation in liquid medium for challenge in Atlantic salmon. The results indicate that bacteria cultivated in liquid media show similar virulence as bacteria cultivated on solid media (CHAB plates) (unpublished data from VESO Vikan research station) with the advantage of shorter incubation period for the former. This is positive in terms of shortening the preparation time for the challenge trial and reduces the number of bacterial generations in each cultivation, reducing the probabilities to produce genetic changes in the isolate.

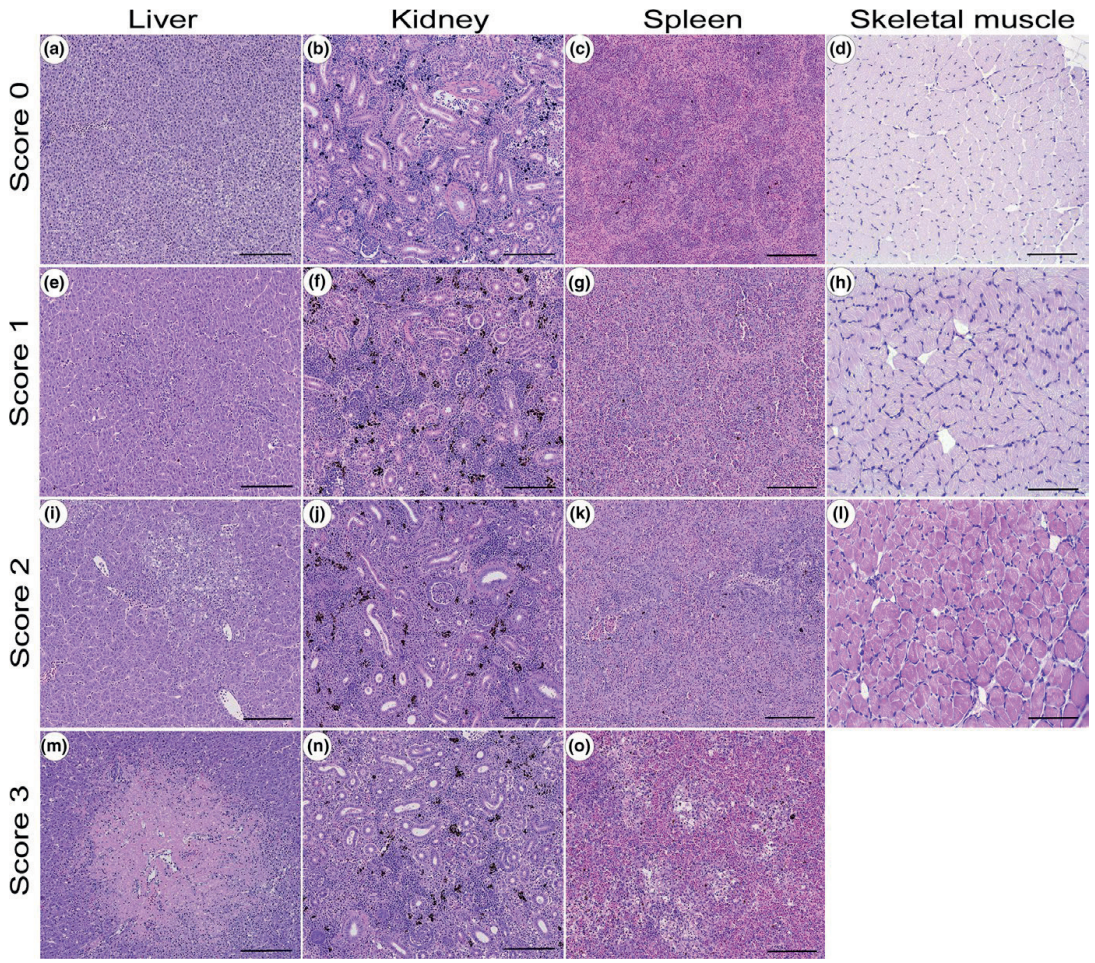

FIGURE 4 Representative histomorphological images (HE). Representative images from liver, kidney, spleen and skeletal muscle with scores 0, 1, 2 and 3 (score 3 was not observed for skeletal muscle in this experiment). Score 0: no visible changes in the tissue. Score 1: focal changes with inflammation in the liver, oedema in head kidney, mild congestion in the spleen and oedema causing interfibre space increase in skeletal muscle. Score 2: focal changes with inflammation and necrosis in liver, inflammation and oedema in head kidney, loss of tissue architecture in spleen and fibre degeneration in skeletal muscle. Score 3: extensive necrosis in liver, necrosis with oedema in head kidney, oedema and congestion and loss of architecture in the spleen. Scale bar = 100 μm

Piscirickettsiosis is known as an aggressive disease that causes high mortality and significant economic losses (Almendras, Fuentealba, Jones, Markham, & Spangler, 1997; Dettleff, Bravo, Patel, & Martinez, 2015; Rozas-Serri et al., 2017). An incubation period of 14 days was observed in i.p. challenged fish in this study which is in agreement with a previous report by Rozas-Serri et al. (2017). In the present study, the incubation period was followed by an aggressive and short outbreak with fish mortality reaching 100% in both challenged groups. In contrast to the former report, fish death of the cohabitant group was in our study recorded significantly earlier (28 dpc vs. 36 dpc). Several factors such as challenge conditions (water quality and temperature) and fish species could influence the disease development (Almendras et al., 1997; Arkush et al., 2005; Díaz et al., 2017). In our challenge experiment, fish were held in full strength sea water (32‰ NaCl) at 15°C. This is in contrast to the experiment of Rozas-Serri et al. (2017) in which the fish were held in brackish water (15‰ NaCl) at 12°C.

It is likely to assume that the challenge dose for cohabitant fish was considerably lower compared with shedder fish that was receiving a high dose of bacteria injected directly into the intraperitoneal cavity. In the present study, the disease development and mortality progressed faster in the i.p. challenged fish compared with the cohabitant fish group (Figure 1), thus indicating that piscirickettsiosis has a dose-dependent pathogenesis. This

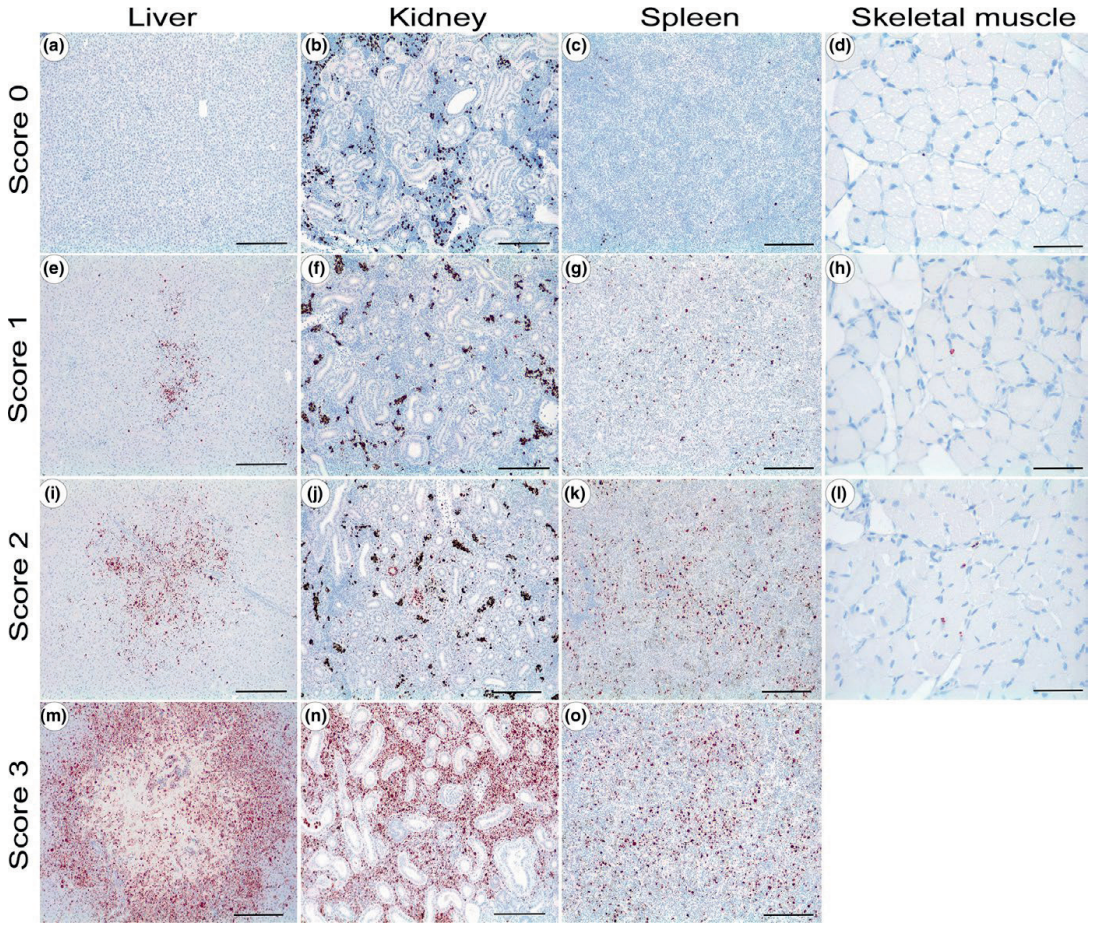

FIGURE 5 Representative immunohistochemistry images of liver, kidney, spleen and skeletal muscle sections from fish challenged by *Piscirickettsia salmonis*. The bacteria are visualized by red staining (AEC). The corresponding histopathological scores are indicated. Score 3 was not observed for skeletal muscle in this experiment. Scale bar = 100 µm in a–c, e–g, i–k and m–o; 50 µm in d, h and l

is in agreement with the results of a study made by Smith et al. (2015) in which a high mortality of rainbow trout was recorded at a short time post-immersion with a high dose (10^5 TCID 50/ml) of *P. salmonis*. The mortality curves of the two fish groups were otherwise similar when comparing the results from 1 and 2 wpc of the i.p. injected fish with 3 and 4 wpc of the cohabitation challenged fish (Figure 1). This observation is supported by similarities in the development of the disease. No major differences in

TABLE 2 Presence of *Piscirickettsia salmonis* detected by IHC in different tissues after challenge by intraperitoneal injection or cohabitation

	Intraperitoneally injected fish				Cohabitant fish			
wpc	Liver	Spleen	Head kidney	Skeletal muscle	Liver	Spleen	Head kidney	Skeletal muscle
1	–	–	–	–	–	–	–	–
2	+	+	+	+	–	–	–	–
3	n/a	n/a	n/a	n/a	–	+	+	–
4	n/a	n/a	n/a	n/a	+	+	+	+
5	n/a	n/a	n/a	n/a	+	+	+	+

Note. wpc: week post-challenge; +: presence of bacterial DNA in at least one out of twelve fish; –: not detected; n/a: not available.

TABLE 3 Presence of *Piscirickettsia salmonis* DNA detected by qPCR in different tissues after challenge by intraperitoneal injection or cohabitation

	Intraperitoneally injected fish					Cohabitant fish				
wpc	Liver	Spleen	Head kidney	Skeletal muscle	Plasma	Liver	Spleen	Head kidney	Skeletal muscle	Plasma
1	+	+	+	–	–	–	–	–	–	–
2	+	+	+	+	+	–	+	+	+	–
3	n/a	n/a	n/a	n/a	n/a	+	+	+	+	–
4	n/a	n/a	n/a	n/a	n/a	+	+	+	+	+
5	n/a	n/a	n/a	n/a	n/a	+	+	+	+	+

Note. wpc: week post-challenge; +: presence of bacterial DNA in at least one out of twelve fish; −: not detected; n/a: not available.

pathological changes were found when comparing samples from 1 and 2 wpc of the i.p. challenged fish with samples from 3 and 4 wpc of the cohabitants. The severe pathological changes registered in 5 wpc were probably due to terminal development of the disease (Figure 2).

Several trials with different EM90-like isolates have been conducted at VESO Vikan. In conclusion, the virulence varies significantly between isolates, also with regard to the capacity of horizontal transfer of bacteria by cohabitation. The mortality rate in challenge trials with *P. salmonis* is typically high, and a lower challenge dose will often lead to lack of horizontal transfer of the infection (VESO Vikan, unpublished data). The concentration of the inoculum used in this trial was based on previous experience with the isolate at VESO Vikan, and the mortality obtained was in line with previous results.

Surprisingly, the detection of *P. salmonis* DNA in tissue analysed by qPCR and by IHC suggests less relation between the number of bacteria in the tissues and the necropsy and HE-staining results. The bacteria were first detected by qPCR in cohabitant fish 15 days before mortality was recorded in this group. Thus, the cohabitants were exposed to *P. salmonis* from one day before onset of the outbreak in the i.p. injected fish, suggesting that *P. salmonis* is excreted in numbers to reach the infective dose only a short period of time before fish death occurs. On the other hand, pathological changes such as pale focal nodes in the liver were detected by necropsy and HE-staining from 1 wpc. These early changes could be fibrin clots present in the organ, suggesting liver injury and/or disease, and the pathological findings present at later time points (3 and 4 wpc) were more probably due to necrosis. It is tempting to suggest that the bacteria were excreted by the i.p. fish already from the early stage of the challenge inducing an immune response in the cohabitant fish, thus explaining the longer incubation period observed in the cohabitation outbreak. The challenge dose of *P. salmonis* through the waterborne transmission route should in the future be elucidated by water samplings during challenge experiments. *Piscirickettsia salmonis* is described to secrete extracellular molecules that are suggested to cause tissue damage in fish (Rojas et al., 2013). A cytotoxic effect of the bacteria could explain the observed pathological changes in different organs before bacterial detection by qPCR or IHC. The bacterium may produce and secret toxins in remote tissues. In this case, toxins could have been secreted from the site of initial infection, for example the gut or the gills. Nevertheless, another possible explanation to the conflicting results may be the insufficient sensitivity of the methods to detect the bacteria.

Pathological changes were observed in all tissue analysed and previously been described in fish naturally and experimentally infected by *P. salmonis*. (Almendras et al., 2000; Fryer & Hedrick, 2003; Fryer et al., 1992; Mauel & Miller, 2002; Rozas-Serri et al., 2017; Yáñez et al., 2016). Also, and in accordance with the present results, it is well known that not all observed pathological signs are necessarily present in all diseased fish (Fryer & Hedrick, 2003; Mauel & Miller, 2002). The intermittent presence of the pathological changes could be explained by individual differences between fish and a low number of sampled fish that result in low representation of the fish population.

The authors of several previous studies of field cases have reported the liver as the organ with most prominent tissue damage (Almendras et al., 2000; Arkush et al., 2005; Cvitanich, Garate, & Smith, 1990; Fryer & Hedrick, 2003; Mauel & Miller, 2002; Olsen et al., 1997; Rozas-Serri et al., 2017). In contrast, the study by Almendras et al. (2000) reported no gross lesions in the liver, which emphasizes the variation of pathological manifestations of piscirickettsiosis. The necropsy results of this study reveal that the most affected tissue during early pathogenesis was the spleen presenting splenomegaly from 1 wpc in both challenge groups. Significant liver damages were observed later in the challenge experiment around and after onset of disease outbreak.

In this study, the qPCR protocol was not normalized to quantify *P. salmonis* DNA. For this reason, the results were only qualitative and a quantitative comparison of bacterial load in different tissues is not applicable. *Piscirickettsia salmonis* was detected in all types of examined tissues by qPCR and IHC confirming septicaemia. The qPCR and IHC results demonstrate that *P. salmonis* has a higher preference for several visceral organs such as liver, spleen and head kidney than for skeletal muscle tissue and plasma. Bacteria were present in spleen, head kidney and liver of the analysed fish as early as 1 wpc in the i.p. injected fish and in the head kidney of 50% of the analysed fish in the cohabitant fish 2 wpc. The observed differences in tissue tropism between challenge models could strengthen the assumption about a dose-dependent pathogenesis of piscirickettsiosis.

5 | CONCLUSION

This study contributes with information to understand the pathogenicity of the bacteria under controlled conditions and reveals the bacterial tropism to the visceral organs (liver, spleen and head kidney) instead of skeletal muscle and plasma.

This is to our knowledge, the first documentation available comparing two different challenge models against *P. salmonis* under controlled conditions. Cohabitation challenge is typically considered to be superior to challenge by i.p. injection with regard to mimicking natural infection. This study shows that there are only minor differences between the two different challenge models with respect to mortality, pathological and histopathological changes. The mortality onset is faster in i.p. challenge model most likely because the bacteria are injected directly to the individual fish making the bacterial load higher, resulting in a shorter trial with reduced operating expenses. At the same time, a lower number of fish (research animals) are needed for this type of challenge model, contributing to the ethical aspect of using research animals.

Further research is necessary not only to be able to understand the bacterial behaviour but at the same to have better control of the challenge models to test prophylactic measurements against piscirickettsiosis.

ACKNOWLEDGEMENTS

This project was funded by VESO and the Norwegian Research Council, Project number 258783. We would like to acknowledge all VESO Vikan staff for the technical support during the trial, and the histology laboratory staff at Department of Basic Sciences and Aquatic Medicine, NMBU for the technical support related to histology and IHC. Duncan Colquhoun/NVI is acknowledged for providing the antibody for IHC. Thanks to Turhan Markussen, Elisabeth Furuseth Hansen, Simen Foyn Nørstebø, Aud Kari Fauske, Øystein Wessel and Cristian Bravo for valuable input.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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How to cite this article: Meza K, Inami M, Dalum AS, Bjelland AM, Sørum H, Løvoll M. Development of piscirickettsiosis in Atlantic salmon (*Salmo salar* L.) smolts after intraperitoneal and cohabitant challenge using an EM90-like isolate: A comparative study. *J Fish Dis*. 2019;00:1–11. https://doi.org/10.1111/jfd.13004

ARTICLE II

Comparative evaluation of experimental challenge by intraperitoneal injection and cohabitation of Atlantic salmon (*Salmo salar* L) after vaccination against *Piscirickettsia salmonis* (EM90-like).

Running title: Evaluation of *P. salmonis* challenge models after vaccination.

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Acknowledgments

This project was funded by VESO and the Norwegian Research Council, Project number 258783.

We would like to acknowledge all VESO Vikan staff for the technical support during the trial. Duncan Colquhoun/ NVI is acknowledged for providing the antibody for IHC. Thanks to Aud Kari Fauske for technical support in the research vaccine's production employed in this study. Thanks to Alexander Jaramillo, Simen Foyn Nørstebø, Elisabeth Furuseth Hansen, Stine Braaen, Anne Bakke, Ingvild Berg Nyman and Øystein Wessel for valuable inputs.

Conflict of Interests

The authors declare that they have no competing interests.

Abstract

The Chilean aquaculture has been challenged for years by piscirickettsiosis. A common prophylactic measurement to try to reduce the impact from this disease is vaccination, but the development of vaccines that induce satisfactory protection of the fish in the field has so far not been successful. Experimental challenge models are used to test vaccine efficacy. The aim of this study was to evaluate the performance of experimental vaccines after challenge by the two most widely used challenge routes; intraperitoneal injection and cohabitation. A total of 1120 Atlantic salmon were vaccinated

with non-commercial experimental vaccines with increasing amounts of an inactivated *Piscirickettsia salmonis* EM90-like isolate. Differences in mortality, macroscopic and microscopic pathological changes, bacterial load and immune gene expression were compared after challenge by different routes. The results revealed a similar progression of the diseases after challenge by both routes and no gross differences reflecting the efficacy of the vaccines could be identified. The analysis of the immune genes suggest a possible suppression of the cellular immunity by CD8 T-cell and with this stimulation of bacterial survival and replication. Comparative studies of experimental challenge models are valuable with regards to identifying the best model to mimic real-life conditions and vaccines' performance.

Keywords

Piscirickettsia salmonis, Atlantic salmon, vaccines, cohabitation, intraperitoneal injection, challenge model.

1. Introduction

Chilean salmon industry has been challenged by piscirickettsiosis for almost 30 years (Almendras & Fuentealba, 1997; Mauel & Miller, 2002; Carril, Gómez, & Marshall, 2017) and, currently, the disease produce economical losses around USD 700 million yearly (Maisey, Montero, & Christodoulides, 2017). Piscirickettsiosis has also been identified as the main reason for the use of 322.7 tons of antibiotics in Chile in 2018, where 95.5% was intended to treat this disease (SERNAPESCA, 2019). Several vaccines have been developed as a prophylactic measurement against *Piscirickettsia salmonis*, the etiological agent of piscirickettsiosis (Fryer, Lannan, Garcés, Larenas, & Smith, 1990; Garcés et al., 1991), but commercial fish populations remain susceptible to outbreaks of piscirickettsiosis (Rozas & Enríquez, 2014; Otterlei et al., 2016; Maisey et al., 2017). There are currently 32 vaccines against *P. salmonis* registered by the Chilean Agriculture and Livestock Service (SAG), of which 28 are inactivated vaccines, three are subunit vaccines and one is a live-attenuated vaccine (SAG, 2017).

Piscirickettsia salmonis is a pleomorphic, non-motile, facultative intracellular Gram-negative bacterium (Almendras & Fuentealba, 1997; Marshall, Gómez, Ramírez, Nilo, & Henríquez, 2012; Henríquez et al., 2013; Vera, 2016). In addition to Chile, the bacterium has been detected in Canada (Brocklebank, Evelyn, Speare, & Armstrong, 1993), Scotland (Grant, Brown, Cox, Birkbeck, & Griffen, 1996), Ireland (Rodger & Drinan, 1993) and Norway (Olsen, Melby, Speilberg, Evensen, & Håstein, 1997). In Chile, different isolates of *P. salmonis* have been classified by genotyping into two main groups as LF89-like (Fryer, Lannan, Giovannoni, & Wood, 1992) and EM90-like (Mauel, Giovannoni, & Fryer, 1999). Due to the intracellular nature of the bacteria, the pathogenicity and the interaction with the immune system of the host has been difficult to understand.

The efficacy of vaccines is typically evaluated by experimental challenge. The most commonly used challenge methods in fish are immersion, intraperitoneal injection or cohabitation with intraperitoneally injected shedder fish. Experimental challenge models that mimic real life conditions are essential to document the efficacy of vaccines, both for proof of concept studies as well as for release of vaccine batches with market authorization (Cowan, Smith, & Christofilogiannis, 2016; Midtlyng, 2016).

The aim of this study was to compare the disease development and the protection afforded by experimental test vaccines against piscirickettsiosis after challenge through two of the most commonly used routes; by i.p. injection and by cohabitation. The results of the study will provide valuable input to the decision process when selecting the challenge model for documentation of the efficacy of vaccines against piscirickettsiosis.

2. Materials and Methods

2.1 Fish

Experimental Atlantic salmon were produced at the VESO Vikan hatchery (Namsos, Norway). Fish were smoltified by manipulating the photoperiod using artificial light; six weeks of 12 hours light and 12 hours darkness followed by six weeks of 24 hours light. A total number of 1232 Atlantic salmon

with an average weight of 30 grams at vaccination were used. From the total number of fish, 112 were left unvaccinated and the rest were divided into five groups of 224 fish. The fish population was analyzed prior to vaccination and confirmed negative for specific antibodies against *Aliivibrio salmonicida*, *Vibrio ordalii*, *Aeromonas salmonicida* and *Moritella viscosa* (Norwegian Veterinary Institute (NVI), Oslo, Norway). Absence of *P. salmonis*, infectious pancreas necrosis virus (IPNV), infectious salmon anemia virus (ISAV), salmon pancreas disease virus (SPDV), piscine orthoreovirus (PRV) and piscine myocarditis virus (PMCV) was confirmed by qPCR (PatoGen, Ålesund, Norway).

2.2 Bacterial cultivation

A *Piscirickettsia salmonis* EM90-like isolate (VESO Vikan) originally isolated from a field outbreak in Atlantic salmon (*Salmo salar* L.) in 2004 (Calbuco, Los Lagos, Chile) was used for vaccine production and experimental challenge. This *P. salmonis* isolate was aerobically cultured from an ampule stored at -80°C in 10 mL of FN2 liquid medium (unpublished media, original recipe provided by FAVET-INBIOGEN Universidad de Chile) with agitation (150 rpm) at 18°C for four days. A spectrophotometric analysis (Jenway 6300) was used to measure the density of the liquid bacterial culture. Possible contamination was evaluated by visual examination of the liquid culture under a phase contrast microscope. Furthermore, 100 µL of bacterial culture was plated on cysteine heart agar plates (CHAB) supplemented with bovine blood (Norwegian Veterinary Institute, Oslo, Norway) and on blood agar plates with bovine blood and 2% NaCl (Kystlab PreBio, Namsos, Norway). The CHAB plates were incubated aerobically at 18°C and the blood plates were incubated aerobically at 22°C, both for ten days. No bacterial colonies were observed on the blood agar plates. No bacterial colonies other than *P. salmonis* were observed on the CHAB plates.

2.3 Test vaccines

Test vaccines were developed in the Bacteriology laboratory at the Faculty of Veterinary Medicine, Norwegian University of Life Sciences (NMBU, Oslo, Norway). The bacterium, *P. salmonis,* EM90-like,

was cultured in liquid medium FN2 at 18°C with agitation (150 rpm) for four days, followed by bacterial inactivation with 0.6% formaldehyde (Sigma-Aldrich, Darmstadt, Germany). A spectrophotometric analysis (Genesys 20, Thermo scientific) was used to measure the density of the liquid bacterial culture. Four vaccines (A-D) were developed with different concentrations of the target antigen Vaccines A, B, C and D contained 5, 3, 2 and 0.5 times (×) the concentration of the antigen, respectively. Vaccine E (control) consisted of a saline solution of 0.9% NaCl. The bacterin solution was thoroughly mixed with 50% Freund's Incomplete adjuvant (Sigma) and stored at 4°C until vaccination. The vaccines were developed for experimental use only.

2.4 Vaccination and experimental challenge

The trial was approved by the Norwegian Animal Welfare Authority (ID 13703) and conducted at VESO Vikan research facility (Namsos, Norway). The fish were starved 48 hours prior to vaccination. The fish were sedated with 0.001% benzocaine chloride (Sykehusapoteket Oslo, Ullevål, Norway), marked by shortening of the maxilla and/or adipose fin and intraperitoneally injected with 0.1 mL of experimental vaccines A-E. The immunization period was seven weeks (735 day-degrees). The fish were acclimatized in brackish water (25‰) at 15°C for 14 days and starved for 48 hours prior to challenge. At day 0, fish were distributed into four identical tanks (tank 1, 2, 3 and 4) with a capacity of 1100 L and a stocking density not higher than 40 kg m^{-3}. Tanks 1 (challenge by i.p. injection) and 2 (challenge by cohabitation) were used for mortality registration with 35 fish per group. Tanks 3 (challenge by i.p. injection) and 4 (challenge by cohabitation) were used for sampling, and each tank included 77 fish per group. Tanks with fish challenged by cohabitation included 20% shedders. Shedders were anesthetized with 0.001% benzocaine chloride. The bacterial inoculum dose for i.p. injected fish was obtained from a previous study elaborated by Meza et al. (2019), where a theoretical dose of 1.0×10^5 cfu mL^{-1} was used. The fish were kept in brackish water with a salinity of 25‰ at 15°C and 24 hours light. The fish were fed with a commercial diet (Skretting AS) twice a day. The fish were monitored daily, and mortality was registered. Fish in the terminal stage were euthanized with an

overdose of benzocaine chloride and registered as dead. Bacteriological examination of dead fish was performed daily by inoculating head kidney samples on blood agar with 2% NaCl and incubation at 22°C for 48 - 72 hours.

Statistical analysis of mortality curves was executed by JMP® Pro (SAS Institute Inc.) program version 13.0.0. Survival analysis was performed by application of Log-rank ($p < 0.05$) and Wilcoxon test ($p < 0.05$).

2.5 Sampling and necropsy

Fish for sampling were selected randomly by netting from tank 3 and 4 and euthanized with an overdose of benzocaine chloride (0.002%). Six unvaccinated and 30 vaccinated fish (six fish per vaccine group (A-D) and six from control group (E)) were sampled prior to challenge on day 0. After challenge, samples were collected weekly (7, 14, 21, 28 and 35 dpc) by selection of six fish per group (A-E) per tank.

2.5.1 Blood samples

Blood samples were obtained immediately after euthanizing the fish and before the necropsy was performed. Blood was drawn from the dorsal vein (*Vena caudalis*) and/or dorsal artery (*Aorta caudalis*) by puncturing the fish lateroventrally behind the anal fins. Vacutainer® blood collection tubes containing ethylenediaminetetra-acetic acid (EDTA) anticoagulant were used (BD, New Jersey, USA). After sampling, the plasma was obtained by centrifugation of the tubes at 797 g at 4°C for 10 minutes, followed by transfer to eppendorf tubes and storage at -80°C.

2.5.2 Necropsy

Necropsy was performed immediately after blood sampling. The pathological changes were classified as ascites (A), pale focal nodes in the liver (PFNL), swollen kidney (SK), splenomegaly (SP), hemorrhages in the digestive tract (HDT), distended ventricle with gelatinous content (DV/GC) and

general hemorrhages (GH). The pathological changes were registered as percentage of the occurrence among the six sampled fish per group.

2.5.3 Tissue samples

Tissue samples were dissected from the spleen, liver and head kidney. The samples for real-time quantitative PCR (qPCR) were submerged in RNA*later*® (Sigma-Aldrich) and stored at 4°C overnight, followed by -20°C until DNA extraction. Samples for immunohistochemistry and histology were fixated in 4% formaldehyde for 24 hours followed by transfer to 70% ethanol and storage at 4°C.

2.6 Histology and Immunohistochemistry

All sampled fish (n=209) were subjected to histological and immunohistochemical analysis at Pharmaq Analytiq (Oslo, Norway). Formalin-fixed samples from liver, head kidney and spleen were processed for histological analysis and immunohistochemistry according to standard procedure (Suvarna, Layton, & Bancroft, 2018). For haematoxylin and eosin- staining (HE), paraffin-embedded tissues were sectioned at 2 µm thickness. HE-sections were evaluated in a blinded manner by two independent scientists with a scoring system from 0 to 3. The criteria for scoring the samples are shown in Table 1. After scoring the samples, the average of both values was calculated. Only representative samples from each score were evaluated by immunohistochemistry (IHC). For IHC, tissues were sectioned at 4 µm thickness and mounted on poly-L-lysine-coated slides (KF FROST, VWR International BV, Amsterdam, The Netherlands). Protocol for immunohistochemistry was adapted from Dalum et al. (2016) including a primary antibody against *P. salmonis* (provided by Duncan Colquhoun, Norwegian veterinary institute (NVI)). The slides were incubated at 58°C overnight prior to deparaffinization in xylene. Heat induced epitope retrieval was performed at 121°C for 10 minutes in 0.01 M citrate buffer, pH 6, followed by washing in 0.01 M phosphate-buffered saline (PBS, pH 7.3). The sections were incubated for 40 minutes at 37°C in pre-heated PBS with 0.05% phenyl-hydrazine (Aldrich chemistry, MO, USA) followed by washing with PBS. Slides were blocked with a solution of

0.05 M tris-buffered saline (TBS, pH 7.6) with 0.2% goat normal serum and 5% bovine serum albumin (BSA) for 20 minutes. The primary polyclonal rabbit antibody was diluted 1:40 000 in TBS with 1% BSA. The antibody was labelled with polymer-HRP anti-rabbit (Dako EnVision + System-HRP) and developed with 3-amino-9-ethyl carbazol (AEC) producing a red staining when reacting with tissue-bound primary antibody. Counterstaining was performed with haematoxylin followed by washing in distilled water. A descriptive observation was performed.

Table 1. Score system for haematoxylin-eosin slides.

Score	Liver	Spleen	Head kidney
0	No visible changes	No visible changes	No visible changes
1 **Mild changes**	Focal changes dominated by inflammation	Mild congestion	Edema hematopoietic tissue
2 **Moderate changes**	Focal changes dominated by necrosis	Loss of tissue architecture due to edema	Inflammation and edema hematopoietic tissue and thickening Bowman's capsula
3 **Extensive changes**	Bridging or diffuse necrosis surrounded by inflammation	Edema and congestion, loss of tissue architecture, perivascular aggregation of immune cells and necrosis	Necrosis of hematopoietic tissue with edema and an increase in inflammatory cells, glomerulonephritis, and enlargement of Bowman's space

2.7 DNA extraction and qPCR

A total of 627 samples were analyzed for bacterial DNA by quantitative PCR (qPCR). Out of these, 18 tissue samples were from unvaccinated fish, and 609 tissue samples were from vaccinated fish (90 from unchallenged fish, 144 from i.p. injected fish and 375 from cohabitant fish). Tissue samples were weighed in OHAUS® Analytical plus (Sigma-Aldrich) scale, before they were homogenized using sterile steel beads (5 mm) and TissueLyzer II (Qiagen) for 15 seconds at 25 Hz, followed by incubation with protein kinase (Qiagen) at 56°C overnight. The DNA extraction was performed using QIAcube and QIAGEN DNeasy® Blood & tissue kit (Qiagen) according to the manufacturer's protocol. The DNA concentration was measured using NanodropTM ND-1000 spectrophotometer (Thermo Scientific) and diluted to a concentration of 20 ng/µl. Primer and probe sequences targeting *P. salmonis* were obtained from Meza et al. (2019). Primer sequences were 5'-AGG GAG ACT GCC GGT GAT A-3' for

207 forward primer and 5’-ACT ACG AGG CGC TTT CTC A-3’ for the reverse primer. The probe sequence
208 was 5'-TCG CTC CAC ATC GC-3'.
209 Each qPCR reaction contained 10 µL TaqMan qPCR Supermix, 300 nM of specific forward and
210 reverse primer, 100 nM of a specific probe and 60ng DNA template in a total volume of 20 µL. The
211 qPCR thermal profile started with 10 min denaturation at 95°C followed by 40 cycles applying a melting
212 point at 95°C for 15 seconds, annealing and elongation at 58°C for 45 seconds. Samples were analyzed
213 in triplicate. Results with Ct values above 35 were considered negative.
214 For normally distributed data, a two-way ANOVA test ($p < 0.05$) was performed, and for non-
215 normally distributed data, a Kruskal-wallis test ($p < 0.05$) was performed. Statistical analysis was
216 executed by JMP® Pro (SAS Institute Inc.) program version 13.0.0.
217
218 *2.8 RNA extraction and reverse transcription qPCR (RT-qPCR)*
219 A total of 209 spleen samples (36 from unchallenged fish, 48 from fish challenged by i.p. injection
220 and 125 from fish challenged by cohabitation) were analyzed for immune gene expression by RT-qPCR.
221 Tissue samples were weighed in OHAUS® Analytical plus (Sigma-Aldrich) scale. Qiazol lysis reagent
222 (Qiagen) was added in a volume of 650 µL before the tissue was homogenized using sterile steel beads
223 (5 mm) and TissueLyzer II (Qiagen) for 5 min at 25 Hz. After the disruption of the tissue, 130 µL of
224 chloroform was added followed by vigorous shaking by hand for 15 seconds. Samples were incubated
225 at room temperature (RT) for 2-3 min and centrifuged at 12000 g for 15 min at 4°C. The aqueous phase
226 was transferred to a clean tube, and the RNA extraction was performed using QIAcube and QIAGEN
227 RNeasy® mini kit (Qiagen) according to the manufacturer’s protocol. The quality of the RNA was
228 measured using NanodropTM ND-1000 spectrophotometer (Thermo Scientific). After the RNA
229 extraction, cDNA was prepared with QuantiTect® Reverse Transcription kit (Qiagen) according to the
230 manufacturer’s protocol.
231 Primer sequences targeting the immune genes *sIgM, mIgM*, *CD8α* and *CD4-1* as well as the
232 reference gene *EF1αβ* were obtain from previous studies and are listed in Table 2.

Each reaction contained 10 µL Maxima SYBR Green Supermix (Thermo scientific), 300 nM of forward and reverse primer for the reference gene, 450 nM of forward and reverse primer of target gene and 15 ng cDNA template in a total volume of 20 µL. The qPCR thermal profile started with 2 min UDG pre-treatment at 50°C followed by initial denaturation of 10 min at 95°C. This was followed by denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 60 seconds were applied for 40 cycles. Samples were analyzed in duplicates. Results with Ct values above 35 were considered negative. The Ct-values were normalized to the expression of the reference gene *EF1αβ*.

For the statistical analysis, the mean normalized expression of the target genes was calculated from raw Ct values by relative quantification. Kruskal-wallis test ($p < 0.05$) was performed due to the non-normally distributed data. Statistical analysis was executed by JMP® Pro (SAS Institute Inc.) program version 13.0.0.

Table 2. Genes to be quantified by RT-qPCR and the corresponding primer sequences.

Gene		Sequence	Reference
EF1αβ	Forward	5'-TGCCCCTCCAGGATGTCTAC-3'	Løvoll, Austbø, Jørgensen, Rimstad, and Frost (2011)
	Reverse	5'-CACGGCCCACAGGTACTG-3'	
Membrane bound IgM (*mIgM*)	Forward	5'-CCTACAAGAGGGAGACCGA-3'	Iliev, Thim, Lagos, Olsen, and Jørgensen (2013)
	Reverse	5'-GATGAAGGTGAAGGCTGTTTT-3'	
Secreted bound IgM (*sIgM*)	Forward	5'- CTACAAGAGGGAGACCGGAG-3'	Iliev et al. (2013)
	Reverse	5'- AGGGTCACCGTATTATCACTAGTTT -3'	
CD8α	Forward	5'-CGTCTACAGCTGTGCATCAATCAA-3'	Grove et al. (2013)
	Reverse	5'-GGCTGTGGTCATTGGTGTAGTC-3'	
CD4-1	Forward	5'-GAATCTGCCGCTGCAAAGAC-3'	Tadiso et al. (2011)
	Reverse	5'-AGGGATTCCGGTCTGTATGATATCT-3'	

2.9 Bead based multiplex immunoassay

2.9.1 Antigens and bead coupling

Piscirickettsia salmonis EM90-like was cultured in 10 mL of FN2 medium for four days at 18°C and with agitation (150 rpm), after this period the culture was diluted until the concentration reached OD_{600nm} = 1.0. The bacterial culture was centrifuged at 2500 g at 4°C for 10 min, followed by

resuspension of the bacterial pellet in 5 mL of physiological saline. The inactivation of *P. salmonis* was performed by the addition of formalin (0.6%). Samples were kept on ice and sonicated at 40 Hz for 6 × 20 seconds. For detection of specific antibodies, *P. salmonis* whole cell sonicate was coupled to distinct MagPlex® -C Microspheres (Luminex Corp. Austin, TX, USA) according to the manufacturer's protocol using the Bio-Plex® amine coupling kit (Bio-Rad) and as previously described (Lund et al., 2019). Briefly, 1× scale of stock beads at a concentration of 1.25×10^6 were washed in wash buffer using a magnetic separator and resuspended in bead-activation buffer. Beads were activated by addition of N-Hydroxysulfosuccinimide sodium salt and N-(3-Dimethylaminopropyl)-N'-ethylcarbid (Sigma-Aldrich) prepared in bead-activation buffer and incubated for 20 min at RT and at constant rotation. Beads were protected from light at all times. Following bead activation, a range of concentrations of the *P. salmonis* whole cell sonicate varying between 3 µg and 12 µg were coupled onto beads of different bead regions. After incubation for 2 hours (RT, constant rotation), beads were washed twice in PBS, resuspended in blocking buffer and incubated for 30 min (RT, constant rotation). After blocking, beads were washed and re-suspended in storage buffer and kept at 4°C in the dark until used. The bead concentrations were determined using Countess™ automated cell counter (Invitrogen).

2.9.2 Multiplex immunoassay

To determine the optimal *P. salmonis'* antigen concentration for bead conjugation, beads coupled with a range of antigen concentrations varying between 3 µg and 12 µg were tested against different samples included in the study. Optimal sample dilutions were established by initial titrations of positive (vaccinated fish 7 weeks post vaccination (wpv), n = 4) and negative (unvaccinated fish, n = 4) pooled plasma in serial two-fold dilutions. Dilutions providing assay readout values falling between the upper and lower detection limit of the assay were chosen.

Piscirickettsia salmonis coupled beads were diluted in assay buffer consisting of PBS with 0.5% BSA (Rinderalbumin; Bio-Rad Diagnostics GmbH, Dreieich, Germany) and 0.05% azide (Merck, Darmstadt,

Germany), and 5000 beads per region were added to each well on Bio-Plex® Pro™ Flat Bottom Plates. Beads coupled with antigens not included in the vaccination and challenge trial, the A-layer protein from *Aeromonas salmonicida subsp. salmonicida* and *Moritella viscosa* whole cell sonicate (Lund et al., 2019), were included in all wells. Beads were washed three times with 200 µL assay buffer for 30 seconds in the dark and on a shaker at 800 rpm, then kept for 120 seconds in a BioPlex handheld magnetic washer before the supernatant was poured off. Plasma samples were diluted 1:100 in assay buffer, before 50 µL sample was added to each well and in duplicates. Positive and negative plasma pools in duplicates or triplicates were included on every plate, together with antibody controls and blanks (wells added assay buffer instead of sample). The plate was incubated for 30 min at RT in the dark and on a shaker at 800 rpm. All subsequent incubation and washing steps were performed similarly. Following incubation, beads were washed, and all wells were added Anti Salmonid-IgH monoclonal antibody (1:400, clone IPA5F12, Cedarlane, Burlington, Ontario, Canada). After incubation and washing, biotinylated goat Anti-Mouse IgG2a antibody (1:1000, Southern Biotechnology Association, Birmingham, AL, USA) was added to each well, and finally, after incubation and washing, Streptavidin-PE (1:50, Invitrogen) was applied. After the final incubation, beads were washed and suspended in assay buffer. Plates were analyzed using a Bio-Plex® 200 in combination with Bio-Plex® Manager 5.0 software (Bio-Rad). The reading was carried out using a low PMT target value, the DD-gate was set to 5000-25000, and 100 beads from each region were read in each well. Each bead is classified by its signature fluorescent pattern and then analyzed for the mean fluorescence intensity (MFI) of the reporter molecule.

The GraphPad Prism 7 software (Mann-Whitney test, $p < 0.05$) was used to performed statistical analysis.

3 Results

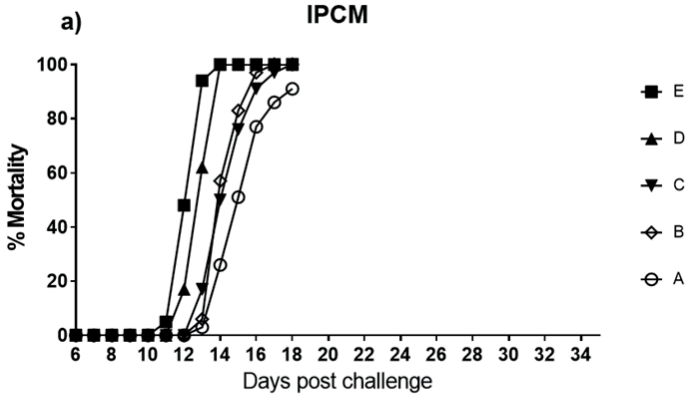
3.1 Mortality and challenge data

The mortality curves of the i.p. challenge model and cohabitation challenge model revealed similar mortality rates, however different incubation periods and outbreak durations (Figure 1). After i.p. challenge, the first fish mortality was recorded in the saline injected control group E at 11 dpc, followed by outbreaks in vaccine group D (12 dpc) and group A, B and C (13 dpc). The incubation period after challenge by cohabitation was calculated from the time mortality occurred in shedder fish (9 dpc) until mortality occurred in the cohabitant fish. After cohabitation challenge, vaccine group B and control group E demonstrated the shortest incubation period with 15 days (24 dpc) followed by vaccine group C and D (16 days, 25 dpc) and vaccine group A (17 days, 26 dpc).

The disease outbreak lasted from two to five days with an average of 3.8 days after i.p. challenge, and from six to nine days with an average of eight days after challenge by cohabitation. All fish groups irrespective of challenge model and vaccine group reached 100% mortality with the exception of vaccine group A after i.p. challenge where three fish were humanely sacrificed the last day of challenge due to severe symptoms of disease.

After i.p. challenge, vaccine group D and control group E reached 100% mortality on 14 dpc, followed by vaccine group B (17 dpc) and vaccine group C (18 dpc). After challenge by cohabitation, vaccine group D reached 100% mortality six days after onset of outbreak (6 days, 31 dpc), followed by control group E (7 days, 31 dpc), vaccine group B (9 days, 33 dpc), vaccine group C (9 days, 34 dpc) and vaccine group A (9 days, 35 dpc). One fish from control group E died at 13 dpc. Microbiological examination did not reveal any bacteria including *P. salmonis*.

The mortality curves of each group were compared with the mortality curve of control group E (saline). After i.p. challenge, there was a significant difference in all the groups in relation with control group E, in contrast with the cohabitation challenge model where there was significant difference in vaccine group A and B in comparison with control group E (Figure 1C). Mortality curves are shown in Figure 1A and Figure 1B.



c)

Statistical comparison againts control group (p values)								
	IPCM				CCM			
Test	A	B	C	D	A	B	C	D
Long-rank	***	***	***	**	**	*	*	n.s.
Wilcoxon	***	***	***	**	**	*	n.s.	n.s.

Figure 1. Cumulative mortality after a) intraperitoneal injection challenge and b) cohabitation challenge against *P. salmonis*. Challenge by IPCM was terminated according to humane endpoint criteria at 91.4% mortality in group A. c) The table shows p values of vaccine group A, B, C, and D compared with control group (E), where p values < 0.05 were presented as (*), < 0.001 were presented as (**) and < 0.0001 were presented as (***). n.s. = not significant. IPCM = intraperitoneal challenge model, CCM = cohabitation challenge model.

3.2 Pathological changes

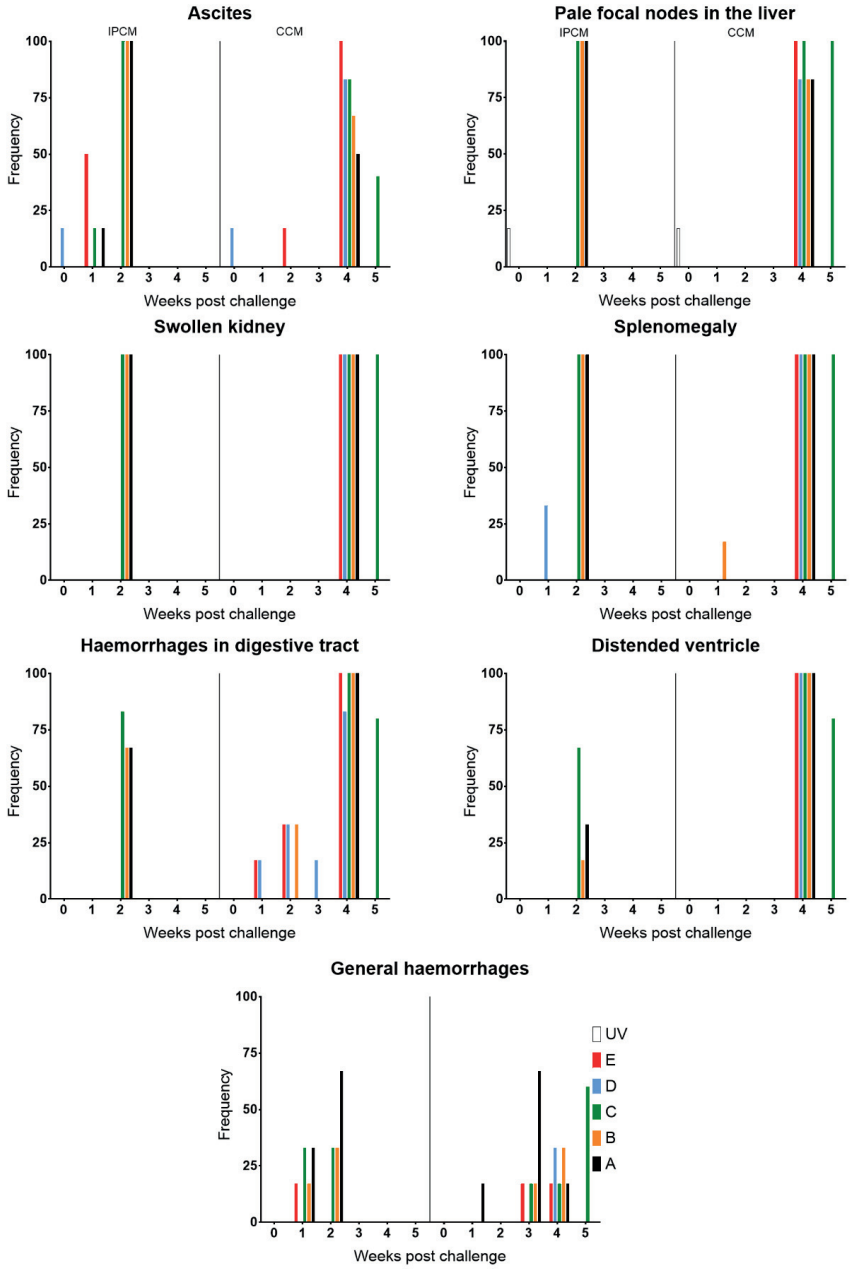
After i.p. challenge, fish from all groups were sampled at 1 wpc, however relatively few pathological findings were detected at this time point. Ascites was found in 50% (3/6) and 17% (1/6) of the fish in control group E and vaccine group A, respectively, and splenomegaly was identified in 33% (2/6) of

342 the fish in vaccine group D. General hemorrhages were detected in 33% (2/6) of the sampled fish in
343 vaccine group A and C, and in 17% (1/6) of the fish in vaccine group B and control group E.
344 At 2 wpc, only fish from vaccine group A, B and C were sampled due to 100% mortality in vaccine
345 group D and control group E. All sampled fish in all examined groups presented ascites, pale focal
346 nodes in the liver, swollen kidney and splenomegaly at this sampling point. Necropsy detected
347 hemorrhages in the digestive tract more frequently in sampled fish of vaccine group C (83% (5/6))
348 compared to vaccine group A and B (67% (4/6)). Similarly, fish with distended ventricle with gelatinous
349 content were detected more frequently in vaccine group C (67% (4/6)) followed by fish in vaccine
350 group A (33% (2/6)) and less frequently in vaccine group B (17% (1/6)). General hemorrhages were, in
351 contrast, more often detected in sampled fish of vaccine group A (67% (4/6)) compared to sampled
352 fish of vaccine group B and C (33% (2/6)).
353 Fish from all groups were sampled at 1 to 5 weeks after challenge by cohabitation. The severe
354 pathological changes reported at 5 wpc reflects that the mortality curve of all groups reached 100%
355 at the sampling date. One of the first reported pathological changes in this challenge model were
356 hemorrhages in the digestive tract. In fact, this pathological sign was observed at an earlier time point
357 in the cohabitation model (1 wpc) compared to the i.p. model (2 wpc). Also, a tendency of a dose-
358 response relationship between pathology and vaccine dose was shown; hemorrhages in the digestive
359 tract were reported in 17% (1/6) of the sampled fish in vaccine group D and control group E at 1 wpc,
360 followed by 33% (2/6) of the sampled fish in vaccine group B, D and control group E at 2 wpc, and in
361 17% (1/6) of the sampled fish in vaccine group D at 3 wpc. General hemorrhages were also detected
362 early in the disease development however only in sampled fish of vaccine group A (17% (1/6)) at 1
363 wpc and in no sampled fish regardless of group at 2 wpc. At 3 wpc, these pathological changes were
364 more frequently identified in sampled fish of vaccine group A (67% (4/6)) compared to examined fish
365 of vaccine group B, C, control group E (17% 1/6)) and vaccine group D (0/6). Splenomegaly was
366 reported in sampled fish of vaccine group B at 1 wpc (17% (1/6)). However, splenomegaly was not

367 detected at 2 and 3 wpc. Similarly, ascites was present in 17% (1/6) of the fish in control group E at 2
368 wpc, but not detected in any groups at 3 wpc.

369 From 4 wpc, swollen kidney, splenomegaly and distended ventricle was detected in all sampled fish
370 in all groups. Hemorrhages in the digestive tract were also a dominating finding at this time point; in
371 all examined fish of vaccine group A, B, C and control group E (100% (6/6)), and 83% (5/6) of the
372 sampled fish of vaccine group D. Similarly, pale focal nodes in the liver were identified in 100% (6/6)
373 of fish in vaccine group C and control group E, and 83% (5/6) of the fish of vaccine group A, B and D.
374 In contrast to the i.p. challenge model, a dose-response relationship between the vaccine antigen and
375 development of ascites was observed at 4 wpc by the cohabitation model. This pathological sign was
376 present in all sampled fish of control group E (100% (6/6)), in 83% (5/6) for vaccine group C and D,
377 67% (4/6) for vaccine group B and 50% (3/6) for vaccine group A at this time point. General
378 hemorrhages were the least frequent finding at 4 wpc and only present in 33% (2/6) of the fish of
379 vaccine group B and D, and in 17% (1/6) of the sampled fish in vaccine group A, C and control group E.

380 Despite no bacterial challenge, 17% (1/6) of the control fish in vaccine group D presented ascites.
381 Also, pale focal nodes were found in 17% (1/6) of unvaccinated (UV) fish at 0 wpc. All vaccinated fish
382 that were examined showed adherences and melanosis in the abdomen (results are not shown). The
383 presence of pathological changes after challenge is shown in Figure 2.



384 Figure 2. Pathological changes after challenge by i.p. injection or cohabitation against *P. salmonis*. The
385 frequency (%) was measured as percentage of the sampled fish that showed pathological changes
386 where 100% corresponded to n = 6. A: vaccine A (5×), B: vaccine B (3×), C: vaccine C (2×), D: vaccine D
387 (0.5×) and E: saline. IPCM = intraperitoneal challenge model, CCM = cohabitation challenge model.
388

3.3 Histology

The average of HE-scoring in liver, spleen and head kidney from fish challenged by i.p. injection or cohabitation is shown in Figure 3. The results demonstrate an increment of the histopathological changes throughout time after challenge by both routes.

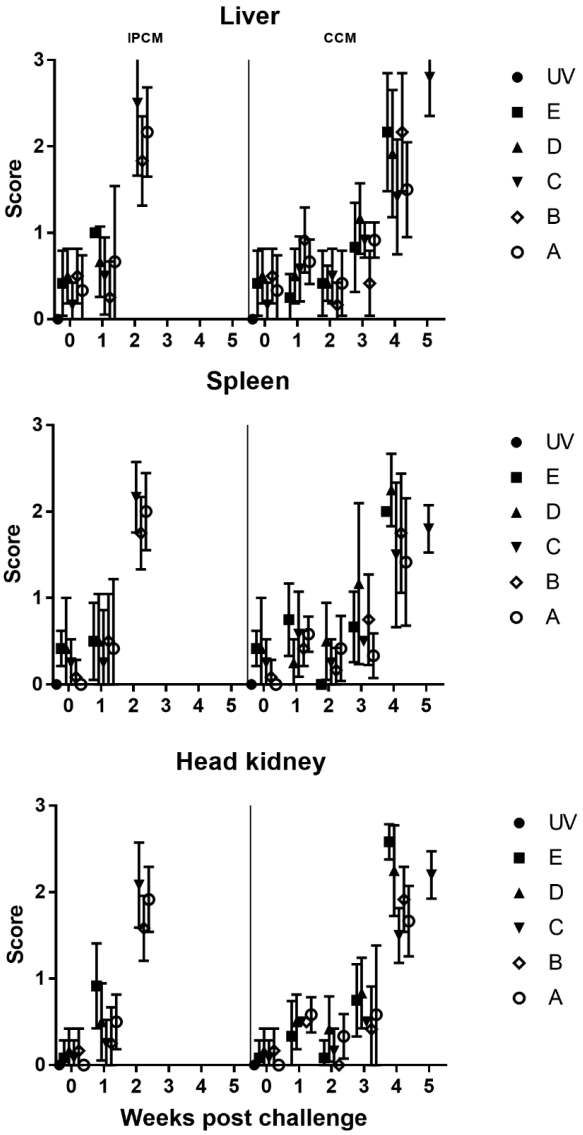
In the unchallenged fish, significant differences in average HE-scoring of liver were found when comparing UV fish with vaccine group B ($p = 0.0081$), vaccine group D ($p = 0.0081$) and control group E ($p = 0.0269$). In spleen there was significant difference when control group E was compared with vaccine group A ($p = 0.0067$), vaccine group B ($p = 0.0341$) and UV fish ($p = 0.0067$) being control group E with the highest HE-score.

After i.p. challenge, no significant difference in histopathological changes in liver, spleen and head kidney were observed between the groups at each time point. The highest average HE-score for the i.p. challenged fish in the liver (2.5), spleen (2.2) and head kidney (2.1) was found in group C at 2 wpc.

After challenge by cohabitation, significant differences were found when comparing HE-scores in liver of un-vaccinated fish with vaccine group A ($p = 0.0379$) and B ($p = 0.0145$) at 1 wpc, control group E presented the lowest HE-score. Significant differences were also identified when comparing vaccine group B with vaccine group A ($p = 0.0289$), vaccine group D ($p = 0.0203$) and vaccine group C ($p = 0.0289$) at 3 wpc; vaccine group B presented the lower HE-score. At 4 wpc, significant differences in HE-scores of head kidney were found when comparing vaccine group A ($p = 0.0034$), vaccine group B ($p = 0.0031$) and vaccine group C ($p = 0.0096$) with control group E, and when vaccine group C was compared with vaccine group D ($p = 0.0236$). The highest average scores for the cohabitation model in the liver (2.8) was found at 5 wpc in vaccine group C (morbid fish). In the spleen (2.3) the highest average score was obtained in vaccine group D and in head kidney (2.6) it was obtained in group E at 4 wpc.

Significant difference between challenge models was only identified in spleen when comparing 1 wpc by i.p. injection with 3 wpc by cohabitation ($p = 0.0448$).

414 Figure 4, Figure 5 and Figure 6 illustrate examples of the HE-scoring (A-D) and their corresponding
415 IHC staining targeting *P. salmonis* (E-H) to demonstrate the bacterial distribution in relation with the
416 tissue lesions (score).



417 Figure 3. Average of HE-scoring per tissue samples from fish challenged by intraperitoneal injection
418 and cohabitation against *P. salmonis*. UV: unvaccinated fish, A: vaccine A (5×), B: vaccine B (3×), C:
419 vaccine C (2×), D: vaccine D (0.5×) and E: control (saline). IPCM = intraperitoneal challenge model, CCM
420 = cohabitation challenge model.

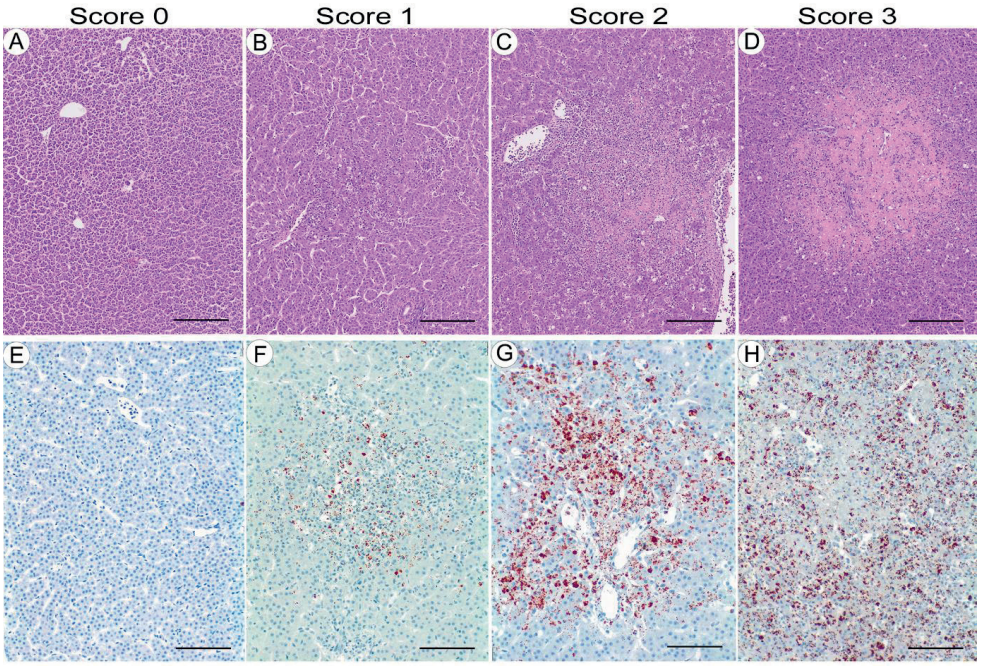


Figure 4. Histopathological manifestation of infection with *P. salmonis* in the liver. (A) and (E) represents negative controls of un-challenged fish. (B) Score 1; Changes dominated by focal inflammation, where only slight numbers of bacteria are seen (F). (C) Score 2; Focal changes consisting of a combination of inflammation and necrosis, where moderate numbers of bacteria are seen (G). (D) Score 3; Focal changes consisting of central necrosis surrounded by inflammation and necrosis, where extensive numbers of bacteria are seen particularly at the margins of the lesions (H). A-D; haematoxylin and eosin staining, scale bar 100 μm. E-H; immunohistochemistry targeting *P. salmonis* (red reaction), scale bar 50 μm.

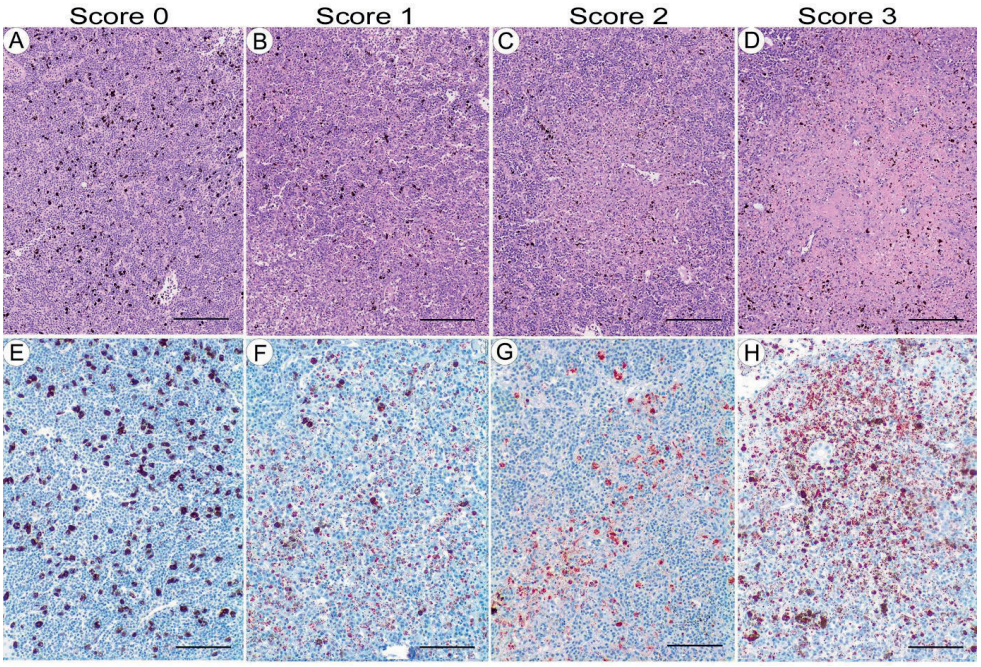


Figure 5. Histopathological manifestation of infection with *P. salmonis* in the head kidney. (A) and (E) represents negative controls of un-challenged fish. (B) Score 1; Changes dominated by mild, diffuse inflammation, congestion and partly disrupted tissue architecture, where only slight numbers of bacteria are seen in a random fashion throughout the tissue (F). (C) Score 2; In addition to changes as seen for score 1, focal changes consisting of a combination of inflammation and necrosis, where moderate numbers of bacteria are seen particularly in the focal changes (G). (D) Score 3; In addition to changes as seen for score 1, focal changes consisting of central necrosis surrounded by inflammation and necrosis, where extensive numbers of bacteria are seen throughout the lesions (H). A-D; haematoxylin and eosin staining, scale bar 100 μm. E-H; immunohistochemistry targeting *P. salmonis* (red reaction), scale bar 50 μm.

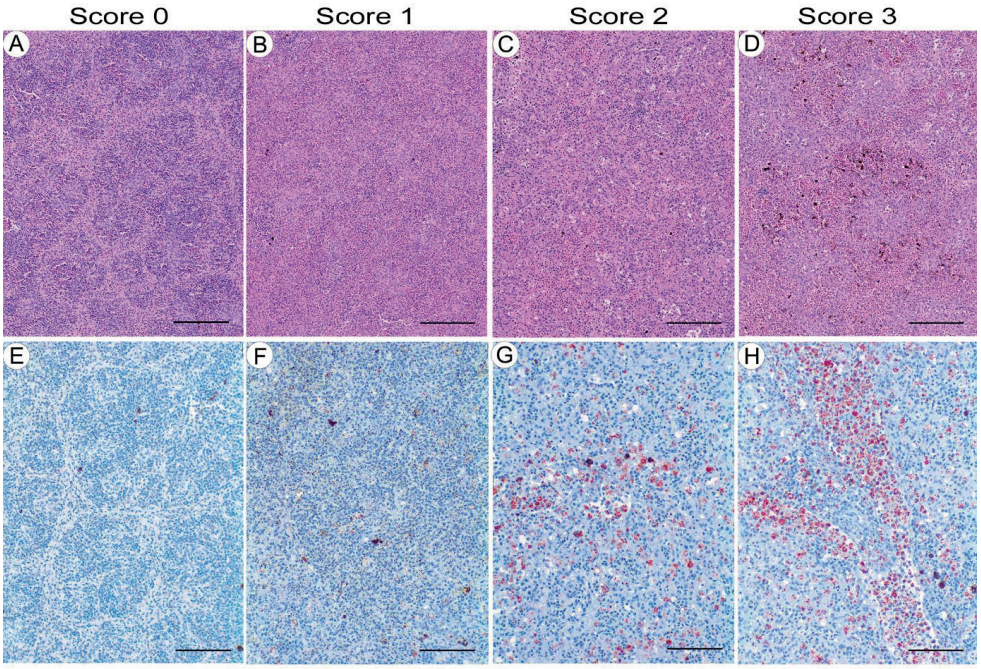


Figure 6. Histopathological manifestation of infection with *P. salmonis* in the spleen. (A) and (E) represents negative controls of un-challenged fish. (B) Score 1; Changes dominated by mild, diffuse inflammation, mild congestion and partly disrupted tissue architecture, where only slight numbers of bacteria are seen in a random fashion throughout the tissue (F). (C) Score 2; In addition to changes as seen for score 1, single cell necrosis is seen throughout the tissue, where moderate numbers of bacteria are seen at highest numbers in necrotic cells as well as lower numbers of bacteria dispersed throughout the tissue. (G). (D) Score 3; In addition to changes as seen for score 1 and 2, focal changes consisting of necrosis with complete disruption of tissue architecture, where extensive numbers of bacteria are seen throughout the lesions as well as lower numbers of bacteria dispersed throughout the tissue. (H). A-D; haematoxylin and eosin staining, scale bar 100 μm. E-H; immunohistochemistry targeting *P. salmonis* (red reaction), scale bar 50 μm.

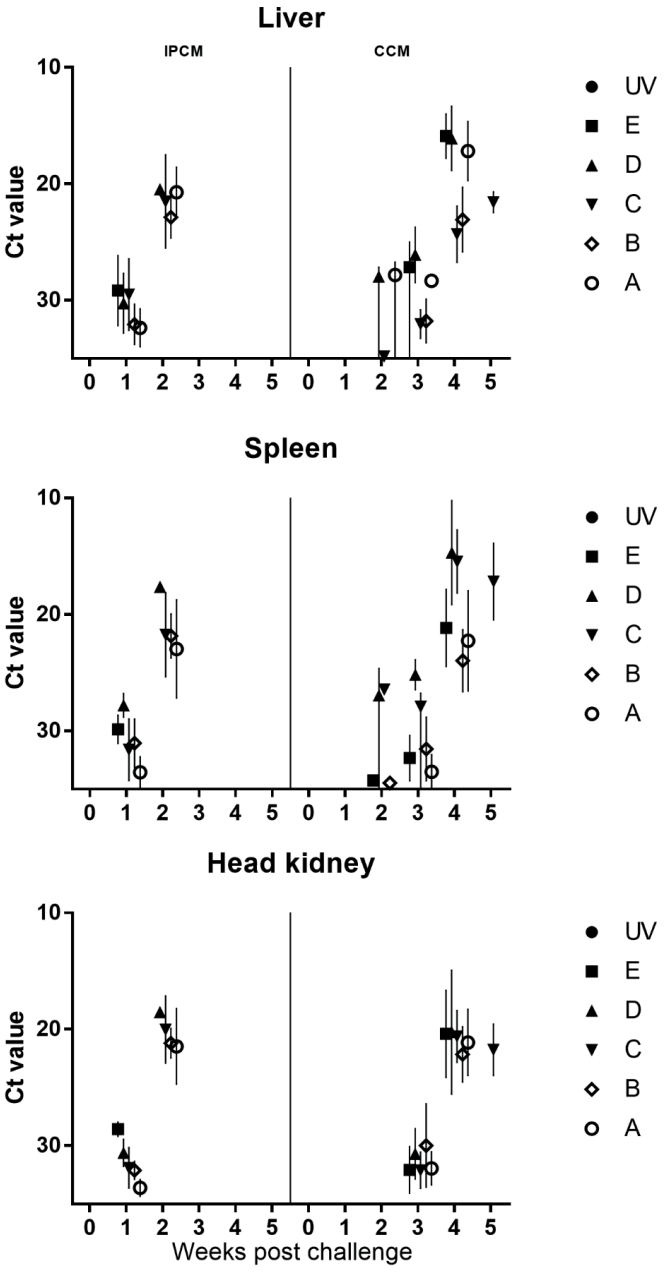
3.4 Bacterial DNA qPCR

The detection of *P. salmonis* bacterial DNA in all visceral tissues is shown in Figure 7. No bacterial DNA was detected in unvaccinated fish and fish sampled at time point 0. After challenge by i.p. injection, bacterial DNA was detected in all groups at week 1 post challenge (wpc) in liver, head kidney and spleen. After challenge by cohabitation, *P. salmonis* was detected as early as 2 wpc in liver (vaccine group A, C and D) and spleen (vaccine group B, C and control group E). The bacterium was not identified in head kidney until 3 wpc (all groups). The highest concentration of *P. salmonis* was

identified in the liver at 2 wpc by i.p. injection with a Ct value of 17.5. After challenge by cohabitation, the highest bacterial concentration was recorded in liver at 4 wpc with a Ct value of 19.4.

In the fish challenged by i.p. injection, the liver did not present significant differences in the bacterial load. In the spleen at 1 wpc significant differences was detected demonstrating that vaccine group D presented higher bacterial load than vaccine group A and vaccine group C, and control group E higher than vaccine group A. In the head kidney there was also a significant difference between groups at 1 wpc where control group E presented a higher bacterial load compared with the rest of the groups. Furthermore, vaccine group D had a higher bacterial load compared with vaccine group A. These differences suggest a vaccine dose-response between the groups (Supplementary table 1). No significant differences were detected between groups at 2 wpc.

After challenge by cohabitation, no significant differences were detected between groups in liver, spleen and head kidney at 1 wpc, 2 wpc, 3 wpc and 4 wpc. Significant differences in bacterial DNA load were identified by comparing samples from the two challenge models before (1 wpc by i.p. injection versus 3 wpc by cohabitation) and after (2 wpc by i.p. injection versus 4 wpc by cohabitation) onset of the disease outbreak. In liver, there was a significant difference in bacterial load before ($p = 0.0382$) and after ($p = 0.0428$) the outbreak. In spleen, there was a significant difference in the bacterial load just before ($p = 0.0011$) the onset of the outbreak. In head kidney, no difference between the challenge models was detected before and after the outbreak.



478 Figure 7. Average of Ct value for *P. salmonis* bacterial DNA per group and tissue after i.p. challenge
479 and cohabitation challenge. UV: unvaccinated fish, A: vaccine A (5×), B: vaccine B (3×), C: vaccine C
480 (2×), D: vaccine D (0.5×) and E: control (saline). IPCM = intraperitoneal challenge model, CCM =
481 cohabitation challenge model.
482

3.5 Expression of selected immune gene transcripts

The fish immune response against *P. salmonis* was analyzed by measuring the expression level of immune genes transcripts encoding the T cell markers (*CD8* and *CD4*) (Medzhitov, 2007) and two genes related to humoral immunity (*membrane bound (m) IgM* and *soluble (s) IgM*). IgM has been described as part of the response to systemic infection (Adams, Thompson, & Roberts, 2016). mIgM is bound to B cells serving as antigen specific receptors and sIgM is produced by plasma cells and secreted in body fluids (Gao et al., 2014). The immune genes were analyzed in samples from spleen tissue due to previous reports of this organ's high expression of *CD4* in Atlantic salmon (Moore, Dijkstra, Koppang, & Hordvik, 2009).

The *mIgM* expression was consistent throughout the time points in both challenge models. The expression of *sIgM*, however, showed more variation throughout the infection. The expression of *CD8α* showed a trend towards reduced expression after challenge through both administration routes, but particularly in fish challenged by cohabitation. *CD4-1* expression increased during the weeks after challenge for both challenge models.

A tendency of a dose response was observed in the expression of *sIgM* and *CD8α* at 2 wpc after i.p. challenge. In both cases the dose-response was reflected by a higher expression in vaccine group A and lower in vaccine group C (Figure 8).

After i.p. challenge, no significant differences were found between vaccine groups in *mIgM* expression at 1 and 2 wpc. Significant differences were, however, found in *sIgM* expression when comparing vaccine group A and vaccine group C where the highest expression were demonstrated in vaccine group C at 2 wpc. The expression of *CD8α* was less pronounced in control group E compared with vaccine group B, C and D at 1 wpc. At 2 wpc, vaccine group A showed significant higher *CD8α* expression compared to vaccine group C (Supplementary table 2).

After challenge by cohabitation, *mIgM* expression was significant different between groups at 3 wpc, demonstrating higher expression level in vaccine group A and control group E when compared to vaccine group B, C and D. At 3 wpc, *sIgM* expression level was significant higher for control group E

compared to vaccine group B, C and D. Similar results were observed for vaccine group A compared to vaccine group B, C and D. At 4 wpc, the expression of *sIgM* was significant higher for vaccine group B compared to vaccine group D. No significant differences were observed in the expression of *CD8α* and *CD4-1* between groups at any time point (Supplementary table 2).

Before challenge, significant differences in the expression of *sIgM* ($p = 0.0351$) and *CD8α* ($p < 0.0001$) were identified between challenge models. The immune gene expression profiles are shown in Figure 8.

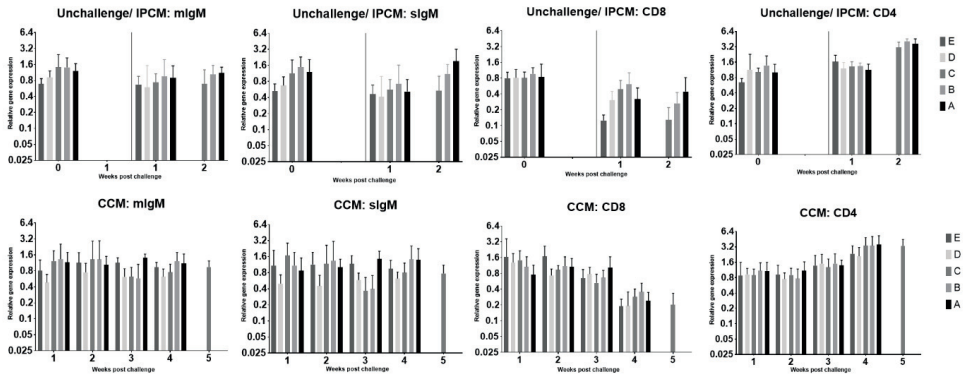


Figure 8. Relative gene expression of *mIgM*, *sIgM*, *CD8α* and *CD4-1* in spleen after challenge of Atlantic salmon with *P. salmonis* by i.p. injection or cohabitation. The gene expression levels are shown in fold change compared with *EF1αβ*. The fish were vaccinated 7 weeks prior to challenge with vaccines A-E where vaccine A (5×), vaccine B (3×), vaccine C (2×), vaccine D (0.5×) and vaccine E (saline) (n = 6). IPCM = intraperitoneal challenge model, CCM = cohabitation challenge model.

3.6 *Multiplex immunoassay*

To determine the optimal antigen concentration for bead conjugation in the immunoassay, a range of concentrations of the inactivated and the non-inactivated *P. salmonis* whole cell sonicate ranging from 3 to 12 µg per 1× scale beads were tested against different samples included in the study. Inactivated *P. salmonis* whole cell sonicate at an amount of 6 µg per 1× scale coupling reaction was chosen for the immunoassay.

Beads coupled with inactivated *P. salmonis* were screened against individual serum samples from fish immunized with experimental vaccine formulations A to D with graded *P. salmonis* antigen content and a control group E (saline). The results demonstrated a dose-response relationship

between antigen content in the administered vaccine and the antibody titers in the samples (Figure 9). A significant difference in measured IgM antibody responses was found between vaccine A and vaccine C, and vaccine A and vaccine D ($p \leq 0.05$). The positive control *P. salmonis* plasma pool was within the same MFI range as antibody values from fish vaccinated with Vaccine A. Antibody levels in saline-injected controls were low and within the same range as the negative *P. salmonis* plasma pool and individual values of samples from non-vaccinated fish.

No significant difference was found between the challenge models concerning the levels of *P. salmonis* specific antibodies after vaccination with vaccine A or B (Figure 10A and B). A slower onset of titer increment was demonstrated after cohabitation challenge compared with i.p. challenge, and also in vaccinated fish in group B compared with vaccine group A. Fish vaccinated in group C and D had significantly lower antibody titers compared to fish in group A in both challenge models (Figure 10C and D). Specific antibody levels in saline-injected fish (group E) remained low throughout the sampling period (Figure 10E). No sampled fish were positive for specific antibodies against non-*P. salmonis* antigens; A-layer from *A. salmonicida* or *M. viscosa* whole cell sonicate, throughout the sampling period (not shown).

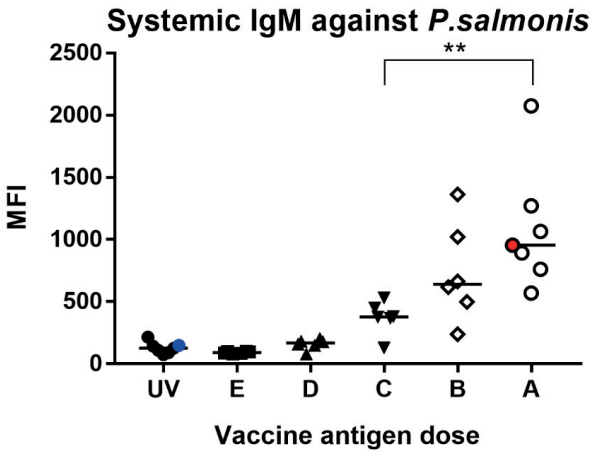


Figure 9. Dose-response systemic *IgM* against *P. salmonis* seven wpv. Vaccines with different antigen concentration (*P. salmonis*). MFI= mean fluorescence intensity. UV: unvaccinated fish, A: vaccine A (5×), B: vaccine B (3×), C: vaccine C (2×), D: vaccine D (0.5×) and E: control (saline).

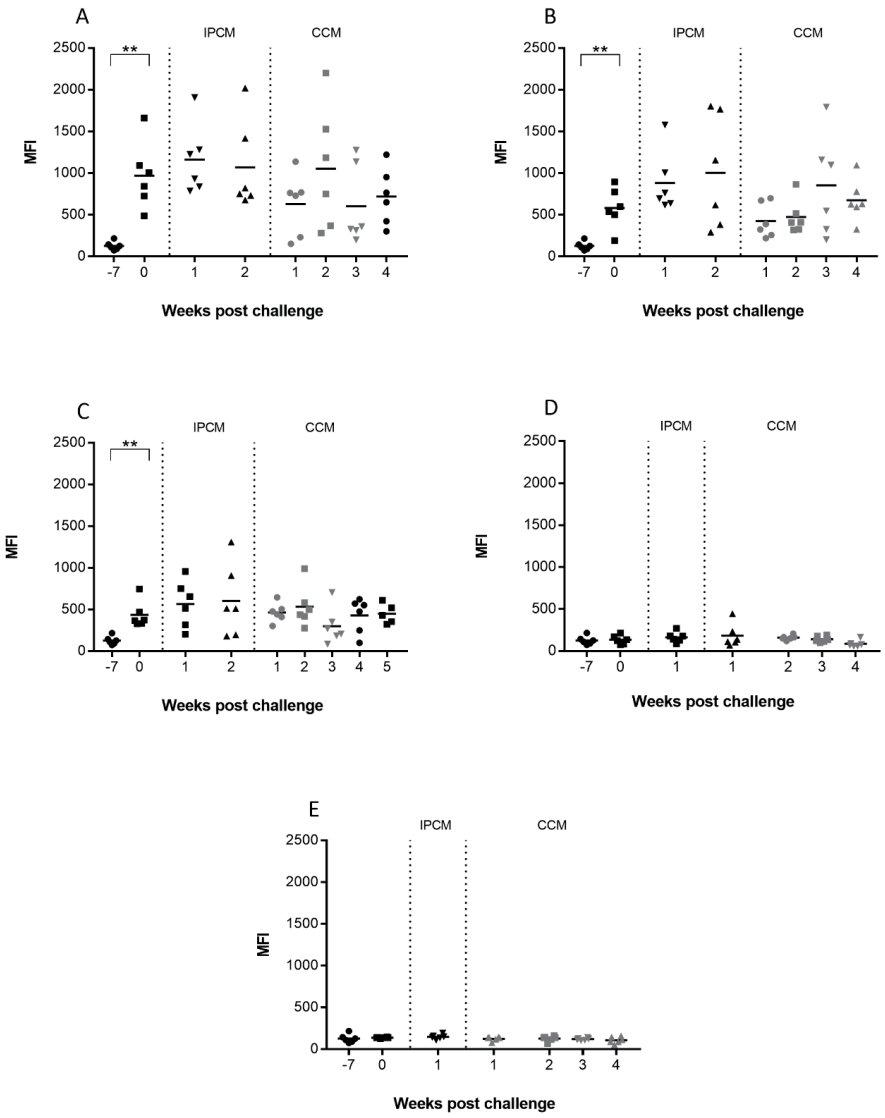


Figure 10. Antibody titers of plasma samples from fish challenged by intraperitoneal injection (IPCM) and cohabitation (CCM) against *P. salmonis*. A, fish vaccinated with vaccine A (5×); B, fish vaccinated with vaccine B (3×); C, fish vaccinated with vaccine C (2×); D, fish vaccinated with vaccine D (0.5×); E, fish injected with saline solution (control). MFI= mean fluorescence intensity.

4 Discussion

The main approach in testing efficacy and potency of fish vaccines is the use of experimental challenge either by i.p. injection, cohabitation or immersion. The three routes of infection each have

their advantages and disadvantages. Challenge by i.p. injection represents an efficient route of infection that shortens the time to develop signs of disease and is often preferred due to reduced time and costs. Challenge by cohabitation or immersion typically leads to a longer period of time to develop signs of disease, but is considered a more natural way of infection that more closely mimics real-life conditions (Nordmo, 1997; Birkbeck, Rennie, Hunter, Laidler, & Wadsworth, 2004). Which model to use depends on the pathogen of interest and is directed by monographs for vaccine testing (EMA, 2011) as well as experience from previous comparative trials. Experimental challenge with *Moritella viscosa* after immunization is, for example, well-known to reveal different degree of protection provided by the vaccines dependent on whether the challenge is done by i.p. injection or immersion (unpublished results from VESO Vikan). In addition, environmental factors such as temperature, light, water flow and water quality strongly influence the development of disease and must be taken into consideration when designing experimental challenge models. Previously reported challenge studies with *P. salmonis* show variation in onset of mortality and disease development compared to the present study, which could be related to differences in water temperature and salinity between trials (Vásquez, 2012; Rozas-Serri et al., 2017; Meza et al., 2019). The aim of the present study was to compare the disease development of two *P. salmonis* challenge models; i.p. injection and cohabitation to elucidate the pathogenesis of *P. salmonis* and evaluate the challenge models' proficiency to differentiate the protection afforded by vaccines against piscirickettsiosis.

P. salmonis is an intracellular facultative bacterium and hence the development of cellular immunity after vaccination is the key in protection against the disease (Beck & Peatman, 2015). CD8α and CD4-1 are co-receptors found on cytotoxic T-cells and T-helper cells, respectively, which are key players in the development of cellular immunity (Medzhitov, 2007; Beck & Peatman, 2015; Secombes & Belmonte, 2016). In the present study, the *CD8α* expression was downregulated and *CD4-1* expression was upregulated in the spleen after challenge by both routes. This is in agreement with the results obtained in the study performed by Rozas-Serri, Peña, Arriagada, Enríquez, and Maldonado (2018). The upregulation of *CD4-1* expression indicates an involvement of T-helper cells in the immune

584 response. Although the kinetics of lymphocyte receptor expression in the current study may provide
585 some insight into the pathogenesis of *P. salmonis*, the results should be interpreted with caution
586 bearing in mind that the expression of cellular markers in the spleen may be influenced by the traffic
587 of lymphocytes in and out of the organ.

588 The pathological changes found in the present study also suggest a hypothesis related to the
589 pathogenesis of *P. salmonis*. Short time after challenge (1 wpc), hemorrhages in the digestive tract
590 were only identified in fish challenged by cohabitation and only in the two fish groups that received
591 less or no antigen (vaccine group D and control group E). This result suggests that the digestive tract
592 is the natural port of entry of *P. salmonis*. In addition, general bleedings were found in highest
593 frequency in fish vaccinated with the highest antigen dose (Group A) at 2 wpc (i.p. injection) and 3
594 wpc (cohabitation). Thus, it is tempting to speculate that these pathological signs are caused by the
595 teleost's acquired immune response after vaccination.

596 All macro- and microscopic pathological changes observed in this study were consistent with those
597 previously described after both natural and experimental outbreaks (Almendras, Fuentealba,
598 Markham, & Speare, 2000; Fryer & Hedrick, 2003; OIE, 2003; Evensen, 2016a; Meza et al., 2019).

599 The fish were considered healthy and free of known pathogens before the study started.
600 Nevertheless, two unchallenged fish were found to have pale focal nodes in the liver and ascites,
601 respectively. This could be related to individual differences between fish, iatrogenic harm caused by
602 vaccination or necropsy, or artefacts during preparation of tissue for analysis. The presence of
603 histological alterations in unchallenged, but vaccinated fish and the presence of adhesion and
604 melanosis in all vaccinated fish might also be due to side effects of vaccine components. Adhesions
605 and melanosis presented in vaccinated fish are well-known side effects derived from oil adjuvant
606 present in the vaccines (Shoemaker, Klesius, Evans, & Arias, 2009).

607 The mortality rates in both challenge models were identical (100%). However, fish challenged by
608 i.p. injection demonstrated a shorter incubation period (11-13 dpc) compared to the fish challenged
609 by cohabitation (15-17 days). Furthermore, the duration of the outbreak was shorter after i.p.

challenge compared to challenge by cohabitation. These differences could be related to the route of infection and challenge dose. Challenge by i.p. injection of bacteria directly into the fish, ensures a high and instant challenge dose. In contrast, when fish are challenged by cohabitation, the bacteria are transmitted from shedder fish. The challenge dose could be adjusted through the number of shedders; nevertheless, the cohabitant fish are exposed to a lower challenge dose for a longer time compared to the fish challenged by injection. The more prolonged duration of the outbreak and thus the extended period for pathological changes and immune responses to develop suggests that challenge by cohabitation could be a more proficient model to study the disease pathogenesis than challenge by injection.

The bacterial load of *P. salmonis* in head kidney, spleen and liver was analyzed by qPCR. A higher bacterial load was found in fish challenged by injection before the disease outbreak and could be related to the higher challenge dose compared to the cohabitation challenge. However, the bacterial load was found to be similar in both challenge models during the disease outbreaks. This could suggest that the fish started to die at the time when the bacterial load reached a certain level, independent of the challenge model. A dose-response relationship was identified between vaccine antigen dose and bacterial load in the different tissues after challenge by injection, especially in the head kidney and spleen, suggesting an ability to use qPCR to discriminate between the potency of different vaccines after injection challenge. In addition, a dose-response relationship between *CD8α* expression and vaccines A, B and C was observed after challenge by injection, with the highest *CD8α* expression identified in the spleen of fish vaccinated with vaccine A. It has been described that *P. salmonis* is able to modulate cell-mediated immunity and thus promoting intracellular replication of the bacteria and its survival inside the host cells (Rozas-Serri et al., 2018). Thus, the acquired immune response developed in fish after vaccination against *P. salmonis* could avoid downregulation of *CD8α*. However, the vaccine did not provide enough protection to develop a strong immune response in the fish challenged by cohabitation. These results point to that the i.p. injection route could be the model of choice for evaluation of protection afforded by vaccines against *P. salmonis.* When compared to the

636 cohabitation or bath challenge models, the injection model will not only reduce the cost and duration
637 of the challenge trial, but also potentially provide improved animal welfare due to the shortened time
638 that fish will be exposed to disease.

639 Inactivated or subunit vaccines have been shown to be ineffective in stimulating cellular immunity
640 and mainly stimulate the production of humoral immunity (Seder & Hill, 2000; Shoemaker et al., 2009).
641 This is also evident in the current study, with results showing increased expression of *sIgM* after
642 vaccination and challenge in parallel to detectable antibody titers against *P. salmonis,* whereas the
643 expression of *CD8α* decreased after challenge. These results warrant further studies. There has been
644 described that increased cell density in vaccines could improve their efficacy (Makrinos & Bowden,
645 2016). Therefore, future studies should include an increased concentration of antigen in the vaccine
646 formulation, for example from 5× to 10×, with the objective to stimulate a protective immune
647 response. Especially the antigen-specific CD8 T-cell response, described as essential for the immune
648 protection against intracellular pathogen (Rozas-Serri et al., 2018). Other alternatives to improve the
649 stimulation of a specific immune response could be by live-attenuated vaccines, which have been
650 described to stimulate a stronger cellular immune response to intracellular pathogens (Evensen,
651 2016b; Tandberg et al., 2017).

652 After vaccination, a dose-response of antibody titers related to the antigen contents in the
653 different vaccine formulations was evident, with the highest antibody levels found in fish vaccinated
654 with vaccine A. This was consistent with the response elicited in rainbow trout (*Oncorhynchus mykiss*)
655 immunized with different antigenic concentrations of *P. salmonis* bacterin (Smith et al., 1997).
656 Following challenge, the antibody levels in fish vaccinated with vaccines A and B further increased,
657 indicating a secondary immune response to the pathogen. Furthermore, after challenge by injection,
658 a dose-response in *sIgM* expression in fish vaccinated with vaccines A, B and C was evident, with the
659 highest *sIgM* expression in the spleen of fish vaccinated with vaccine A. These results show the
660 initiation of a humoral immune response in fish vaccinated with the highest doses of antigen.
661 However, the antibody titers showed no statistical correlation to the protection or lack of protection

induced by vaccination. Taken together, results of antibody kinetics and immune gene expression indicate that vaccine A produces a better secondary response after *P. salmonis* challenge than the other vaccines present in the study. This underlines the possibility in finding prophylactic measures that contribute to the development of a robust primary immune response before the fish are transferred to seawater, which could potentially give a better secondary response when the individuals get in contact with the pathogen in the rearing phase.

In this study, fish were immunized before challenge for a period of seven weeks. That period applied to the i.p. injected fish but for those challenged by cohabitation the period of immunization could be considered as longer. Therefore, this could be thought as a variable that could affect the experiment outcome. However, this was not reflected in the mortality curves where the cohabitants' mortality curves were similar between both challenge models.

Histology and IHC are valuable tools and techniques for disease diagnostic. However, in the case of a septicemia like piscirickettsiosis, these tools are not suitable for interpretation or evaluation of vaccine efficacy. Measurement of bacterial load by qPCR after challenge may be a promising approach to document the efficacy of vaccines. In the present study differences between vaccine groups were seen one week after challenge by injection; vaccine group A presented the lowest bacterial load and control group E the highest. Furthermore, liver displayed high loads of bacteria, and could be suggested as the preferred tissue for bacterial load quantification. Expression of *CD8α* measured by RT-qPCR, could also add some information in the evaluation of vaccines after challenge by i.p. injection.

The statistical variability observed in the present study could have been reduced by increasing the numbers of experimental fish included at each sampling point. Similar, an increment in sampling time points could have elucidated more details related to the pathogenesis by covering critical stages during disease development. However, for ethical reasons, the number of experimental animals is kept to a minimum.

687 Further research is necessary to optimize a challenge model for efficacy testing of vaccines against
688 *P. salmonis*. Traditional efficacy testing based on the assessment of mortality after challenge is not
689 optimal for evaluation of vaccines against piscirickettsiosis due to the low degree of differentiation
690 between vaccine groups. Taken together with the ethical aspects of using mortality as outcome
691 parameter, efforts should be made to find an alternative to mortality-based evaluation of vaccine
692 efficacy. With this, the analysis for documentation of the vaccines can be improved and may probably
693 result in a more selective scale for new vaccines.
694
695 **5 Conclusion**
696 The present study reveals no major differences in the efficacy induced by vaccines after challenge
697 by either i.p. injection or cohabitation. The choice of challenge model will depend on the aim of the
698 study. In case of studying the disease and pathogenicity, the challenge by cohabitation would be
699 preferred. In case of studying vaccine potency and efficacy, the suggestion could be challenge by i.p.
700 injection. Further research is needed to identify alternatives to mortality-based evaluation of the
701 efficacy of vaccines against piscirickettsiosis. Other outcome parameters than mortality, such as
702 bacterial load, antibody titers or immune gene expression may be able to better differentiate between
703 the protection induced by different vaccines.
704
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Supplementary table1. Bacterial load statistical differences between groups in different challenge models; by i.p. injection (IPCM) and by cohabitation (CCM). A, vaccine A (5×); B, vaccine B (3×); C, vaccine C (2×); D, vaccine D (0.5×); E, control (saline).

		Liver					Spleen					Head kidney				
		IPCM														
		E	D	C	B		E	D	C	B		E	D	C	B	
1 wpc	D	n.s.				D	n.s.				D	*				
	C	n.s.	n.s.			C	n.s.	*			C	**	n.s.			
	B	n.s.	n.s.	n.s.		B	n.s.	n.s.	n.s.		B	**	n.s.	n.s.		
	A	n.s.	n.s.	n.s.	n.s.	A	*	**	n.s.	n.s.	A	***	*	n.s.	n.s.	
2 wpc	D					D					D					
	C					C					C					
	B			n.s.		B			n.s.		B			n.s.		
	A			n.s.	n.s.	A			n.s.	n.s.	A			n.s.	n.s.	
		Liver					Spleen					Head kidney				
		CCM														
		E	D	C	B		E	D	C	B		E	D	C	B	
1 wpc	D	n.s.				D	n.s.				D	n.s.				
	C	n.s.	n.s.			C	n.s.	n.s.			C	n.s.	n.s.			
	B	n.s.	n.s.	n.s.		B	n.s.	n.s.	n.s.		B	n.s.	n.s.	n.s.		
	A	n.s.	n.s.	n.s.	n.s.	A	n.s.	n.s.	n.s.	n.s.	A	n.s.	n.s.	n.s.	n.s.	
2 wpc	D	n.s.				D	n.s.				D	n.s.				
	C	n.s.	n.s.			C	n.s.	n.s.			C	n.s.	n.s.			
	B	n.s.	n.s.	n.s.		B	n.s.	n.s.	n.s.		B	n.s.	n.s.	n.s.		
	A	n.s.	n.s.	n.s.	n.s.	A	n.s.	n.s.	n.s.	n.s.	A	n.s.	n.s.	n.s.	n.s.	
3 wpc	D	n.s.				D	n.s.				D	n.s.				
	C	n.s.	n.s.			C	n.s.	n.s.			C	n.s.	n.s.			
	B	n.s.	n.s.	n.s.		B	n.s.	n.s.	n.s.		B	n.s.	n.s.	n.s.		
	A	n.s.	n.s.	n.s.	n.s.	A	n.s.	n.s.	n.s.	n.s.	A	n.s.	n.s.	n.s.	n.s.	
4 wpc	D	n.s.				D	n.s.				D	n.s.				
	C	n.s.	n.s.			C	n.s.	n.s.			C	n.s.	n.s.			
	B	n.s.	n.s.	n.s.		B	n.s.	n.s.	n.s.		B	n.s.	n.s.	n.s.		
	A	n.s.	n.s.	n.s.	n.s.	A	n.s.	n.s.	n.s.	n.s.	A	n.s.	n.s.	n.s.	n.s.	

(*) p values < 0.05, (**) p value < 0.001, (***) p value < 0.0001. n.s. = not significant.

892 Supplementary table 2. Immune genes relative expression differences between groups. A, vaccine A (5×); B, vaccine B (3×); C, vaccine C (2×); D, vaccine D
893 (0.5×); E, control (saline).

	mIgM						*sIgM*						*CD8α*						*CD4-1*					
		E	D	C	B	A		E	D	C	B	A		E	D	C	B	A		E	D	C	B	A
0 wpc	D	n.s.					D	n.s.					D	n.s.					D	n.s.				
	C	n.s.	n.s.				C	n.s.	n.s.				C	n.s.	n.s.				C	*	n.s.			
	B	n.s.	n.s.	n.s.			B	n.s.	n.s.	n.s.			B	n.s.	n.s.	n.s.			B	*	n.s.	n.s.		
	A	n.s.	n.s.	n.s.	n.s.		A	n.s.	n.s.	n.s.	n.s.		A	n.s.	n.s.	n.s.	n.s.		A	n.s.	n.s.	n.s.	n.s.	
	UV	n.s.	n.s.	n.s.	n.s.	n.s.	UV	n.s.	n.s.	n.s.	n.s.	n.s.	UV	n.s.	n.s.	n.s.	n.s.	n.s.	UV	*	n.s.	n.s.	n.s.	n.s.
	IPCM																							
1 wpc	D	n.s.					D	n.s.					D	*					D	n.s.				
	C	n.s.	n.s.				C	n.s.	n.s.				C	*	n.s.				C	n.s.	n.s.			
	B	n.s.	n.s.	n.s.			B	n.s.	n.s.	n.s.			B	*	n.s.	n.s.			B	n.s.	n.s.	n.s.		
	A	n.s.	n.s.	n.s.	n.s.		A	n.s.	n.s.	n.s.	n.s.		A	n.s.	n.s.	n.s.	n.s.		A	n.s.	n.s.	n.s.	n.s.	
2 wpc	D						D						D						D					
	C						C						C						C					
	B			n.s.			B			n.s.			B			n.s.			B			n.s.		
	A			n.s.	n.s.		A			*	n.s.		A			*	n.s.		A			n.s.	n.s.	
	mIgM						*sIgM*						*CD8α*						*CD4-1*					
	CCM																							
		E	D	C	B	A		E	D	C	B	A		E	D	C	B	A		E	D	C	B	A
1 wpc	D	n.s.					D	n.s.					D	n.s.					D	n.s.				
	C	n.s.	n.s.				C	n.s.	n.s.				C	n.s.	n.s.				C	n.s.	n.s.			
	B	n.s.	n.s.	n.s.			B	n.s.	n.s.	n.s.			B	n.s.	n.s.	n.s.			B	n.s.	n.s.	n.s.		
	A	n.s.	n.s.	n.s.	n.s.		A	n.s.	n.s.	n.s.	n.s.		A	n.s.	n.s.	n.s.	n.s.		A	n.s.	n.s.	n.s.	n.s.	
2 wpc	D	n.s.					D	n.s.					D	n.s.					D	n.s.				
	C	n.s.	n.s.				C	n.s.	n.s.				C	n.s.	n.s.				C	n.s.	n.s.			
	B	n.s.	n.s.	n.s.			B	n.s.	n.s.	n.s.			B	n.s.	n.s.	n.s.			B	n.s.	n.s.	n.s.		
	A	n.s.	n.s.	n.s.	n.s.		A	n.s.	n.s.	n.s.	n.s.		A	n.s.	n.s.	n.s.	n.s.		A	n.s.	n.s.	n.s.	n.s.	
3 wpc	D	*					D	*					D	n.s.					D	n.s.				
	C	*	n.s.				C	*	n.s.				C	n.s.	n.s.				C	n.s.	n.s.			
	B	*	n.s.	n.s.			B	*	n.s.	n.s.			B	n.s.	n.s.	n.s.			B	n.s.	n.s.	n.s.		
	A	n.s.	**	**	**		A	n.s.	*	*	*		A	n.s.	n.s.	n.s.	n.s.		A	n.s.	n.s.	n.s.	n.s.	
4 wpc	D	*					D	n.s.					D	n.s.					D	n.s.				
	C	n.s.	n.s.				C	n.s.	n.s.				C	n.s.	n.s.				C	n.s.	n.s.			
	B	n.s.	*	n.s.			B	n.s.	n.s.	*			B	n.s.	n.s.	n.s.			B	n.s.	n.s.	n.s.		
	A	n.s.	*	n.s.	n.s.		A	n.s.	n.s.	n.s.	n.s.		A	n.s.	n.s.	n.s.	n.s.		A	n.s.	n.s.	n.s.	n.s.	

894 (*) p values < 0.05, (**) p value < 0.001, (***) p value < 0.0001. n.s. = not significant.

ARTICLE III

Cultivation and characterization of a *Piscirickettsia salmonis* EM90-like isolate used for *in vivo* challenge of Atlantic salmon (*Salmo salar* L.)

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Abstract

Piscirickettsia salmonis is one of the most widespread fish pathogens in the Chilean aquaculture and the causal agent of piscirickettsiosis, a disease that is responsible for big economical losses in the salmon industry. *P. salmonis* is a facultative intracellular bacterium classified in two different genogroups, LF89 and EM90. A majority of the published studies of *P. salmonis* are related with the reference strain LF89. The aim of this study was to characterize phylogenetically and phenotypically a *P. salmonis* EM90-like isolate cultured in FN2 liquid medium. The isolate has previously been used in experimental challenge trials of Atlantic salmon (*Salmo salar* L.). The growth dynamics of the bacteria in different media was studied, and the enzyme activity and the protein expression profile of the bacterium was described after cultivation in a new liquid medium. Optimization of cultivation is essential to ensure that the bacterial culture remains pure, maintains bacterial yield and virulence. Some differences were observed in the protein expression profile after cultivation of *P. salmonis* on CHAB agar plates compared with liquid FN2 medium. Furthermore, differences in protein expression were identified when comparing fresh bacteria with bacteria inactivated with formalin. A

morphological difference in the color of the colonies was observed when comparing the *P. salmonis* EM90-like isolate cultured on CHAB agar plates with the bacteria cultured in FN2 medium. Results from *in vivo* challenge trials with fish indicated that the virulence of the bacteria was conserved independent of cultivation method.

Keywords

Piscirickettsia salmonis, phylogeny, phenotype, western blot, broth medium.

1. Introduction

Piscirickettsia salmonis is a Gram-negative, non-motile and facultative intracellular bacterium. The bacterial cells are pleomorphic, however mainly coccoid in shape (Almendras et al., 1997; Henríquez et al., 2013; Otterlei et al., 2016). *P. salmonis* is the etiological agent of piscirickettsiosis (Fryer et al., 1990), which is the most significant disease in the Chilean salmon industry (Rozas-Serri et al., 2018) causing an annual loss of around USD 700 millions (Maisey et al., 2017).

The bacterium was initially described as strictly intracellular (Mauel and Miller, 2002) and typically cultivated in the chinook salmon cell line CHSE 214 (Henríquez et al., 2013). In 2007, cultivation of a *P. salmonis* LF89-like isolate on cell-free agar plates was reported (Mikalsen et al., 2008). Subsequently, several free-cell media were successfully developed (Henríquez et al., 2013; Mandakovic et al., 2016; Mikalsen et al., 2008). The transition from cultivation in cell cultures to cultivation on cell-free agar was considered a milestone that significantly reduced the time and cost spent on *in vitro* cultivation.

There are two genogroups of *P. salmonis*; LF89 and EM90. The genogroups differ in geographic location, antibiotic resistance, host specificity and clinical manifestations (Nourdin-Galindo et al., 2017). The EM90 genogroup is widely spread and responsible for

most of the disease cases in the geographical area where the salmon farms are (Saavedra et al., 2017). The LF89 genogroup is located more concentrated in the Los Lagos region in the southern part of Chile, and accounts for less than 50% of the reported piscirickettsiosis cases (Saavedra et al., 2017). Whole genome sequences have been published for an LF89-like isolate (Bravo and Martinez, 2016), as well as partial sequences have been reported for EM90-like isolates (Nourdin-Galindo et al., 2017).

Antibiotics and vaccines are well-known measures to reduce the impact of piscirickettsiosis in the Chilean industry (Rozas-Serri et al., 2018). Antibiotics have been the preferred treatment of the disease *in situ* and have also been used as a prophylactic measurement. In addition, several vaccines have been developed with the objective to prevent the disease (SAG, 2017). The efficacy of vaccines is evaluated using experimental challenge of immunized fish under controlled conditions. For experimental challenge with *P. salmonis,* it is essential to establish consistent and reproducible cultivation of the bacteria. The bacteria thrive in nutrition rich media, which may lead to contamination with unrelated non-pathogenic microorganisms. It is beneficial to optimize the cultivation procedure by reducing the time of growth needed to reach a high concentration of the bacterium, ensuring a pure inoculum and maintaining the bacterial virulence.

P. salmonis is considered a particularly tedious and difficult bacterium to work with (Birkbeck et al., 2004). Thus, the optimization of cell-free media is an important contribution to further studies of the bacteria. The aim of this study was to characterize phylogenetically and phenotypically a *P. salmonis* EM90-like isolate used for *in vivo* studies. Furthermore, a new cell-free medium, FN2, has been described.

2. Materials and Methods

2.1. Bacterial strain and culture conditions

The *P. salmonis* EM90-like strain (VESO Vikan) was originally isolated from Atlantic salmon during a disease outbreak in Calbuco, Los Lagos, Chile in 2004. The bacteria were stored at -80°C until use. The isolate was cultured on cysteine heart agar (CHAB) plates (Norwegian Veterinary Institute, Oslo, Norway), FN2 agar plates, and in FN2 broth at 18°C for 4-7 days depending on the type of medium. Broth cultures were incubated with an agitation of 150 rpm.

2.2. FN2 culture medium

The FN2 cell free medium included 10 g peptone (Sigma-Aldrich), 5 g yeast extract (Merck & co., Inc.), 12.5 g NaCl, 5 g glucose (Merck & co., Inc.), 50 mL (5%) fetal bovine serum (FBS) (Sigma-Aldrich), 0.5 g L-cysteine (Sigma-Aldrich), 55.6 mg $FeSO_47H_2O$, 10 mL (1%) minimum essential media (MEM) (Sigma) and distilled water to a total volume of 1 L.

Peptone, yeast extract, NaCl and distilled water were sterilized by autoclaving and cooled to room temperature (RT). Glucose, L-cysteine and $FeSO_47H_2O$ were sterile filtrated (0.2µm) and added to the solution together with MEM and FBS after autoclaving. For FN2 agar preparation, 15 g of agar for microbiology (VWR chemicals) was added to the initial solution prior to autoclaving.

2.3. Growth curve experiment

One mL of *P. salmonis* EM90-like culture was transferred from a freeze stock ampule and cultivated in 10 mL FN2 broth at 18°C with agitation (150 rpm) for four days. The bacterial culture was further diluted in FN2 medium to an optical density of 0.2 ± 0.02 at 600 nm (OD_{600nm}) using a Genesys 20 spectrometer (Thermo scientific) and split in six replicates of 10 mL each. The cultures were further incubated at 18°C with agitation (150 rpm), and the OD_{600nm} was measured every 4 hours for a total of 160 hours. An average and standard deviation (SD) of the six OD_{600nm} measurements was calculated, and growth curves were plotted using OD_{600nm}

measurements as a function of time. Control of contamination was develop by plating 100 µL of culture on CHAB and blood agar plates every 24 hours.

2.4. Bacterial enzyme profiling

From freeze stocks, *P. salmonis* EM90-like bacteria were thawed and inoculated in FN2 broth at 18°C for seven and four days, respectively. Bacteria cultured in FN2 broth were pelleted by centrifugation (4000g) for 10 min at 4°C, resuspended in saline water (0.9% NaCl) until the turbidity was equivalent to a McFahrland standard of 5-6, and finally applied to an API® ZYM strip. The incubation temperature and length were adjusted to 18°C for 96 hours. Otherwise, the manufacturer's instructions were applied. The API® ZYM gallery allowed a rapid and semiquantitative detection of 19 enzymatic reactions including alkaline phosphatase, esterase (C 4), esterase lipase (C 8), lipase (C 14), leucine arylamidase, valine arylamidase, cysteine anylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucoidase. Each reaction was classified as positive, moderate or negative according to the manufacturer's instructions.

2.5. Bacterial sequencing

2.5.1. Bacterial cultivation and identification of P. salmonis EM-90

The *P. salmonis* strain was cultured using the broth culture medium FN2. The culture was performed in triplicate, with a negative control (culture medium only) and incubated at 22°C with gentle agitation. Growth dynamic was periodically evaluated by measuring the OD_{600nm} (Epoch, BioTek Instruments, USA). At an OD_{600nm} between 0.6-0.8, the culture was harvested and stored at -80°C for further analysis. For confirmation of bacterial identity a qPCR assay with primers and a specific probe was used according to Dettleff et *al.* (2015).

132

133 *2.5.2. Genomic DNA extraction*

134 The bacterial genomic DNA was extracted with the NucleoSpin DNA purification kit
135 (Macherey-Nagel) according to the manufacturer's instructions. The concentration was
136 measured using dsDNA BR Assay kit (QUBIT) and the purity was measured using an
137 absorbance ratio of 260/280.

138

139 *2.5.3. Genomic library preparation and sequencing*

140 The DNA quality was verified through capillary electrophoresis using High Sensivity DNA
141 genomic kit (Fragment analyzer). Once the quality was checked, the library was constructed
142 using the Nextera XT DNA kit (Illumina) following the manufacturer's instructions.
143 Subsequently, the size of the library was assessed by capillary electrophoresis using High
144 Sensivity NGS Fragment Analysis kit (Advanced Analytical). Library quantification was
145 performed by qPCR using a commercial kit (Kapa Biosystems, USA) and by Qubit using
146 dsDNA High Sensitivity kit (Life Technology). The sequencing was done in a MiSeq
147 equipment (Illumina Platform) using V3 version and 600 cycles paired end kit in the FAVET-
148 INBIOGEN laboratory.

149

150 *2.5.4. Analysis of sequence data*

151 Raw reads were trimmed based on quality of the sequence using Trimmomatic software
152 (Bolger et al., 2014), using the default parameters. Then, trimmed sequences were assembled
153 using Spades v3.5.0 (Nurk et al., 2013).

154

155 *2.5.5. Phylogenetic analyses.*

156 The analysis involved 11 whole genome sequences, retrieved from a subset of genomes
157 available online for *P. salmonis* from NCBI (accession numbers; ASM30029v4;
158 ASM40151v2; ASM75641v3; ASM75643v3; ASM75848v1; ASM76407v1; ASM102993v1;
159 ASM193259v1; ASM193279v1; ASM255662v1) in addition to the *P. salmonis* isolate used in
160 the current study. These retrieved genome sequences represent the two genogroups that have
161 been previously assessed using isolates from disease outbreaks in Chile (Bravo and Martinez,
162 2016). Alignments were obtained using the Realphy pipeline using the merge option (Bertels
163 et al., 2014). Estimates of evolutionary divergence between the sequences (Jukes-Cantor
164 distances) and phylogenies were reconstructed using the Bayesian algorithm BEAST 2.5
165 (Bouckaert et al., 2019) in order to obtain the posterior distribution of the tree topology
166 assuming uninformative priors. The posterior distribution was inspected using tracer, in order
167 to check for local convergence.
168
169 *2.6. Western blot*
170 *2.6.1. Sample preparation*
171 The *P. salmonis* EM90-like isolate was cultured from two ampules stored at -80°C. The first
172 ampule was divided in three bottles with 10 mL FN2 broth each and from the second ampule
173 100 µL were plated on CHAB and FN2 agar plates. The bacteria were incubated at 18°C for
174 up to 96 hours for the broth culture and up to six days for the agar plates before harvesting.
175 Inactivation by formalin was done in two of the bottles; one was subject to cold inactivation
176 (4°C) and the other was inactivated at RT by adding 0.6% formalin.
177 Plated colonies (FN2 and CHAB) were dissolved in PBS (8.0 g/L NaCl, 0.2 g/L KCl, 1.44 g/L
178 Na2HPO4, 0.24 g/L KH2PO4, pH 7.4) and washed twice by centrifugation (12000g, 5 minutes,
179 4°C) before resuspension in PBS. Broth medium (FN2) with inactivated (cold and RT) bacteria
180 were collected by centrifugation (12000g, 5 minutes, 4°C) and washed three times in PBS (pH:

7.4). All samples were adjusted to an OD_{600nm} of 0.90 (Genesys 20, Thermo Scientific) in PBS before collection by centrifugation (12000g, 5 minutes, 4°C). For protein isolation the resulting pellets were re-suspended in 50 µl 2% Triton-X 100 (Sigma) and 50 µl 2× Sample Preparation Buffer (125mM Tris-HCl pH: 6.8; 4% SDS; 20% glycerol; 20mM EDTA; 150mM DTT). Samples were mixed by vortexing and incubated at 37°C for 15min.

2.6.2. SDS-PAGE

SDS-PAGE was performed on a Criterion XT 12% Bis-Tris Precast Gel (BioRad) run with XT MES buffer (BioRad) driven by PowerPac™ HC transformer (BioRad) at 200V for 50 minutes. Precision Plus Protein Western C ladder (BioRad) was used as standard.

Total protein staining was performed according to GelCode™ Blue Safe Protein Stain protocol (Thermo Scientific).

Separated SDS-PAGE gel was enclosed in Trans-Blot® Turbo™ Midi PVDF Transfer Pack 0.2µm (Bio-Rad) and proteins were transferred by mixed molecular weight program (MW) on the Trans-Blot® Turbo™ Transfer System (BioRad) as described by the manufacturer. The PVDF membrane was washed 2 × 10 minutes with TBS-T buffer (10mM Tris-HCl pH:7.4; 150mM NaCl; 0.05% Tween 20) and blocked with TBS-T added 5% skimmed milk (Sigma) for 1 hour at RT. Staining with primary specific antibody (kindly provide by Duncan Colquhoun, Norwegian Veterinary Institute) diluted 1:60 000 in TBS-T added 1% skimmed milk was performed at 4°C overnight. Washing with TBS-T 3 × 10 minutes in RT was followed by staining with secondary antibodies (HRP-conjugated anti-rabbit) and Precision plus StrepTactin HRP both diluted 1:60 000 for 1 hour at RT. Further washing with TBS-T 4 × 10 minutes in RT was followed by chemiluminescent detection by Amersham ECL Select Detection reagents (GE Healthcare Life Sciences). Total protein stain and Western blot were imaged on ChemiDoc™ XRS+ system (BioRad).

206

207 *2.7. In vivo challenge trial*

208 The challenge trial was conducted at VESO Vikan research facility (Namsos, Norway). Fish
209 were acclimatized in brackish water with a salinity of 25‰ at 15°C for 14 days prior to trial
210 start and starved for 48 hours prior to challenge. At day 0, the fish were distributed into
211 duplicated tanks (tank A and tank B). The tanks had a capacity of 130 L, and the stocking
212 density was not higher than 40 kg/m^3 for the trial. Each tank included 10 i.p. injected fish. The
213 fish were sedated with 0.001% benzocaine chloride (Sykehusapoteket Oslo, Ullevål). The
214 bacterial inoculum was prepared for tank A in FN2 broth and for tank B in CHAB agar plates.
215 The bacterial inoculum for i.p. injection of fish was adjusted to OD_{600nm} = 1.0 and further
216 diluted by four tenfold dilutions giving a theoretical dose of 1.0×10^5 cfu/ml. Each fish was
217 injected with 0.1 ml of the bacterial inoculum on day 0. The fish were kept in brackish water
218 with a salinity of 25‰ at 15°C and 24 hours light. The fish were fed with a commercial diet
219 (Skretting AS) twice a day from day 1 post-challenge (dpc). The fish were monitored daily,
220 and dead fish were registered.

221

222 **3. Results**

223 *3.1. Bacterial colony morphology and growth rate*

224 Visible growth of *P. salmonis* appeared on CHAB plates after five days incubation. The *P.*
225 *salmonis* colonies on CHAB plates were pinpoint size, grey-white, shiny and frequently
226 confluent (Figure 1A). In contrast, bacterial colonies were visible on FN2 plates as early as on
227 day three of incubation. On these agar plates, *P. salmonis* colonies were translucent with a pale-
228 yellow color that resembled the medium (Figure 1B) and they were equal in size to the bacterial
229 colonies observed on CHAB plates.

The growth curve of the *P. salmonis* EM90-like isolate cultured in FN2 broth is shown in Figure 2. In the current experiment, the lag phase lasted for 40 hours followed by the logarithmic phase that continued for 56 hours. The bacterial growth reached stationary phase after 96 hours and lasted for 32 hours before onset of the decline phase. The highest bacterial concentration was measured after 104 hours representing an OD_{600nm} of 5.65. No bacterial colonies other than *P. salmonis* were observed on CHAB agar and no bacterial colonies were observed on blood agar plates.

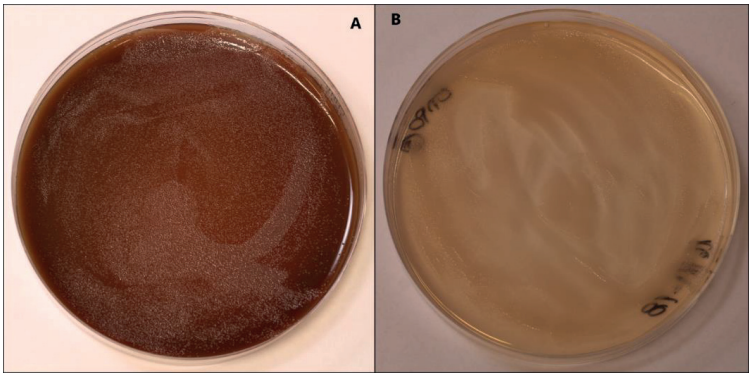


Figure 1. *P. salmonis* grown on CHAB plates (A) and FN2 plates (B). The bacteria were incubated at 18°C for 6 days.

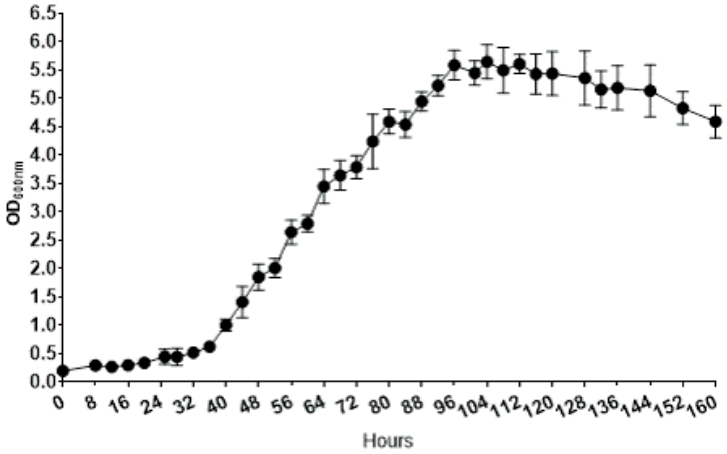
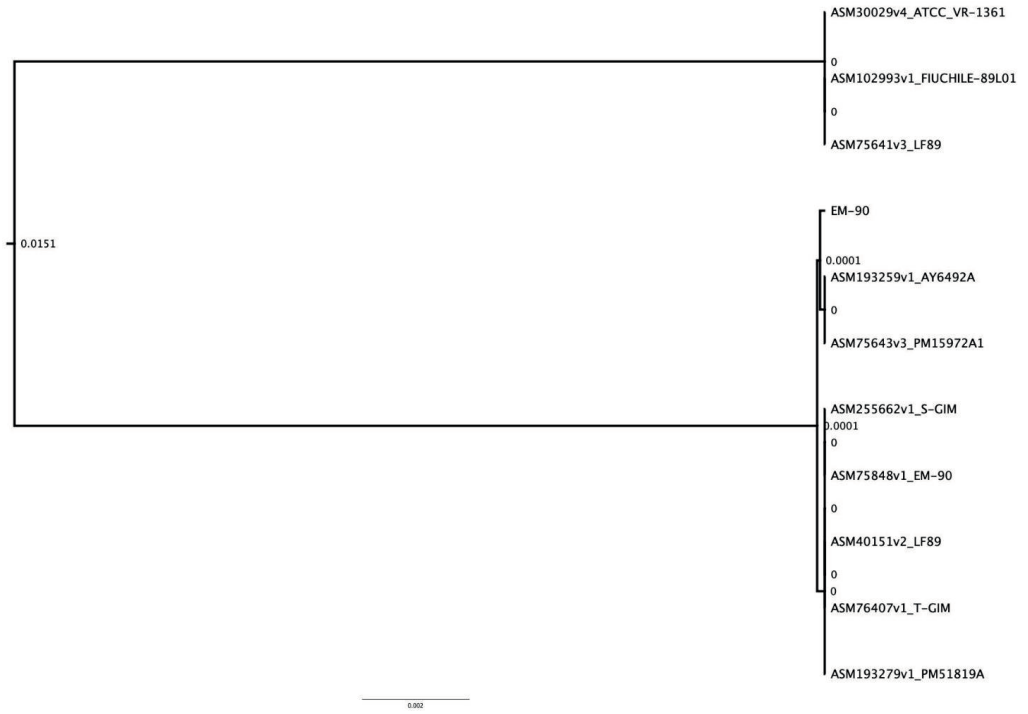


Figure 2. Growth curve of the *P. salmonis* EM90-like isolate cultivated in FN2 broth. Vertical bars indicate the standard deviation.

3.2. Genome sequencing and comparison

The estimates of evolutionary divergence between sequences showed very little divergence inside each genogroup. The tree topology showed complete separation between the LF89 and EM90 genogroups (Figure 3). The differences between the EM90 and LF89 genogroups were about three percent of the number of base substitutions per site. The standard error estimates were obtained by a bootstrap procedure (50 replicates), and were essentially zero, which is expected considering the number of sites considered in the whole genome.



255

256 Figure 3. Whole-genome phylogenetic analysis of eleven selected *P. salmonis* genomes
257 including the *P. salmonis* EM90-like isolate. The tree was constructed using the Bayesian
258 phylogenetic analysis using BEAST. The phylogenetic tree was obtained after considering 10
259 genomes available at NCBI (ASM30029v4; ASM40151v2; ASM75641v3; ASM75643v3;
260 ASM75848v1; ASM76407v1; ASM102993v1; ASM193259v1; ASM193279v1;
261 ASM255662v) and the current EM-90 isolate.

262

263 *3.3. Profiling of bacterial enzyme production*

264 The API®ZYM kit was used for semi-quantitative detection of 19 enzymatic reactions of the
265 *P. salmonis* EM90-like isolate cultured in FN2 broth. The enzymes registered with positive
266 reactions (++) were alkaline phosphatase, leucine arylamidase, valine arylamidase, acid
267 phosphatase and naphthol-AS-BI-phosphohydrolase. Those that were considered to be

moderate reactions (+) were esterase, esterase lipase and cystine arylamidase. The remaining enzymes; lipase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucoidase were negative (-). The results are summarized in Table 1.

Table 1. Enzymatic activity of *P. salmonis* EM90-like bacteria cultivated in FN2 broth. (++) positive reaction; (+) moderate reaction; (-) negative reaction.

Enzyme	**Reaction**
Alkaline phosphatase	++
Esterase (C 4)	+
Esterase lipase (C 8)	+
lipase (C 14)	-
Leucine arylamidase	++
Valine arylamidase	++
Cysteine aylamidase	+
Trypsin,	-
α-chymotrypsin	-
Acid phosphatase	++
Naphthol-AS-BI-phosphohydrolase	++
α-galactosidase	-
β-galactosidase	-
β-glucuronidase	-
α-glucosidase	-
β-glucosidase	-
N-acetyl-β-glucosaminidase	-
α-mannosidase	-
α-fucoidase	-

3.4. Protein profiling by Western blot

Differences in protein expression were revealed after cultivation of the *P. salmonis* EM90-like isolate in different media followed by SDS-PAGE and Western blotting (Figure 4). Twelve major bands between approximately 13 and 100 kDa were identified in all three bacterial cultures that were non-inactivated by formalin prior to SDS-PAGE (Figure 4, lane A-C). An

additional band representing a protein of approximately 43 kDa was identified in bacteria cultivated on FN2 agar plates (Figure 4, lane B). In comparison, a double protein band of approximately 47 kDa was only identified after cultivation in FN2 broth (Figure 4, lane C). In addition, the lack of a protein band of approximately 10 kDa was identified in bacteria cultured on FN2 agar plates (Figure 4, lane B).

Bacteria cultivated in FN2 broth at 18°C followed by inactivation with formalin at RT (Figure 4, lane D) or 4°C (lane E) displayed less conserved protein bands compared to the non-inactivated bacteria grown in FN2 broth (Figure 4, lane C). The main difference was the presence of the double band of approximately 47 kDa that was expressed in bacteria cultivated in FN2 broth (Figure 4, lane C), but that was absent in the inactivated bacteria (Figure 4, lane D and E). Additionally, the bands of approximately 45 and 20 kDa that were present in the non-inactivated bacteria, were absent in both the inactivated groups. Furthermore, in the bacteria inactivated at 4°C, the band of approximately 10 kDa was absent unlike in the bacteria cultivated in FN2 broth and on CHAB plates where this band was present. The Western blot also revealed that the temperature during formalin-inactivation of the bacteria affected the protein expression. A protein band of approximately 60 kDa was identified in the three non-inactivated bacteria cultures as well as in the culture that was inactivated at 4°C. After inactivation at RT, however, the band was absent. Inactivation at RT (Figure 4, lane D) demonstrated in general poorly conserved protein bands and a thick smear of approximately 200-250 kDa. In contrast, formalin-inactivated bacteria at 4°C (Figure 4, lane E) demonstrated more conserved protein bands, although with similar agglomeration of proteins of approximately 250 kDa.

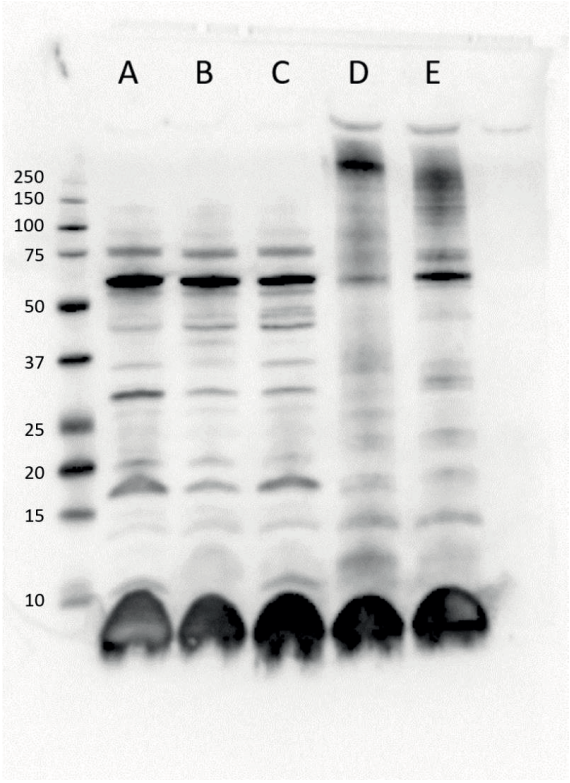


Figure 4. Protein expression profile of the *P. salmonis* EM90-like isolate cultured in different growth media (A-E) and inactivated in formalin at different temperatures (Lane D and E). A = CHAB plates, B = FN2 plates, C = FN2 broth, D = FN2 broth inactivated with formalin at room temperature, E = FN2 broth inactivated with formalin at 4°C. Ladder in MW, kDa.

3.5. *In vivo challenge of fish*

To evaluate the potential impact of cultivation media on bacterial virulence, *P. salmonis* was cultured in FN2 broth and on CHAB plates, and used for *in vivo* challenge of Atlantic salmon split into tank A and B, respectively. The mortality curves in Figure 5 show that the disease developed faster and more progressive in tank A compared to tank B. In tank A, dead fish were registered from day 11 post challenge (dpc) reaching 100% mortality after one day (12 dpc).

In tank B, mortality was registered from 13 dpc and reached 100% after three days on 16 dpc. Even though the initial mortality rate was slightly higher in tank B, the mortality did not reach 100% until 16 dpc. On the other hand, the mortality rate in tank A increased rapidly and reached 100% mortality on 11 dpc.

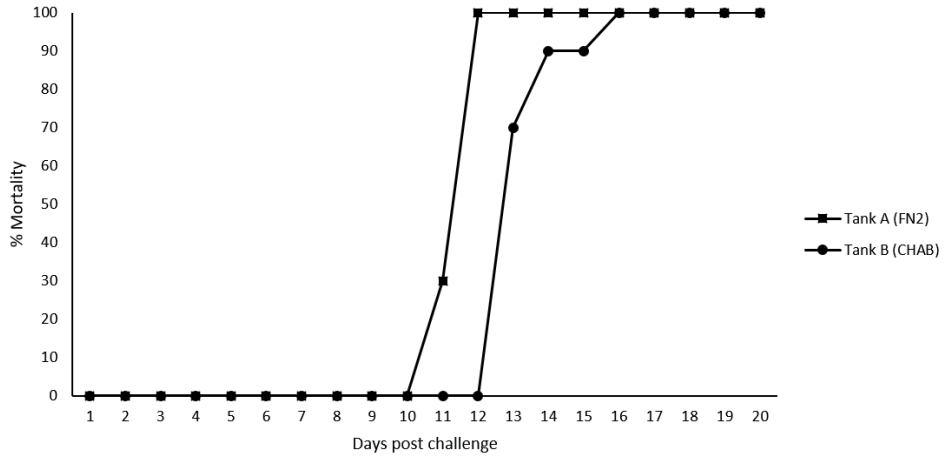


Figure 5. Mortality curve after challenge of Atlantic salmon (*Salmo salar* L.) with a *P. salmonis* EM90-like isolate. Ten fish were included in each tank. In tank A, the inoculum was prepared with bacteria cultured in FN2 broth. In tank B, the inoculum was prepared with bacteria cultured in CHAB plates.

4. Discussion

Previous studies on *P. salmonis* have commonly focused on the LF89 reference strain. The LF89 (ATCC(R) VR 1361) was established as a reference strain in 1992 (Fryer et al., 1992) and was the first isolate of *P. salmonis* to be described. The EM90 genogroup is, however, described to be the most widespread genogroup of *P. salmonis* as well as responsible for the majority of piscirickettsiosis outbreaks (Saavedra et al., 2017). EM90-like strains are therefore frequently used in experimental challenge trials, which aim to mimic real life conditions in

commercial aquaculture. The aim of this work was to study the genetic background of a Chilean isolate of *P. salmonis* that has previously been used in experimental challenge trials at VESO Vikan (Meza et al., 2019a; Meza et al., 2019b), and to describe the isolate's phenotypical and virulence properties. The phylogenetic study confirms that the isolate used in the previous challenge trials performed by Meza et al. (2019a) belongs to the EM90 genogroup, where it clusters with eight previously sequenced *P. salmonis* EM90 genomes, this is in accordance with what has been seen previously (Bravo and Martinez, 2016). The two different branches representing the two genogroups; EM90 and LF89, are shown in Figure 3. The results are in agreement with a report published by Nourdin-Galindo et al. (2017) where *P. salmonis* isolates were classified as LF89 or EM90.

The morphology of bacteria grown on agar plates observed in this study was in agreement with previous findings where grey-white pinpoint and no distinctive colonies were described (Mikalsen et al., 2008; Otterlei et al., 2016). The incubation time needed for bacterial colonies to become visible on solid agar media has previously been described to be between four and eight days when cultivated with 5% sheep blood, 0.1% cysteine and 1%glucose (BCG) and/or CHAB agar plates (Yañez et al., 2012). Similar results were observed in the current study, and interestingly, bacterial colonies cultured in FN2 broth or FN2 agar were found to require a shorter incubation time to achieve appropriate growth compared to bacteria grown on CHAB plates. Thus, the use of FN2 broth for cultivation of *P. salmonis* may reduce the time required to obtain a sufficient amount of bacteria for challenge trials. Meza et al. (2019a) demonstrated that the incubation time of bacterial pre-challenge cultures was reduced from ten to four days when using FN2 broth medium instead of CHAB plates. This difference in growth rate could be due to the composition of the growth media. In contrast to the CHAB medium, the FN2 recipe lacks the addition of blood and hemoglobin. Furthermore, the FN2 medium contains a high concentration of NaCl, which is higher than the physiological concentration (0.9%), but

360 closer to the NaCl concentration of brackish water (0.5-35‰). The FN2 medium also contains
361 less L-cysteine and glucose than the CHAB medium. It may be suggested that some
362 components in blood are able to reduce or inhibit the bacterial growth, but on the other hand,
363 the lower NaCl concentration in the CHAB medium may also account for the reduced growth
364 of *P. salmonis*. High concentration of selected nutrients may not always be beneficial for
365 organisms. This could be the case for L-cysteine and glucose, which may enhance bacterial
366 growth at a lower concentration.
367 The bacterial growth dynamics are particularly relevant for preparation of inoculums for
368 challenge trials, since the bacteria must be harvested during the logarithmic phase of the growth
369 curve. This is important to maintain the viability and virulence of the bacteria. The *P. salmonis*
370 presented in this study demonstrated a shorter lag phase and a logarithmic and stationary phase
371 that were extended compared to the report of Yañez et al. (2012). There are two important
372 factors that affect the bacterial inoculum for challenging fish; bacterial viability and bacterial
373 concentration. The different studies on bacterial growth rate should, however, be compared
374 with caution due to the many factors that must be taken into consideration. An LF89-like *P.*
375 *salmonis* isolate which was studied by Yañez et al. (2012) was reported to have a lower optical
376 density of 1.80 at OD_{620nm} compared with the current study of $OD_{600nm} = 5.65$. The differences
377 may be related to the cultivation medium. The optimal initial concentration, based on
378 theoretical concentration, for inoculum preparation of *P. salmonis* for challenge of fish is
379 $OD_{600nm} = 1.0$ of the bacterial culture was estimated to be 1.0×10^{10} cfu/ml (Berger, 2014).
380 This concentration was obtained after cultivation of *P. salmonis* in the FN2 medium for 40
381 hours compared with 96 hours of incubation in the AUSTRAL-SRS medium. The shorter
382 incubation time required for cultivation of *P. salmonis* in FN2 medium is beneficial to avoid
383 contamination of the bacterial culture. If the aim is to obtain a high bacterial concentration
384 FN2, broth should be preferred.

385 The enzymatic expression profiles obtained in this study revealed some differences when
386 compared to the report on an EM90-like isolate (NVI-5786) made by Mikalsen et al. (2008).
387 The results showed moderate reaction of lipase and no reaction of the cysteine arylamidase.
388 The differences may arise from different cultivation media, but they may also be related to the
389 different isolates tested.
390 Vaccines against intracellular pathogens are in general difficult to develop. New strategies are
391 therefore sought to obtain protection against those types of infections (Evensen, 2016). It has
392 been suggested that vaccines based on live-attenuated bacteria could induce better stimulation
393 of the immune system compared to the inactivated vaccines (Tandberg et al., 2017). Live-
394 attenuated vaccines aim to stimulate the cellular pathways of the immune system based on T-
395 cell responses (Itano et al., 2006; Secombes and Belmonte, 2016). This response may be
396 particularly helpful to the defense against intracellular pathogens mainly due to its relation with
397 the antigen presentation (Secombes and Belmonte, 2016).
398 Based on the results from the Western blotting we hypothesized that formalin inactivation
399 could cause denaturation of proteins, which may possibly affect their antigenic properties. The
400 efficacy of vaccines for some pathogens, may thus be compromised by the reduction in the
401 immunogenicity caused by the formaldehyde treatment (Montero, 2003; Soto, 2007). In the
402 Western blot analysis performed in this study, there is one band of approximately 60 kDa
403 representing a protein expressed in all the non-inactivated bacteria as well as the bacteria
404 inactivated at 4°C. These results are in agreement with the study developed by Tandberg (2018)
405 where Chilean, Norwegian and Canadian isolates were compared, and a 60 kDa protein; GroEl,
406 was detected. This protein is known for its immunogenic capacities of stimulating the innate
407 and adaptive parts of the immune system (Ranford and Henderson, 2002). In the same study,
408 and similar to our observations of non-inactivated bacteria, an SDS-PAGE separating bacterial
409 cell membrane fractions and outer membrane vesicle proteins revealed the presence of a protein

of approximately 45 kDa. This band was, however, absent in the formalin-inactivated bacteria in the present study. This strengthens the suggestion that formalin-inactivation affects structures in the bacterial cell membrane. A proteomic analysis is necessary with the objective to identify the function of the 45 kDa protein as well as the proteins representing the double band of 47 kDa presented in the bacteria cultured in FN2 broth.

The results from the *in vivo* challenge confirmed that the bacteria cultured in FN2 broth conserved their virulence and induced a mortality rate similar to the bacteria cultured on CHAB agar plates. The fish challenged with *P. salmonis* cultured in FN2 broth started to die two day before those challenge with CHAB agar plates, this could be related with the faster growth rate of the bacteria culture in broth.

5. Conclusion

The phylogenetic studies confirm that the *P. salmonis* isolate used in previous *in vivo* challenge trials belongs to the EM90 genogroup.

The virulence of the *P. salmonis* isolate used in this study was not affected by changing the cultivation media from solid CHAB agar to liquid FN2 medium. Cultivation in liquid medium lead to a reduction in the incubation time needed for cultivation of bacteria for *in vivo* challenge of fish. This will reduce the risk of contamination.

A study related with proteomics derived from the Western blot could be valuable with regards to determinate the function of bacterial proteins and how they may be involved in the immunogenicity against *P. salmonis*.

Conflict of interest statement

The authors declare that they have no competing interests.

Acknowledgments

This project was funded by VESO and the Norwegian Research Council, Project number 258783.

We would like to acknowledge Duncan Colquhoun/ NVI for providing the antibody for western blot. Thanks to Aud Kari Fauske for her technical support during this study.

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ISBN: 978-82-575-1635-2
ISSN: 1894-6402

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