Immune responses and efficacy mediated by vaccination with a live attenuated vaccine against *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

Hanne Jacobsen

Master of Science in Aqua medicine

Department of Biological Sciences

University of Bergen, Norway

June 2024

2024

Immune responses and efficacy mediated by vaccination with a live attenuated vaccine against *Piscirickettsia salmonis* in Atlantic salmon (*Salmo salar*)

University of Bergen

Acknowledgements

This master thesis in Aqua medicine was carried out by the Fish immunology Group at the Department of Biological Sciences at the University of Bergen, in collaboration with PHARMAQ AS.

I wish to express my deepest gratitude to Anita Rønneseth; I could not have asked for a better supervisor. Thank you for always being supportive, helpful during laboratory work and throughout the writing process, and always having your door open. Further, I would like to thank my co-supervisor Rolf Hetlelid Olsen for your invaluable knowledge, help during the writing process and always being available should a question arise. To my other co-supervisor, Marius Karlsen, thank you for help prior to this master thesis and throughout the writing process.

Further, I would like to thank Harald S. Lunde for help with laboratory work and technical guidance, your help has been most appreciated. Thank you, Marte Follesø Sønnervik, for help with laboratory work and sampling. Claudia Santander, thanks for translating and finding important information for me, Additionally, thanks to Fredrik Bjørnestad and Marie Stette for assisting during the vaccination. Furthermore, I extend my gratitude to my colleagues at PHARMAQ AS for rooting for me.

My heartfelt thanks to my fellow classmates for five unforgettable years together, our shared experience and memories made are greatly appreciated. Time flies when you`re having fun. Finally, I would like to thank my family and friends for their continuous support and encouragement throughout these five years.

Bergen, June 3. 2024

List of abbreviations

Abbreviations	Full name
Ab	Antibody
AF	Adipose fin
Ag	Antigen
AJ	ALPHA JECT
APC	Antigen-presenting-cell
BcR	B cell receptor
cDNA	Complementary Deoxyribonucleic acid
CD4+	Cluster of Differentiation 4 positive
CD8+	Cluster of Differentiation 8 positive
CHSE	Chinook salmonid embryo
C.m.	Cumulative mortality
Cq	Quantification cycle
DC	Dendritic cell
dd	Day-degree
DNA	Deoxyribonucleic acid
dpc	Days post challenge
dpv	Days post vaccination
EHE	Euthanized Humane Endpoints
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European Medicines Agency
ER	Endoplasmic reticulum
FAB	Fragment Antigen-Binding
FC	Fragment Crystallizable
FCS	Fetal Calf Serum
FP	Forward primer
g	Gram
HK	Head kidney
HKL	Head Kidney leucocyte
hpe	Hours post exposure
IA	Inactivated

IF	Immunofluorescence
Ig	Immunoglobulin
IL	Interleukin
ILAB	Industrial and Aquatic Laboratory
i.m.	Intramuscular
i.p.	Intraperitoneal
IPNV	Infectious pancreatic necrosis virus
ISAV	Infectious salmonid anaemia virus
IQR	The Interquartile range
L	Liter
LM	Left maxillary
M	Molar
mg	Milligram
MHC	Major histocompatibility complex
mL	Millilitre
mm	Millimetre
MNE	Mean normalize expression
mRNA	Messenger – Ribonucleic acid
Nab	Neutralizing antibody
ng	Nanogram
Nm	Nanometre
NLR	NOD-like receptors
NOD	Nucleotide oligomerization domain
NRT	Negative reverse transcriptase
OD	Optical density
OMP	Outer Membrane Protein
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
ppt	Parts per thousand
PRR	Pattern-recognition receptor
pv	Post vaccination
qPCR	Quantitative Polymerase Chain Reaction

RIG-I	Retinoic acid-inducible gene I
RLR	RIG-like receptor
RM	Right maxillary
RNA	Ribonucleic acid
RP	Reverse primer
RPS	Relative percent survival
RT	Reverse transcriptase
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SO	Suboptimal
SPC	Summary of product characteristics
SRS	Salmonid rickettsial septicaemia
TAP	Transporter associated with antigen processing
Tbet	T-box transcription factor
Tc	T-cytotoxic cell
TcR	T cell receptor
Th	T-helper cell
TLR	Toll-like receptor
VIE	Visible Implant Elastomer
µl	Microliter

Abstract

Salmonid Rickettsial Septicaemia (SRS), caused by *Piscirickettsia salmonis*, has plagued Chilean salmonid aquaculture since 1989. The bacterium evades immune responses by hiding in antigen-presenting cells, rendering inactivated vaccines ineffective. This study aimed to identify immunological markers of successful vaccination using a live attenuated vaccine, ALPHA JECT LiVac® SRS, by evaluating vaccine efficacy, analysing antibody responses in vaccinated fish and measuring immune gene regulations after *in vitro* exposure of leucocytes.

Fish were vaccinated with a live attenuated vaccine, a multivalent inactivated vaccine, or both. The live vaccine was also tested in suboptimal and inactivated state to highlight potential errors in use. To assess protection under suboptimal conditions, two rearing temperatures were tested. Vaccine efficacy trial using intraperitoneal challenge model, antibody response measured with ELISA and analyse *in vitro* exposed leucocytes for gene expression using RT-qPCR.

Fish vaccinated with the live vaccine or with a combination including the live vaccine showed good protection, while the group at suboptimal temperature and groups with inactivated vaccines experienced high mortalities. Levels of antibodies in plasma targeting *P. salmonis* increased over time in all vaccinated groups indicating that high antibody response does not necessarily correlate with protection against SRS.

Gene expressions were measured from isolated leucocytes 48 days post vaccination, *in vitro* exposed to *P. salmonis* and harvested after 6 hours. Despite there being a large difference in protection between groups, no clear differences in regulation of immune genes were found after measuring gene expression of RPS20 (reference gene), sIgM (secreted IgM antibody), Caspase-1 (indicating intracellular location and recognition by Nod-like receptors), GATA3 (indicating differentiation of Th2 response), IL-18 (indicating differentiation of cytotoxic T-cells), Perforin (indicating presence of activated cytotoxic T-cells), Tbet (indicating differentiation of Th1 response) and IL-4/13a (indicating B-cell differentiation to plasma-and memory cells). The study did not identify suitable markers for use in verification of vaccination.

Sammendrag

Salmonid rickettsial septikemi (SRS), forårsaket av *Piscirickettsia salmonis* har plaget laksefisk i den chilenske akvakulturen siden 1989. Bakterien unngår immunresponser ved å gjemme seg i antigenpresenterende celler, noe som gjør inaktiverte vaksiner ineffektive. Denne studien hadde som mål å identifisere immunologiske markører for en vellykket vaksinasjon ved bruk av en levende attenuert vaksine, ALPHA JECT LiVac® SRS, ved å evaluere vaksineeffekt, analysere antistoffresponser i vaksinert fisk og måle immunreguleringer etter *in vitro* eksponering av leukocytter.

Fisk ble vaksinert med en levende attenuert vaksine, en multivalent inaktivert vaksine eller i kombinasjon. Den levende vaksinen ble også testet i suboptimal og inaktivert tilstand for å fremheve potensielle feil ved bruk. For å vurdere beskyttelse under suboptimale forhold ble to temperaturer benyttet. Effekt av vaksine ved bruk av intraperitonalt smittemodell, antistoffrespons målt av ELISA og analysere *in vitro* eksponerte leukocytter for genuttrykk ved bruk av RT-qPCR.

Fisk vaksinert med den levende attenuerte vaksinen eller i kombinasjon med den levende attenuerte vaksinen viste god beskyttelse, mens gruppen ved suboptimal temperatur eller grupper med inaktivert vaksine opplevede høy dødelighet. Nivåer av antistoffer i plasma rettet mot *P. salmonis* økte over tid i alle vaksinerte grupper, noe som indikerer at høy antistoffrespons ikke nødvendigvis korrelerer med beskyttelse mot SRS.

Genuttrykk ble målt fra isolerte leukocytter 48 dager etter vaksinasjon, *in vitro* eksponert for *P. salmonis* og høstet etter 6 timer. Til tross for at det er store forskjeller i beskyttelse mellom grupper, ble det ikke funnet noe klare forskjeller i regulering av immungener etter måling av genuttrykk av RPS20 (referanse gen), sIgM (løselig IgM antistoff), Caspase-1 (indikerer intracellulær tilstedeværelse og gjenkjennelse av Nod-lignende reseptor), GATA3 (indikerer differensiering av Th2 respons), IL-18 (indikerer differensiering av cytotoksiske T-celler), Perforin (indikerer tilstedeværelse av aktiverte cytotoksiske T-celler), Tbet (indikerer differensiering av Th1 respons) og IL-4/13a (indikerer B-celle differensiering til plasma-og minneceller). Studien identifiserte ingen egnede markører for bruk i verifisering av vaksinasjon.

Table of contents

List of Figures

List of Tables

1. Introduction

The first published report of successfully immunization of fish came in the early 1940s, when rainbow trout (*Oncorhynchus mykiss*) were vaccinated with an inactivated oral vaccine against *Aermonas salmonicida* (Duff, 1942). After the first fish vaccine was licenced in 1976, fish vaccines have been developed at the same rate as the aquaculture industry (Gudding and Van Muiswinkel, 2013). Vaccination is today one of the most important preventive measures in disease control and has been indispensable to reduce the use of antibiotics in Norwegian salmon farming (Sommerset et al., 2005). Traditional commercial fish vaccines are effective and cheap to make, and often consists of inactivated whole-cell emulsions of the bacterium in an oil-adjuvant and an emulsifier, which gives sufficient protections against several extracellular bacterial infections (Ma et al., 2019). Some fish diseases have however been difficult to control through vaccination using traditional inactivated vaccines. For some intracellular pathogens, this is largely due to these vaccines inability to stimulate necessary cell mediated- immune (CMI) responses such as activation of cytotoxic T-cells (Tc). For these diseases other vaccine strategies are needed such as the use of live vaccine strains, DNA or mRNA vaccines. Of the latter two only DNA vaccines are so far available for fish. Additionally, as measurements of specific antibody response do not always reflect protection in these cases, other methods than experimental challenge to evaluate successful vaccination and vaccine protection is demanded in view of the 3Rs.

1.1 *Piscirickettsia salmonis*

Rickettsia-like bacteria were first identified in 1939 in pufferfish (*Tetraodon fehaka*), which originally came from the river Nile in Egypt (Rozas and Enríquez, 2014). Piscirickettsiosis or salmonid rickettsial septicaemia (SRS), is a highly infectious bacterial disease to salmonid fish. Piscirickettsiosis as a disease was first described in 1989 in Chile in Coho salmon (*Onchorhynchus kisutch*) (Cvitanich et al., 1991), and since then Piscirickettsia-like bacteria have been recognised in different farmed fish species worldwide, in both freshwater and seawater (Mauel and Miller, 2002). The disease is caused by the gram-negative facultative intracellular bacterium *P. salmonis,* which belongs to the genus *Piscirickettssia* (Fryer et al., 1992) in the subdivision of gamma-Proteobacteria together with the *Coxiella*, *Francicella* and *Legionella* genera (Mauel et al., 1999). The bacteria are generally non-motile, aerobic and

predominantly coocid, non-capsulated and usually are found in pairs with a diameter of 0.5-1.5 µm. In addition to being highly fastidious (Fryer et al., 1992; Fryer and Hedrick, 2003). Otterlei et al. (2016) developed a new growth medium for detection of *P. salmonis* called SRS-BA. Optimum growth temperature for the bacterium in culture can range between 19-22 ^{o}C (Otterlei et al., 2016). The bacterium is highly temperature sensitive and seems to favour the warm water temperatures in Chile (Fryer et al., 1990). In seawater, SRS outbreaks rarely occur below 10 ^{o}C, but usually at 12-15 ^{o}C and above. There are significantly more outbreaks in late season when water temperatures rise. Otterlei et al. (2016) was the first study revealing the presence of two genetic groups of *P. salmonis* in Chile, determined through a phylogenetic analysis comprised of 18 distinct isolates, leading to the identification of two clades. In addition, it was revealed that fish diseased with SRS can be infected with two different strains in the same outbreak (Otterlei et al., 2016). The two clades are referred to in the literature as EM90-like and LF89-like type strains. Based on analysis of 73 *P. salmonis* isolates, Schober et al. (2023) suggest dividing *P. salmonis* into two genogroups referred to as EM and LF genogroup, and a separate branch for Norwegian and Canadian isolates. In addition, the EM genogroup can be divided into subgroups ranging from EM1 to EM4. Experimental challenge using the LF89-like strain and EM90-like strain has shown difference in pathogenesis where the EM90-like resulted in a higher cumulative mortality and caused systemic and haemorrhagic disease affecting several tissues, whereas the LF89-like caused lesions in kidney and liver (Rozas-Serri et al., 2017).

1.2 SRS in farmed fish

SRS has been registered in several fish farming countries like Chile, Norway, Canda, Ireland, and Scotland (Rozas and Enríquez, 2014). SRS has however, since first discovered in 1989, been the most important disease causing severe economical and ethical issues in Chilean salmonid farming, resulting in high use of antibiotics (Miranda et al., 2018). SRS related mortalities have been reported already two weeks after seawater transfer in salmonid fish (Cvitanich et al., 1991). Substantial economic losses due to infectious diseases, mainly because of SRS, is annually costing the Chilean aquaculture up to $ 700 million on antibiotics and antiseptics (Flores-Kossack et al., 2020).

In contrast to Chile, Norway has today only few sporadic cases of fish infected with *P. salmonis*, and the disease is no threat to Norwegian fish farming. Still, *P. salmonis* was isolated from 51

Atlantic salmon farms along the west coast of Norway between 1988 and 1992. Outbreaks of disease was at the time often reported in combination with high biomasses in sea cages, fish in relatively poor conditions and in combination with algae blooms (Olsen et al., 1997).

1.2.1 Clinical signs and pathology

Coho salmon suffering from SRS has been described as lethargic, located close to the water surface and without appetite (Bravo and Campos, 1989). Further, diseased fish are described to have darkened skin pigmentation and pale gills (Bravo and Campos, 1989). By necroscopy a swollen kidney, splenomegaly and in some cases mottled livers can be observed, in addition to low haematocrit values (Bravo and Campos, 1989). Haemorrhagic skin lesions to varying degrees can also be observed and, in some cases, fish can appear healthy with no clinical signs of disease (Bruno et al., 2013). Gross pathology manifests as pale anaemic gills, ascites, nodules in the liver, fibrinous epicarditis and a swollen grey kidney. Histopathological changes can occur in most organs, for instance heart, kidney, liver, and spleen. Gills can show hyperplasia with occasional necrosis. The haematopoietic tissue within the kidney can show a lot of necrosis, oedema and increase in inflammatory cells. Liver lesions include focal to diffuse hepatitis with necrosis, which often lead to granulomas. This can also occur in the spleen (Bruno et al., 2013).

1.2.2 Transmission

The transmission route of *P. salmonis* is not completely understood, but it has been reported after experimental challenge that the onset of infection occurs by the bacteria penetrating the salmon skin and/or gills (Smith Schuster et al., 2004). However, according to Rozas-Serri et al. (2017), the gills are the main entry route of *P. salmonis*. The bacteria may be excreted in bile, faeces, and urine from live infected fish (Rozas and Enríquez, 2014). Furthermore, experimental trials have demonstrated horizontal transmission of *P. salmonis* in coho salmon in both freshwater and seawater (Cvitanich et al., 1991). So far, no vector or reservoir of *P. salmonis* has been identified (Rozas and Enríquez, 2014), and the main route of infection is believed to be by horizontal transmission of *P. salmonis* from fish to fish, both within and across farms (Fryer and Hedrick, 2003).

1.2.3 Prevention and control

In depth knowledge of the pathogen is essential to secure effective prophylaxis and disease control measures. The first commercially licenced vaccine against SRS was an inactivated vaccine in oil-adjuvants and were available to the Chilean marked in 1999 (Bravo and Midtlyng, 2007). The vaccine seemingly lacked sufficient protection against SRS, as the usage of antibiotics remained stable (Bravo et al., 2005). Florfenicol is the antibiotic mainly used in seawater stage to reduce SRS mortality (Price et al., 2016). In 2022, 341 500 kg antibiotics were used in the Chilean aquaculture (sernapesca, 2023). In comparison to 523 kg of antibiotics, which none were used against *P. salmonis,* in the Norwegian aquaculture in 2023 (Fhi, 2024). The overuse of antibiotics has made the Chilean salmon farming industry one of the highest consumers of antibiotics, leading to huge problems with antibiotic resistance (Miranda et al., 2018).

1.3 Immune responses in fish

The immune system of fish consists of innate and adaptive immune responses (Uribe et al., 2011). The innate immune system is the first line of defence against an infection. When a pathogen is detected, it will react swiftly in an unspecific manner and will not become more efficient upon a second encounter with the pathogen (Uribe et al., 2011). The innate immune cells will activate the adaptive immune response The latter recognises pathogens by specific antigen receptors, and specific immunity is achieved through the generation of memory cells and the production of antigen specific antibodies. The adaptive immune system reacts slowly and specific, thus will react quickly once re-exposed to the same or similar pathogens (Uribe et al., 2011).

Depending on the infective agent being an intracellular or an extracellular pathogen fundamentally different immune responses are called into play. Innate immune cells are equipped with pattern recognition receptors (PRRs) to detect pathogens (Magnadóttir, 2006). These PRRs recognise molecules frequently found on pathogens, the pathogen associated molecular patterns (PAMPs), for example flagellin which is a subunit protein of the flagellum (Tsoi et al., 2006). PRRs can be divided into three groups namely the secreted PRRs, the phagocytic PRRs and the signalling PRRs (Pietretti and Wiegertjes, 2014). Depending on the nature of the pathogen different PRRs are activated. The phagocytic PRRs are membrane-bound

to phagocytic cells, such as macrophages, and ligand binding phagocytosis will be induced. The secreted PRRs flow freely in the bloodstream, and has functions in complement activation, opsonisation, agglutination, and neutralization of pathogens. Signalling PRRs will upon binding initiate cell signalling through cytokine secretion (proinflammatory cytokines and interferons) and induce apoptosis (Murphy and Weaver, 2017). These include the toll-like receptors (TLRs), present on the cell surface membrane and on intracellular membranes of endosomes, and the nucleotide oligomerization domain (NOD)-like receptors (NLRs) and the retinoic acid-inducible gene I (RIG - I) - like receptors (RLRs) present in the cytosol of the cell (Pietretti and Wiegertjes, 2014). The RLRs mainly recognize viral RNA and DNA, while the NLRs recognize both intracellular bacteria and virus ligands (Murphy and Weaver, 2017). Activation of NLRs may induce formation of the inflammasome complex which will upon bacterial infections activate caspase-1, which is an enzyme that activates pro-forms of interleukin (IL) 1β and IL-18 (Hornung et al., 2009).

When a pathogen infects a host, the pathogen can thus be phagocytized by immune cells through recognition of PAMPs by PRRs. Some of the phagocytic cells are also antigen presenting cells (APCs) (Murphy and Weaver, 2017). The APCs are characterized by their ability to execute phagocytosis, break down the pathogen and present antigen derived peptides on the major histocompatibility complex (MHC) class II, in addition to giving co-stimulatory signals for activation of CD4+ T-cells. Examples of APCs in fish are macrophages, dendritic cells (DC), and B-cells (the latter in contrast to mammalian B-cells) (Li et al., 2006). Antigen presentation in fish occurs in the secondary lymphoid tissues, head kidney (HK) and spleen (Magnadóttir, 2006). When a pathogen is phagocytised, it goes through the endocytic pathway, it is taken into the cell as a phagosome, where it is exposed to lytic enzymes by fusion with lysosomes which creates an acidic environment and results in degradation of the pathogen (Murphy and Weaver, 2017). The phagolysosome containing the degraded antigen is then fused with an endosome containing MHC class II molecules and pathogen antigen derived peptides will bind to MHC class II and the complex is transported to the cell membrane. The MHC class II-peptide complex can be recognised by CD4+ T-helper cells, CD4 will recognise the MHC class II and the T-cell receptor (TcR) of the specific cell may recognise the peptide. Two more signals are required to activate the T-cell differentiation, a co-stimulatory signal formed when binding of B7.1/B7.2 ligand on the APC and the CD28 protein on the T-cell, as well as secreted cytokines as a result of PRR activation. These secreted cytokines decide which subset of T-helper (Th) cell the CD4+ T-cell (naïve T-cells) will differentiate into (Murphy and Weaver, 2017). Further, the T-box

transcription factor (Tbet) regulates the development of Th1-cells and the nucleotide sequence GATA3 regulate the differentiation of Th2-cells (Kanhere et al., 2012). Different Th cells have different immunological functions, for example Th1-cells will aid in the differentiation of CD8+ cytotoxic T-cells (Tc), Th2-cells will aid in the differentiation of plasma cells, Th17-cells are pro-inflammatory cells secreting IL-17 and T-regulatory (T-reg) cells have function in supressing the immune response (Buchmann and Secombes, 2022). The Th phenotypes are further characterized by the cytokines they produce. For example, Th1-cells produce IFN-γ to promote cellular immune responses against intracellular microorganisms, IL-12 to activate macrophages and IL-2 to activate Tc, Th2-cells produce IL-4/13a and IL-5 to promote humoral immune responses (Buchmann and Secombes, 2022).

B-cell activation can happen either in a T-cell dependent or a T-cell independent manner (Buchmann and Secombes, 2022). An activated B-cell can proliferate and differentiate to memory cells and plasma cells, where the latter produces antibodies (Murphy and Weaver, 2017). In fish, a distinction can be made between three different immunoglobulins, immunoglobulin M (IgM), immunoglobulin T (IgT) and immunoglobulin D (IgD), IgM is the major antibody present in blood (Hordvik, 2015). T-cell dependent activation of B-cells demands initial binding of antigen to the B-cell receptor (BcR), and further binding between CD40 (on the B-cell) and CD40L (on the Th2-cell) in addition to recognition of MHC class II-peptide complex presented on the B-cell by the TcR (Buchmann and Secombes, 2022). T-cell independent activation of B-cell also demands binding directly of antigen to the BcR, and either binding to an innate receptor or by cross binding of several BcRs. It is believed that T-cell independent activation to a lesser degree will stimulate the production of B memory cells (Buchmann and Secombes, 2022).

When an intracellular pathogen infects a cell, the pathogen can be recognized by intracellular PRRs (Buchmann and Secombes, 2022). Recognition by TLRs and RLRs will result in secretion of type I IFNs (Pietretti and Wiegertjes, 2014). These IFNs signals to nearby cells to produce antiviral proteins with function to disturb and inhibit the pathogen's ability to replicate intracellularly, they also stimulate nearby cells to upregulate the production of MHC class I (Murphy and Weaver, 2017). In the cytosol of the infected cell the pathogens get degraded and are transported to the endoplasmic reticulum (ER) via a transporter associated with antigen processing (TAP). In ER the MHC class I complex are produced with variable binding seats. All nucleated cells can produce MHC class I. When MHC class I has bound a pathogen derived

peptide is transported through the Golgi apparatus to the cell surface. MHC class I is recognised by the CD8 of CD8+ T-cells and the specific TcR may recognise the presented antigen peptide (Murphy and Weaver, 2017). Upon biding and following activation by Th1-cells, the CD8+ T-cells can differentiate to an active Tc with the ability to kill infected cells through the release perforins which will make pores in the membrane of infected cells and granzymes which will enter through the pores and kill the infected cells. CD8 + T-cells can also be activated by cross presentation, by DC, these cells can present exogenous peptides derived from an extracellular pathogen on MHC class I (Murphy and Weaver, 2017). How the innate and adaptive immune responses come into play when infected with an intracellular and extracellular antigen is illustrated in Figure 1.

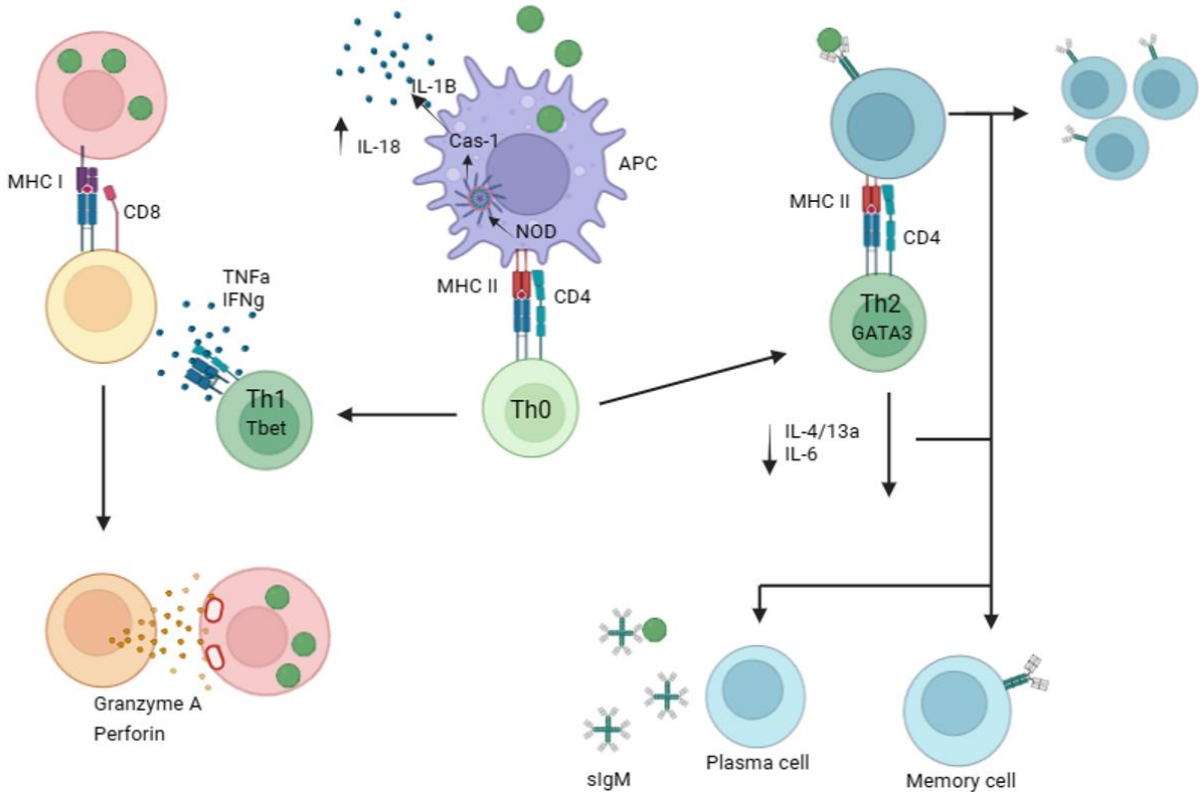


Figure 1- *An overview of immune responses stimulated by an intracellular or extracellular pathogen. The intracellular antigen recognized by NOD-like receptors, creating an inflammasome which will activate Caspasis-1 that activates IL-1β. The APC presents antigen on MHC class II to a naïve CD4+ T-cell and depending on the antigen stimulates either a Th1- or a Th2 response. An upregulation of gene expression for Tbet or GATA3 will then occur. The Th2-cell recognise the antigen presented by the B-cell and activates the B-cell by secreting IL-4/13a and IL-5. The plasma cell secretes antibodies (IgM) specifically targeting the binding antigen. An infected cell presents antigen on MHC class I to a CD8 + T-cell, to activate the Tc-cell a Th1-cell secrete INFg, TNFa and IL-18. The CD8+ T cell can then differentiate to a cytotoxic T-cell (Tc) with killing functions by secretion of perforin which makes holes in the cell membrane and granzyme A which enters trough the holes and induces apoptosis. – Figure made in BioRender: Scientific Images and Illustrations Software and modified from (Haes et al., 2012).*

1.3.1 Immune evasion strategies of *P. salmonis*

As s facultative intracellular bacterium which replicates within the hosts own immune cells *P. salmonis* have several strategies to ensure its survival and to evade the host`s CMI responses (Rozas-Serri., 2022). Previous studies have confirmed the bacterium`s ability to infect, survive and replicate within membrane-bound cytoplasmatic vacuoles of macrophages and polymorphonuclear leucocytes (Rojas et al., 2009). However, it remains uncertain whether this also occur in other APCs, such as DCs and B-cells.

P. salmonis express highly efficient virulence factors, inducing an anti-inflammatory environment inside infected macrophages (Rozas-Serri et al., 2018). Some of these virulence factors include *P. salmonis`* ability to evade phago-lysosomal degradation (Pérez-Stuardo et al., 2019), in addition to inhibit change of pH in the lysosomal compartment in the host cell (Gómez et al., 2013). The bacterium then escapes into the cytosol and replicate in the host cell cytoplasm (McCarthy et al., 2008). By hiding within macrophages *P. salmonis* can resist the host antibody responses (Isla et al., 2014).

1.4 Vaccination and live attenuated vaccines

The purpose of vaccination is to stimulate the immune system to obtain immunity by a rapid adaptive secondary immune response. Vaccines can be administered to fish by injection intraperitoneal (i.p.) or intramuscular (i.m.), orally through feed or through mucosal surfaces through immersion (bath or dip).

A live attenuated vaccines consists mostly of weakened microorganisms that is no longer pathogenic (Ma et al., 2019). The loss off pathogenicity can either be achieved by natural mutation or by artificially induced mutations. Live attenuated vaccines can also consist of non-pathogenic bacteria with shared immunogenic components to the target strain. Vaccination with a live attenuated vaccine leads to a “controlled infection” similar to the immune responses that occur in the host during an infection, without being pathogenic to the host (Shoemaker et al., 2009). When administrating a live attenuated vaccine (diluted in sodium chloride) to the body cavity of a fish the microorganism will replicate intracellularly and extracellularly, stimulating the immune responses. APC will flow to the injection site and through detection of phagocytic PRRs and phagocytosis, the antigen peptides are presented on MHC class II. Simultaneously infected cells will present antigen derived peptides on MHC class I, differentiation of both Th1

- and Th2-cells may occur. Th2-cell activate B-cells to differentiate and proliferate to memory cells and plasma cells. Th1-cells stimulate activation of Tc and memory CMI responses (Murphy and Weaver, 2017).

Today there are 20 commercial vaccines against SRS available on the Chilean marked for 2024, 19 of these vaccines are inactivated whole-cell vaccines and one is a live attenuated vaccine (Sag, 2024). However, none of these vaccines provide full protection against SRS (Rozas and Enríquez, 2014), but it is not unlikely that the live attenuated vaccine can delay SRS outbreaks under field conditions to an extent.

1.5 Aims of the study

SRS, along with sea lice (*Caligus rogercresseyi*), is a major issue in Chilean aquaculture (Sernapesca, 2023). Since the introduction of the live attenuated vaccine in 2016, its use has been widely adopted. However, the vaccine is sensitive to errors, so it is critical to follow the summary of product characteristics (SPC) to achieve a successful vaccination. It is likely that the live bacterium must infect cells to activate the CMI responses, resulting in adequate protection against the disease. Detecting antibody responses post vaccination (pv) has been challenging due to their low levels.

This study aims to identify an immunological marker to confirm successful vaccination with the live attenuated vaccine.

Specifically, the aims of this study were to:

- Measure the effect of the live attenuated vaccine after challenge with *P. salmonis.*
- Measure antibody response after vaccination using different vaccines.
- Detect differences in immune responses when using different vaccines and different vaccine regimes (temperature).

2. Materials and methods

2.1 Fish and rearing conditions

The wet lab study was conducted at the Industrial and Aquatic Laboratory (ILAB) in Bergen, Norway. This master thesis comprise parts of a lager experiment carried out by PHARMAQ AS. The study was applied for and approved by the Norwegian Food Safety Authorities with identification number ID: 30056. Atlantic salmon parr was provided by ILAB. The fish was healthy, had no signs of disease and had a valid health certificate. The parr was acclimatized to the rearing conditions for one week prior to vaccination in tanks with either 150 L or 450 L volume, due to a limited number of available tanks. The fish were kept on 0 ‰, a photoperiod of 12:12 (day: night), water temperature during immunization of either 12 °C or 8 °C and acclimatized to 15 °C at the day of challenge. Oxygen saturation of outlet water was above 75 % throughout the period. Environmental parameters in holding tanks are shown in Table 1. The fish were fed dry pellet feed (Skretting supreme, 3 mm pellet) according to appetite by automatic feeders. The fish were starved for at least 24 hours prior to handling; vaccination, sampling, and challenged with *P. salmonis* pv.

Table 1 *- An overview of the environmental parameters in the holding tanks during the vaccination/immunization period and at challenge. The fish were kept in both 150 L and 450 L tanks during vaccination, due to different water temperatures and logistics.*

Environmental parameters	Vaccination/ Immunization	Challenge
Temperature	8 (± 1°C) / 12 (± 1°C)	15 (± 1°C)
Salinity	0 ‰	0 ‰
Oxygen saturation outlet water	75-100 (%)	75-100 (%)
Photoperiod (day: night)	12:12	12:12
Tank volume	150 L / 450 L	450 L

2.2 Preparation of vaccines

Atlantic salmon parr were vaccinated with the following vaccines/ study substances, either alone or in combination: ALPHA JECT® 5-1 (AJ 5-1) (batch: 659412), ALPHA JECT LiVac® SRS (LiVac) (batch: 636947) and PBS (lot: RNBL7484) as control shown in Table 2.

Table 2 *- An overview of the vaccines used in the vaccination.*

Preparate name	Vaccine type	Antigen(s)	Dose
ALPHA JECT LiVac® SRS	Live attenuated	*P. salmonis*	0.1 mL
ALPHA JECT® 5-1	Inactivated in oil-adjuvants	*P. salmonis* *Vibrio ordalii* *Aermonas salmonicida subsp. salmonicida* Infectious pancreatic necrosis virus (IPNV) Infectious salmon anaemia virus (ISAV)	0.1 mL

To achieve a successful protection after vaccination with LiVac, it is important to thaw the vaccine correctly and according to the SPC. The vaccine was incubated in a water bath at 25 °C until all ice was melted, approximately 3 minutes. After thawing, the vaccine was diluted in 1000 mL of sodium chlorine (NaCl) solution (Ecoflac® plus 0.9 % NaCl, B. Braun, lot: 2220193602) using a transfer cap (Ecoflac® Connect, B. Braun). The container was pumped back and forth in the container three times and then inverted five times to ensure it was mixed thoroughly before use. The LiVac vaccine was used under optimal, suboptimal (SO), and in an inactivated (IA) state. The SO state of LiVac was achieved by applying a suboptimal thawing procedure, where the vaccine was incubated 10 minutes longer in a 25 °C water bath compared to what is recommended in the SPC. The IA state of LiVac was achieved by heat inactivation (held in a 70 °C water bath for 10 minutes).

Baseline samples from eight fish were collected prior to vaccination. Plasma samples for measurements of presence of specific antibodies reactive to *P. salmonis*, as well as tissue sampled from HK and liver on RNAlater for quantitative polymerase chain reaction (qPCR) assay. The weight of 30 randomly selected fish were recorded at the time point of vaccination.

2.2.1. Vaccination

Vaccination was performed by i.p. injections of 0.1 – 0.2 mL vaccine (volume dependent on number of injections), using calibrated pistol-grip syringes (Socorex 1810). The fish were anesthetized in a bath of Tricaine Pharmaq 100 mg/L (lot: 619789) and natrium bicarbonate (lot: 713827) for the vaccination and ID-marking procedure. Fish were ID-marked to enable identification of fish belonging to the different vaccine groups included in this study. The following marking methods were used: shortening of the right maxillae (RM), shortening of the left maxillae (LM), shortening of the adipose fin (AF), shortening of right maxillae and adipose fin (RM+AF) and shortening of the left maxillae and adipose fin (LM+AF), and by use of VIE (Visible Implant Elastomer) (Northwest Marine Technology, inc.) tags set subcutaneously in anal fin (Figure 2). I addition, one fish group were unmarked indicated as NONE (Table 3).

Table 3 *- An overview of the vaccines/study substances and volumes injected at vaccination, as well as ID-mark, water temperature, tank location and which tank were used. The "/" used in the column for ID-Mark indicates that the fish were marked with two different ID-marks for logistics reasons.*

Vaccine/study substance	**Volume (mL)**	**ID-mark**	**Temp. (**°C)	**Tank location and number**
PBS	0.1	AF/VIE-red	12	Cell 6, T5-T8
AJ 5-1	0.1	RM	12	Cell 6, T5-T8
LiVac	0.1	NONE/AF	12	Cell 6, T5-T8
AJ 5-1 + LiVac	0.1 + 0.1	LM	12	Cell 6, T5-T8
AJ5-1 + LiVac (SO)	0.1 + 0.1	RM+ AF	12	Cell 6, T5-T8
AJ 5-1 + LiVac (IA)	0.1 + 0.1	LM+AF	12	Cell 6, T5-T8
AJ 5-1 + LiVac	0.1 + 0.1	LM/ RM + VIE - yellow	8	Cell 7, T1-T3

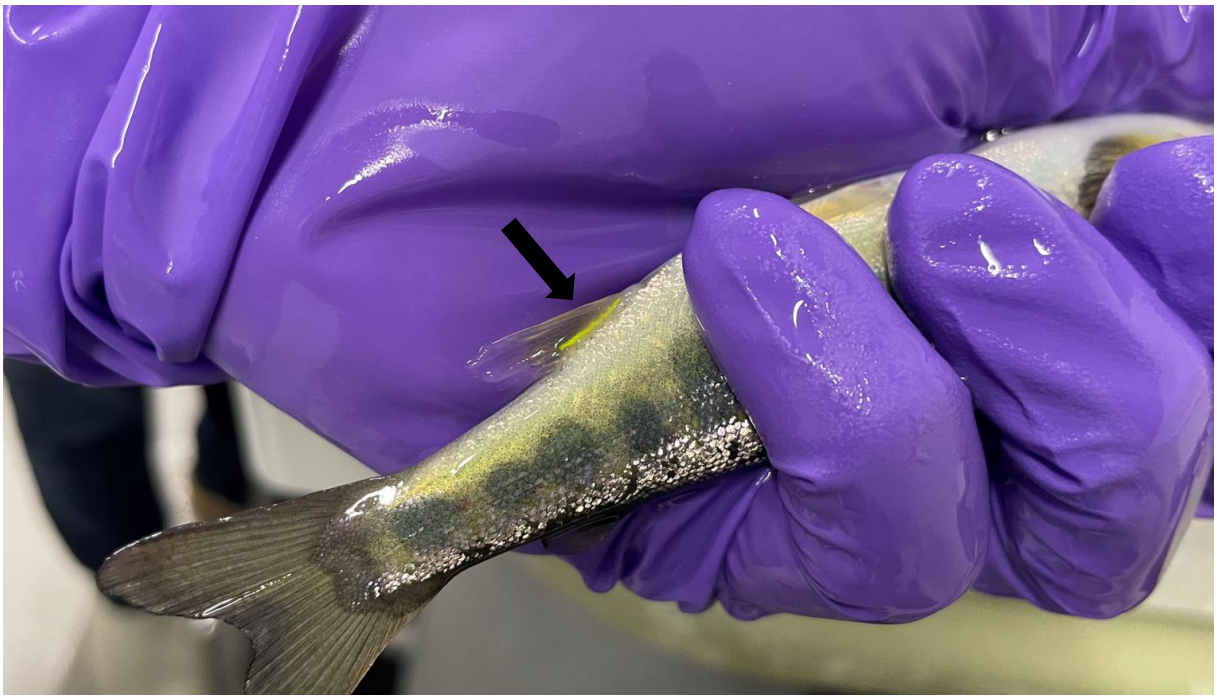


Figure 2 *– Fish marked during vaccination with use of a yellow coloured VIE (Visible Implant Elastomer) tag set subcutaneous in the anal fin (black arrow) of the parr.*

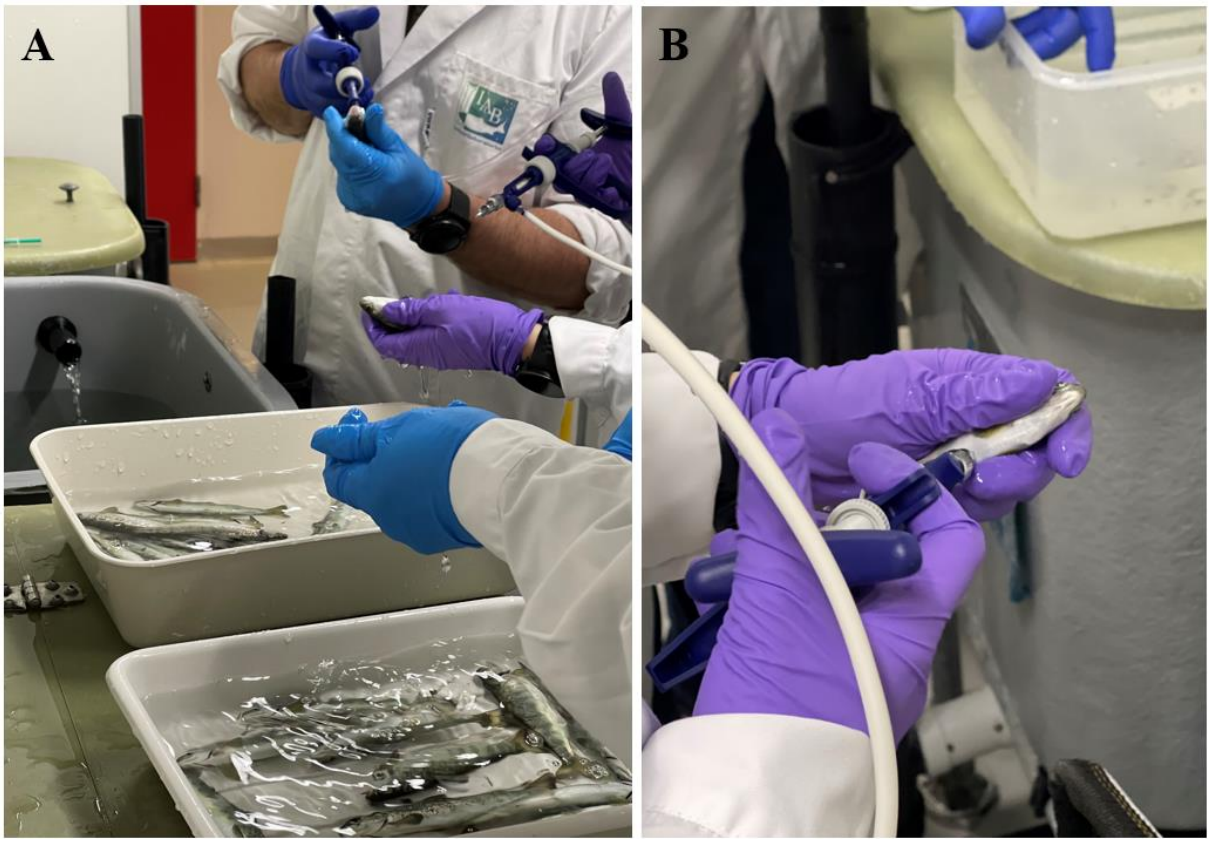


Figure 3 *– The vaccination process. (A) fish under anaesthesia being marked and vaccinated, (B) fish being injected with vaccine intraperitoneally.*

2.3. Vaccine efficacy trial with *P. salmonis*

According to the SPC of ALPHA JECT LiVac® SRS, the fish is fully immunised against SRS 456 day-degrees (dd) pv. A challenge with *P. salmonis* can then be performed to measure the protection of the vaccine. The challenge material was supplied by PHARMAQ AS and is described in Table 4.

Table 4 *- An overview containing details of the challenge material.*

Challenge material	Pre- handling	Storage	Concentration ($TCID_{50}$/mL)	Genogroup
Piscirickettsia salmonis	Thawed at room temperature	-80 °C or dry ice	5.4 x 10^6	EM90-like

2.3.1. Dose titration challenge test

A dose titration challenge test was first conducted on unvaccinated fish using three different dilutions of *P. salmonis* to determine the dose for challenge of vaccinated fish. The challenge material was diluted with PBS to three different dilutions, 1:33, 1:66 and 1:99. The pre-challenge was performed by i.p. injections of 45 fish (n=15/group) injected with 0.1 mL. All fish were anaesthetized with Tricaine and natrium bicarbonate, ID-marked and then challenged.

2.3.2. *P. salmonis* challenge of vaccinated fish

Vaccinated fish were challenged with *P. salmonis*, using the same isolate as tested in the dose titration challenge test. The challenge was performed on 360 fish i.p. injected with 0.1 mL with a 1:10 dilution based on the results from the dose titration challenge test (resulted in a high infection dose). All fish were anaesthetized using Tricaine and natrium bicarbonate, sorted by group according to ID markings, before they were challenged by i.p. injection and transferred to duplicate tanks per group.

After challenge the fish were monitored daily. Dead and moribund fish were collected and recorded in a mortally log and frozen at -20 °C. To reduce suffering, moribund fish were euthanized with an anaesthetic overdose and logged as Euthanized Humane Endpoints (EHE). The criteria for reaching Humane Endpoint (HE) were fish with observable clinical signs, lethargic and loss of equilibrium. Termination of the study was conducted after a minimum of 2 consecutive days without mortality in the negative control group (PBS). At termination, all surviving fish were euthanized by an anaesthetic overdose and counted by group.
Mortality was illustrated as cumulative mortality in percentage, and the relative percent survival value at 60 % mortality of control group and at termination of the experiment (RPS) calculated using Formula 1.

Formula 1 *– Formula used to calculate relative percent survival at the end of the study.*

$$RPS = \left(1 - \left(\frac{\%\ mortality\ vaccine\ group}{mortality\ PBS}\right)\right) x\ 100\ \%$$

2.4 Sampling

Sampling was performed at 1-, 7-, 14-, 24- and 48 days-post vaccination (dpv) from 10 fish of each vaccine group. An illustration of the experimental study design is shown in Figure 4 and specific information about the samples are shown in Table 5. At sampling timepoint 1-, 7- and 14 dpv a total of 210 fish were sampled (HK and liver on RNAlater) for real time qPCR. While at sampling timepoints 24-and 48 dpv a total of 140 fish was sampled (HK kept on L-15+ medium) for leucocyte isolation. In addition, blood samples were drawn for plasma extraction at sampling timepoints 14-, 24- and 48 dpv. At each sampling timepoint the distribution of vaccine in the abdominal cavity was monitored and presence of ascites registered.

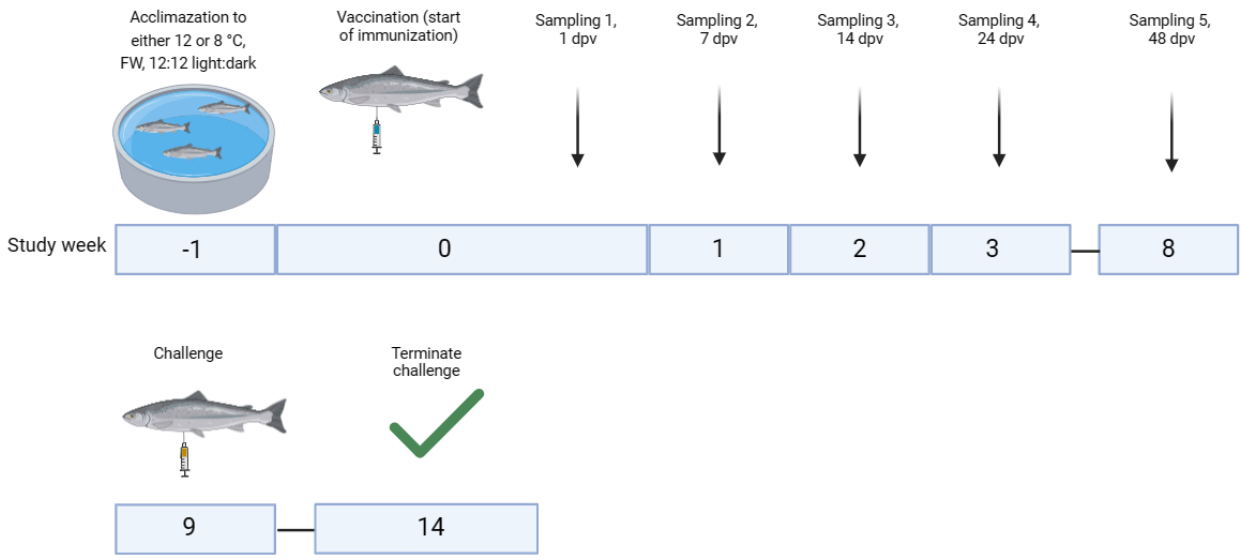


Figure 4 *- Study design of the experiment, illustrating the timepoint prior to vaccination, vaccination, different sampling timepoints and challenge with* P. salmonis. *- Figure made in BioRender: Scientific Images and Illustrations Software.*

Table 5 *- An overview of sampling performed days post challenge and number of fish/groups sampled at each timepoint. *=Baseline samples prior to vaccination performed on unvaccinated fish.*

Days post vaccination	**RNAlater**		**Plasma**	**L-15**	**n/fish**
	Liver	**HK**		**HK**	
0*	10 unmarked	10 unmarked	10 unmarked	-	8
1	10/ group	10/ group	-	-	70
7	10/ group	10/ group	-	-	70
14	10/ group	10/ group	10/ group	-	70
24	-	-	10/ group	10/ group	70
48	-	-	10/ group	10/ group	70

2.5 Serology

At sampling timepoint 14-, 24- and 48 dpv, fish were euthanized with an anaesthetic overdose of Tricaine and natrium bicarbonate. The blood was extracted using needle and blood collection tubes containing an anticoagulant, lithium heparin (Vacuette® Tube, Greiner Bio-One). The needle was injected in the caudal vein posterior of the anal fin and blood was collected in the tubes. The blood tubes were immediately placed on ice, until all the blood samples were collected. The blood tubes were place in a centrifuge (MULTIFUGE 3s) and centrifuged at 300 x g for 15 minutes. The plasma was then pipetted to 1,5 mL microtubes and stored at -20 °C.

2.5.1. Preparations of antigen (Ag) for Enzyme-linked immunosorbent assay (ELISA)

Whole bacterial antigens were harvested in late exponential growth phase by PHARMAQ AS. A pre-culture of *P. salmonis* (EM90-like isolate) was started from a frozen culture that had been cultivated on insect cells with serum, resulting in some cell debris and serum in the culture. The 10 mL pre-culture was cultivated in cell-free liquid medium for three days at 20 °C until optical density (OD) reached 0.7, and then diluted to a 1:10 ratio. 100 mL cell-free liquid medium were inoculated with 4 mL pre-culture and incubated at 20 °C for three days until OD 2.5 with a 1:25 dilution. Resulting in a 1:250 dilution of the cells. When desired OD was achieved, 100 mL bacterial culture was centrifuged at 6 000 x g for 10 minutes at 10 °C. The supernatant was removed, and the pellet was resolved in a microtube with 1 mL supernatant. The suspension was again centrifuged at 6 000 x g for 10 minutes at 10 °C. The supernatant was discarded and the pelleted with bacteria was frozen at – 20 °C and shipped on dry ice to Bergen.

The frozen pellet was incubated at - 80 °C for 24 hours before freeze-dried in an ALPHA 1-2 LDpluss (Martin Christ Gefriertrocknungsnlagen GmbH) freeze dryer. The freeze-dried antigen, 1 mg, was suspended in 1 mL PBS with 5mM EDTA (0,005mol/L) (Appendix A, 7.2) and sonicated 2 x 1 minute at 60 µA (Sonics Vibra Cell). After sonication, the stock-solution was diluted to 1 mg/mL in PBS with 5mM EDTA.

2.5.2 Detection of *P. salmonis* specific antibodies by ELISA

Three different concentrations of coating Ag (freeze dried *P. salmonis*) were tested for coating of 96-well ELISA plates (Thermo Scientific), 5 mg/mL, 10 mg/mL, and 15 mg/mL. The Ag stock solution (section 2.5.1) was diluted in PBS. All tests were conducted using plasma collected 48 dpv from 8 fish from each of the vaccine groups, LiVac and AJ5-1, and 5 fish from the PBS control group, using dilutions of 1:50 and 1:100 in duplicates.

The same plasma samples as used for determining the coating Ag concentration were also used to make titration curves to find the best dilution of plasma to be used for analysis for presence of *P. salmonis* specific antibodies. Coating Ag concentration were 10 µg/mL, based on the pre-test. The plasma samples were diluted in two-fold dilution series in PBS+T (Appendix A, 7.2), 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600 and 1:3200.

Production of specific antibodies reactive to *P. salmonis* in vaccinated fish were measured by ELISA. The coating Ag was diluted to 10 µg/mL in PBS-T and 150 µl Ag suspension was used for coating each well of 96-well plates. The plates were incubated at 4 °C overnight. Thereafter, the plates were washed three times with 200 µl PBS-T/well using a 405TM LS Microplate Washer (BioTek), before 200 µl blocking solution 3 % skim milk (Appendix A, 7.2) were added to the wells. The plates were then left to incubate at room temperature for 1 hour, washed three times and then added 100µl diluted (1:100) salmon plasma in PBS-T. Two parallel wells were included for each plasma sample and wells containing PBS-T instead of salmon plasma were used as negative controls. The plates were incubated overnight at 15 °C. Furthermore, the wells were washed three times and then 50 µl of rabbit anti-salmon IgM diluted (1:2000) in PBS-T were added to each and the plates were incubated for 1 hour at room temperature. The wells were washed three times before 50 µl of goat anti-rabbit Ig conjugated with HRP diluted (1:2000) in PBS-T were added to each well and incubated for 1 hour at room temperature. The wells were again washed three times before 50 µl of peroxidase substrate solution (o-Phenyleneiamidine) (Appendix A, 7.2) were added to each well. After 6 minutes 50 µl of stop-solution 2.5M H_2SO_4 (Appendix A, 7.2) were added to each well to stop the reaction. The OD was then measured at 492 nm in a spectrophotometer (spark® (Tcan) using SPARK software.

2.6 Test for presence of neutralizing antibody (Nab)

CHSE-cells were incubated with *P. salmonis* and plasma from vaccinated fish to investigate the presence of neutralizing antibodies.

2.6.1 Cultivation of CHSE-cells

Chinook Salmon Embryo (CHSE) cells were cultured at 20 °C without CO_2 in Nunc cell culture flask 75 cm^2 (Nuce 156472). The CHSE-cells were stored in a 1 mL cryotube in a nitrogen tank, they were thawed in hand until approximately half of the content was thawed. 1 mL of culture medium (Appendix A, 7.3) was added to the cryotube and when thawed, added to a 15 mL tube containing 6 mL of culture medium. The tube was centrifuged (Centrifuge 5702 R) at 200 x g for 5 minutes. The supernatant was removed, and the cells resuspended in 15 mL culture medium and transferred to a cell culture flask 75 cm^2. The flask was incubated at 20 °C without CO_2 (the card screwed tight) over night. The cells adhered to the bottom of the flask and the culture medium were replaced with 15 mL of new culture medium, before it again was incubated at 20 °C without CO_2. The cells were observed daily in a microscope (Leica DMIL LED) to assess density and assure no apoptosis had occurred.

When a monolayer of cells was achieved, the cells were divided to four flasks. The culture medium was disposed and 5 mL of Hank`s balanced salt solution (HBSS, lot: RNBK 2366) was added to the flask containing the cells. The HBSS removes calcium from the cells and after swirling the flask to even the solution, it was disposed. To detach the cells from the bottom of the flask, 500 µl of Trypsin Versene (Lanzo) was added. The microscope was used to ensure detachment of the cells. Thereafter, 2 mL culture medium was added to the cells, and they were evenly distributed using a glass pipette to four cell culture flasks 75 cm^2, culture medium was again added so that the total volume was 15 mL.

When enough CHSE-cells had been cultivated, two cell flasks were distributed to four 24 wells plates (Nunc™ Cell-culture Treated Multidishes, Thermo Fisher) per flask. The cell medium inside the flasks were disposed and the same procedure was done as when splitting the cells. After trypsin was added and the cells had loosened, 5 mL of culture medium was supplied, and the cells thoroughly suspended. Thereafter, 45 mL of culture medium was added and 500 µl was added to each well on the plates. The plates were sealed using sealing tape (Biorad) and left for further incubation.

2.6.2 Preparation of plasma and *P. salmonis* for exposure of CHSE-cells

Prior to the Neutralizing antibody (Nab) test the plasma was heat inactivated to ensure that all complement proteins were destroyed. 20 µl plasma from each fish sampled from each vaccine group was pooled in Eppendorf tubes and inactivated at 44 °C in a heatblock (QBH2 Grant) for 20 minutes. After inactivation, two-fold dilution series was made of pooled plasma form each vaccine group. 60 µl plasma and 2940 µl challenge medium (Appendix A, 7.3) were added to the first tube (1:50 ratio). 1500 µl from the first tube was added to a second tube, in addition to 1500 µl challenge medium (1:100 ratio). After six dilutions (1:50 – 1:1600), 1500 µl from the last tube was disposed.

A *P. salmonis* isolate, 2,6 x 10^9 cells/mL (TCID50 = 5,4x10^6), was diluted to 6,5 x 10^6 cells/mL in challenge medium.

The dilutions of inactivated plasma and *P. salmonis* (6,5 x 10^6 cells/mL) were mixed (1:1 v/v) and incubated at room temperature for one hour.

2.6.3 NAb test

For exposure of CHSE-cells 150 µl of the solution containing the mixed plasma and *P. salmonis* were added to each well for 1 hour incubation. Sealing tape was wrapped around the plates to create an anaerobe environment. After incubation, 100 µl of challenge medium was added to each well.

As controls CHSE-cells supplied with *P. salmonis* only were included in addition to wells containing non-exposed CHSE-cells. The plates were incubated for 10 days at 15 °C.

2.6.4 Crystal violet staining

Crystal violet staining was performed for the CHSE-cells. The supernatant was discarded using a glass Pasteur pipette. The wells were washed 1 x with PBS before 300 µl of crystal violet colour (Appendix A, 7.3) was carefully added to each well using a pipette and incubated at room temperature for 10 minutes. After incubation, the wells were washed five times with 1.5 mL PBS, placed at 4 °C and later studied using a microscope with camera (Leica DMIL Led with Leica MC 170 HD).

2.7 Test of isolation of leucocytes from liver

A pretest was performed to evaluate isolation yield and survival of leucocytes from liver. These tests were conducted as it initially was planned to vaccinate and challenge the fish at another facility which meant the samples had to be sent by mail and arrive the next day. The aim was to examine the survival of the leucocytes when liver was stored in a container with L-15+ medium at 4 °C for 24 hours. It was also an aim to evaluate if leucocytes harvested from liver could be used for *in vitro* exposure experiments with *P. salmonis*. Protocol used for isolation of leucocytes is described in section 2.8.

2.8 Isolation of leukocytes on Percoll gradients

HK leucocytes (HKLs) were isolated using Percoll gradients. HKLs from 10 fish from each vaccine group were sampled at sampling timepoints 24 - and 48 dpv.

HK was aseptically dissected and added to Gentle Macks tubes (GentleMACS ™ C Tubes, lot: 5220329005) supplied with 2 mL L-15+ medium (Appendix A, 7.4). The HK were homogenized using a GentleMacs dissociator (MACS miltenyi biotec) and a pre-set program for salmon HK. Two Percoll solutions of different densities were added to a 10 mL centrifuge tube (NUNC ™ Cell culture tubes). Firstly, 4 mL of 1,075g/ml Percoll solution (Appendix A, 7.4) were added to the bottom and then 3 mL of 1,060g/ml Percoll solution (Appendix A, 7.4) was carefully laid on the top using a glass Pasteur pipette. Secondly, 2 mL of the L-15+ solution containing the dissolved HK tissue was added on top of the Percoll gradient and the tubes were centrifuged for 35 minutes at 400 x g at 4 °C (Allegra® X-15R centrifuge).

After centrifugation components of low density, such as cell debris remained on top, while components of higher densities than the Percoll gradients were sedimented on the bottom of the tube. The leucocytes appeared as a cloudy band in the middle of the tubes.

The leucocyte fraction was collected from the 1.060-1.075 g/mL interface and the 1.075 g/mL layer of each gradient using glass Pasteur pipette. Firstly, cell debris and top layer was removed. Thereafter, the 0.060 g/mL layer down to 1-2 cm above the leucocyte fraction was removed. Further, the leucocyte fraction and the 1.075 g/mL layer down to 0.5 cm above the packed erythrocytes were added in a new 10 mL tube. L-15 + medium was supplied to the tubes until full, the tubes were inverted 3 times and then centrifuged for 10 minutes at 200 x g at 4 °C. The

resulting supernatant was discarded, and the pellet containing the HKLs were resuspended in 0.5 mL of L-15+ medium without gentamicin and kept on ice.

2.8.1 Counting of leucocytes

After isolation of HKLs each sample were analysed using a CASY cell counter (CASY® Innovatis). Prior to counting each sample were diluted in a CASY-cup by adding 10 µl of the sample to 10 mL of isotone salt solution (CASY® ton). The CASY cell counter provides information about the aggregation factor, viability, and number of cells. The viable cell count/mL given by the CASY cell counter were used to adjust the original leucocyte suspension to 1 x 10^6 cells/mL (cytospin preparations) or 5 x 10^7 cells/mL (*in vitro* exposure to *P. salmonis*) by adding L-15+ medium without gentamicin.

2.8.2 Cytospin preparations

Cytoclips with glass slides, filter card and a re-usable sample chamber were correctly aligned before adding 100 µl of leucocyte suspension (1 x 10^6 cells/mL) into the sample chamber. The preparations were centrifuged (Cytospin 4, Thermo scientific) for 3 minutes at 1000 rpm before cytospin preparations containing the leucocytes were allowed to air-dry overnight.

2.8.3 Colour rapid staining

The cytospin preparations were coloured a using colour rapid kit (Color rapid-set, lot: 8824, Lucerna-chem). The colour stains the leucocytes in a dark blue/purple colour. Three solutions were required for staining, a fixative solution (Methanol), staining solution 1 (Eosinophilic) and staining solution 2 (basophilic thiazine dye). The slides were dipped 5x in their respective order and drained on tissue paper between each solution. The preparations were then held upside down and carefully rinsed under running tap water to remove excess colour. The preparations were air-dried overnight before studied in a microscope (Nikon Labophot 2A).

2.9 *In vitro* exposure of HKLs with virulent *P. salmonis*

The leucocyte concentration, given by the CASY cell counter, was used to calculate the dilution of the samples in L-15+ medium without gentamicin. The same was done for the *P. salmonis* isolate using a CASY cell counter for bacteria. Resulting in all the leucocyte samples having the same concentration, 5 x 10^6 cells/mL. In addition, the bacterium sample was diluted to a concentration of 5 x 10^7 bacteria/mL. Table 6 gives an overview of the number of fish included from each vaccine group.

To each well of 24-wells plates (Nunc™ Cell-culture Treated Multidishes, Thermo Fisher) places 250 µl leucocyte suspension (5 x 10^6 cells/mL) and 250 µl *P. salmonis* (5 x 10^7 bacteria/mL) was added. The plates were incubated for 6 and 24 hours at 15 °C before harvested. Figure 5 illustrates the setup of parallels for each fish, whereas half of the leucocytes were exposed to *P. salmonis* and the other half were unexposed, and harvested at two different timepoints.

Table 6 *- An overview of the number fish from each vaccine group and controls used for the in vitro exposure. Leucocytes from each fish were divided in four parallels, leucocytes in two wells were exposed to* P. salmonis*, and the other two used as controls. From each fish exposed (one well) and control (one well) leucocytes were harvested at two sampling timepoints, 6- and 24 hours post exposure of the bacterium. See Figure 5 for illustration.*

Vaccine combination	Temperature	Number of fish
PBS	12 °C	5
AJ5-1	12 °C	6
LiVac	12 °C	6
AJ5-1 + LiVac	12 °C	6
AJ5-1 + LiVac (IA)	12 °C	6
AJ5-1+LiVac	8 °C	6

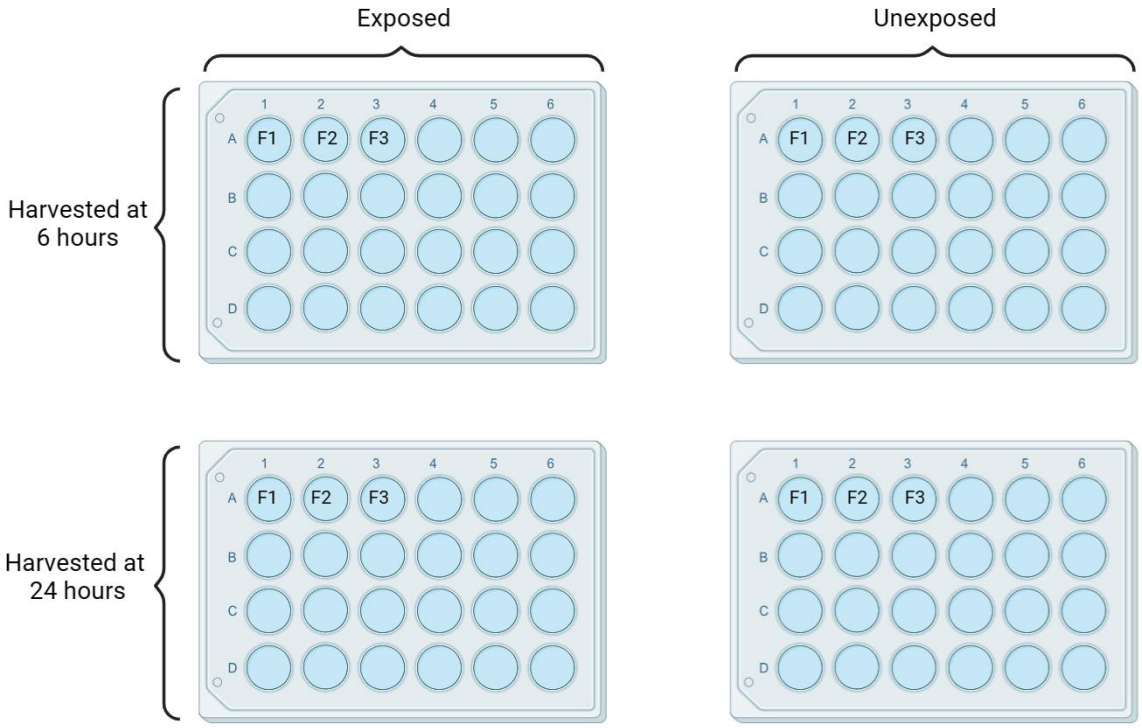


Figure 5 *–Illustrating how leucocytes from each fish were divided into four parallels during the in vitro exposure of P. salmonis. Leucocytes from each fish in two wells were exposed to* P. salmonis*, two wells were used as controls. From each fish exposed (one well) and control (one well) leucocytes were harvested at two sampling timepoints, 6- and 24 hours post exposure of the bacterium. - Figure made in BioRender: Scientific Images and Illustrations Software.*

Lysis buffer (Sigma-Aldrich) was mixed with Dithiothreitol (DTT) (Appendix A, 7.5) prior to sampling. Firstly, the plates were centrifuged (Allegra® X-15R centrifuge) at 200 x g for 10 minutes. Thereafter, the supernatant was carefully removed using glass pipette before 250 µl lysis buffer with DTT was added to each well. The lysis buffer was carefully pipetted up and down in the well until the solution was viscous. Lasty, the lysis solution was transferred to an Eppendorf tube and frozen at – 80 °C until RNA isolation.

2.9.1 Isolating RNA from leucocytes

Total RNA was isolated from tubes containing isolated leucocytes in lysis buffer by using the GenElute mammalian Total RNA Kit (RTN350-1KT, Sigma-Aldrich). All the steps were performed in room temperature and by using RNase-free water, RNase-free Eppendorf tubes and RNase-free pipettes with filters.

The 250 µl lysis buffer/leucocyte suspension was pipetted to a GenElute Filtration Column and centrifuged (Biofuge pico) at 14 000 x g for 2 minutes. The column was then removed and 250 µl of 70 % ethanol were added to the filtered lysate and mixed thoroughly. The ethanol-lysate mix was then pipetted to a GenElute Binding Column and centrifuged at 14 000 x g for 30 seconds. The liquid that went through the binding column were then removed. 500 µl of wash solution 1 was pipetted to the binding column and again centrifuged at 14 000 x g for 30 seconds, before the binding column were transferred to a new collection tube. 500 µl of wash solution 2 (containing 60 mL of 100 % ethanol) were added to the binding column and centrifuged for 30 seconds, before the liquid that went through the column was removed. Again, 500 µl of wash solution 2 were added to the binding column and centrifuged for 2 minutes. The biding column was then transferred to a 1,5mL Eppendorf tube and 30 µl heated RNase-free water (70°C) were then added and centrifuged for 1 minute. The isolated RNA was then kept on ice.

2.10 Real time RT-PCR

The purified RNA was reverse transcribed into complementary DNA (cDNA) by use of the enzyme reverse transcriptase. Then the cDNA was amplified and monitored in real time.

2.10.1 Test of assays

Tests of potential assays were conducted prior to qPCR of the *in vitro* exposed leucocytes. Analysis on agarose gels was used to control products size. qPCR was performed and Quantitative threshold (Cq) values were plotted in English version of Microsoft Excel from Microsoft 365. Formula 4-7 was used in Excel to evaluate the different assays for the target genes presented in Table 7.

Formula 2 *– Formula used to calculate slope.*

$$Slope = SLOPE(known\_y; known\_x)$$

Formula 3 *– formula used to calculate the amplification factor.*

$$Amplification\ factor = 10^{(\frac{-1}{slope})}$$

Formula 4 *– Formula used to calculate efficacy.*

$$Efficacy = (Amplification\ factor - 1)\ x\ 100$$

Formula 5 *– Formula used to calculate correlation coefficients.*

$$Correlation\ (R^2) = RSQ(known\_y; known\_x)$$

Table 7 *– An overview of the genes analysed in this master thesis, containing the genes evaluation and their abbreviation.*

Target gene	RPS20	sIgM	Cas-1	GATA3	IL-18	Perforin	T-bet	IL-4/13a
Forward primer seq, 5'-3'	ATCACCACCAGAAAGACACCCT	ATGAGGACTGGAGCAATGGGACAG	AGCCAGTGAGAAGATGATTGCCAGA	CGCCTGCGGACTCTATCACAAGATG	TCTGCCAAACCACTGGAGCAACCT	TCCGTGCTCAATGCGAAGTTCTCC	CGCAGGCTCTCCTCAACAACTATCC	GCAAGGAGTTCTTCTGCGAAGCTGA
Reverse primer seq, 5'-3'	GAGGTGATCTGCTTGACAATCTCA	ACAGATGGACGCTGTGGATCTCCT	TGGTGCTCCTCCTGCCGTGATAA	GCCACAGTGTTGTCGTGCTCGTT	AGTACCAGCTACACCACAGCGATGA	AGCGGCCATCCAGAGTGAAGTCA	TGACGGGTCCAGAACGCTGATGT	GCCTGGTTGTCTTGGCTCTTCACA
Correlation, R^2	1,000	0,995	0,991	0,997	0,999	0,996	0,995	0,996
Slope	-3,178	-3,135	-3,187	-3,282	-3,283	-3,090	-3,300	-3,264
Amplification factor	2,06	2,08	2,06	2,02	2,02	2,11	2,01	2,02
Efficiency	106,39 %	108,45 %	105,95 %	101,69 %	101,66 %	110,67 %	100,91 %	102,45 %
Product size, bp	122	121	132	131	133	145	131	136
Reference	Rønneseth et al. 2013	This study	This study	This study	This study	This study	This study	Fish immunology group at University of Bergen
Accession	BTO60032	Y12391	XM_014214559	NM_001171800	NM_001141408	XM_014172502	XM_014158543	NM_001204895

2.10.2 Isolation of RNA from tissue

HK were aseptically sampled from 2 unvaccinated fish (to test assays) and transferred to Eppendorf tubes containing 500 µl of RNAlater® stabilising solution. The tubes were incubated at 4°C overnight before stored at -20 °C. Each tissue sample were measured to 40 g and added to FastPrep® Tubes (MP BIOMEDICALS) together with three steel beads and 500 µl lysis buffer (4 mL lysis solution + 64 µl thawed DTT). The tissues were homogenisied in Fastprep®-24 Classic (MP BIOMEDICALS) for 15 seconds x (6.0 m/sec, quick prep). Total RNA from tissue was isolated using the GenElute mammalian Total RNA Kit and was done following the same procedure as for isolating RNA from leucocytes, apart from a few changes (Section 2.8). An additional step was added since the GenElute Filtration Column can only hold 700 µl, the ethanol-lysate mix at 1000 µl was thus executed in two steps. Finally, for elution, the binding columns were supplied with 50 µl heated RNase-free water.

2.10.3 Analysis of RNA with NanoDrop

The concentration and purity of the RNA samples were analysed using a Nanodrop2000 spectrophotometer (Thermo Scientific). The RNA concentration was measured as ng/ µl, with purification assessed through the following ratio of absorbance: 260 nm to 280 nm (A260/280) and 260 to 230 (A260/230). 1,5 µl of RNase-free water was added as a blank sample onto the spectrophotometer arm to calibrate. Then 1.5 µl of isolated RNA was added to the spectrophotometer to measure the concentration and purity of the sample. Nanodrop measurement was conducted both before and after DNase treatment.

2.10.4 Agarose gel electrophoresis

A test on a 1 % agarose gel was conducted both before and after DNase treatment to assess and to ensure a successful DNase treatment and that all genomic DNA was broken down. The 1 % agarose gel was made from 25 mL 1x TAE (Appendix A, 7.6) and supplied with 0.25 g agarose (SeaKem® LE Agarose, Lonza) in a 100 mL glass flask (Appendix A, 7.6). The flask containing agarose and TAE-buffer were weighed before microwaved oven until the agarose was completely dissolved. The flask was then weight again, and 1x TAE was added until the pre-microwaved weight had been achieved. When the solution had reached approximately 50 °C 2.5 µl GelRed Nucleic acid stain (1: 1000 diluted) (Biotum) was added. The liquid was poured

into a container supplied with a gel ring and a 12-well comb and left to dry for 30 minutes. The comb was removed, and the gel placed in a gel tray and 1xTAE was added to the electrophoretic unit until all wells were covered. 1.5 µl of 1Kb + (Appendix A, 7.6) were carefully pipetted to the first well as a reference marker. Thereafter 2 µl RNA mixed with 0.5 µl 5x loading dye (Appendix A, 7.6) and pipped in the remaining wells. The gel electrophoresis was performed at 90V for 40 minutes. Finally, a picture was captured using Gel doc ™ EZ imager to documents the quality of the RNA samples.

2.10.5 DNase treatment

DNase treatment was performed to remove all genomic DNA from the RNA samples. Firstly, the RNA concentration was measured before making the master mix. The master mix consists of 10x reaction buffer and DNase I (1:1) ratio and the amount of master mix added to each sample vary depending on the RNA concentration. In addition, when DNase treatment was performed on RNA isolated from tissue, RNase-free water was supplied to the master mix. The samples were incubated at room temperature for 15 minutes. Stop-solution was then added to the samples before they were incubated for 10 minutes at 70 °C using a heat-plate. The stop-solution protects the RNA against hydrolysis at high temperatures. After the samples were cooled, a second nanodrop measurement and agarose gel analysis were performed before cDNA synthesis. See Appendix A, 7.6 for examples of DNase treatment.

2.10.6 cDNA synthesis

cDNA was made using the qScript cDNA Synthesis Kit (QuantaBio). A negative reverse transcriptase (NRT) sample was prepared from the RNA sample that contained the highest total RNA in ng. This additional sample was included to monitor that no genomic DNA was present in the RNA samples.

The master mix consists of qScript reverse transcriptase and 5x qScript Reaction Mix in a 1:4 ratio. NRT was mixed in an Eppendorf tube and consisted of 5x qScript Reaction Mix, RNase-free water, purified RNA, but no RT enzyme.

All samples were run in a polymerase chain reaction (PCR)-machine (2720 Thermal cycler) following time and temperatures given in Table 8. The PCR monitor amplifies the RNA sequences and is highly sensitive and requires minimal template for detection.

Table 8 *- An overview of the incubation program of the PCR-machine (2720 Thermal cycler).*

Temperature	Time
22 °C	5 min
42 °C	30 min
85°C	5 min
4°C	∞

2.10.7. Quantitative PCR

qPCR is a widely used method to detect, amplify and monitor specific nucleic acid in real-time.

Firstly, each cDNA sample were diluted to 2.5 ng/µl in RNase-free water with a final volume of 106 µl and pipetted to tubes in strips. A robot machine (Pipetmax Gilso) was used to distribute 4 µl template into PCR plates (PCR plate full skirt LP, white, SARSTEDET AG & Co).

Three master mixes containing the ratio of 12µl SYBR green jump start, 1 µl Forward primer (FP), 1 µl reverse primer (RP) and 0.5 µl RNase-free water was made (Appendix A, 7.6 for example) for each triplicate sample and pipetted into tubes in strips with a final volume of 30 µl. The robot pipette machine was used to distribute 6 µl of master mix into the wells of the PCR plate. A sealing tape (Adhensive qPCR seal, Sarsted AG & Co) was applied to the plates to secure the content. The PCR plates were centrifuged (PCR-plate centrifuge II) for 15 seconds at 2200 x g to collect the solution in the bottom of the wells. The qPCR was performed using (CFx96^{TM} Real-time cycle). The PCR program shown in Table 9 was used. The cycle quantification (Cq) values achieved from the real-time PCR and converted to Mean Normalized expression (MNE) by using Formula 6 in Microsoft Excel from Microsoft 365. Furthermore, the interquartile range 1.5 x IQR (IQR– rule for outliers) used in the same program (Formula 7). In addition, a strict limit was set between replicates allowing no greater variation between replicated than 0.5. Resulting in filtered group size of 3-5 fish, with a few exceptions (Appendix B, Table 22). The results were illustrated using the program Graphpad Prism Windows version 10.02.

Formula 6 *– Formula used to calculate mean normalized expression.*

$$MNE = \frac{E_{ref}\overline{Cq}_{ref}}{E_{target}\,\overline{Cq}_{target}}$$

Formula 7 *– Formula used to as 1,5 x IQR rule for outliers between quartiles.*

$$IQR = Q_3 - Q_1$$

Table 9 - *An overview of the incubation program for the CFx96™ Real-time cycle*

Program:	Temperature (°C)	Time (min)	Number of cycles
Initial denaturation	94	2:00	1
Denaturation	94	00:15	40
Annealing, extension, and read florescence	60 °C before lowest primer T_m	01:00	

2.11 Statistical analysis

The statistic software R (The R Foundation for Statistical Computing, Vienna, Austria) was used for calculating differences in cumulative mortality (%) in vaccinated fish compared to PBS control group and across the different vaccine groups. The analysis was performed using a chi-square test for 2x2 contingency tables. The cumulative mortalities (%) were compared when the PBS control group had reached 60 % in mortality and at termination of the experiment. Differences in cumulative mortality (%) was statistically significant if p-values < significance level, $\alpha < 0.05$.

A one-way ANOVA analysis for statistics was performed for the ELISA and RT-qPCR results, using Graphpad Prism version 10.02 and Sigma plot 15. The Tukey`s multiple comparisons test was used for both ELISA and the RT-qPCR statistics. The results were considered significant when *p<0.05, ****p<0.0001 and p>0.05 = n.s.

3. Results

Average weight of 30 random fish at vaccination were approximately 26 g, all weights are presented in Appendix B (Table 16).

From all the sampled fish, throughout the samplings, a good distribution of vaccine in the abdominal cavity was observed. By gross observation of body cavity, no elevated amounts of ascites was observed.

3.1 Mortality after challenge with *P. salmonis*

Figure 6 shows cumulative mortality (%) of fish in vaccinated groups and control groups, in duplicate tanks, after challenge with *P. salmonis* (n = 15 / vaccine group / tank). Mortality started at 16 days post challenge (dpc) and at day 36 dpc the experiment was terminated and remaining living fish were euthanized by an overdose of Tricaine. Throughout the experimental period there were no mortality in the fish group vaccinated with LiVac, in contrast to the PBS control group which reached 100 % mortality at day 22 (Tank 2) and day 23 (Tank 1) post challenge. The AJ5-1 + LiVac (8 ^{o}C) vaccinated group reached 93 % and 100 % mortality, whereas the AJ5-1 vaccinated group reached 88 % mortality in both tanks. The AJ5-1 + LiVac (IA) vaccinated group reached a mortality of 69 % and 71% in the tanks, respectively. The AJ5-1 + LiVac vaccinated group reached a mortality of 19 % and 7 %. Whereas the AJ5-1 + LiVac (SO) vaccinated group reached a mortality of 27 % and 33 %, respectively, which could indicate that the suboptimal group was not as suboptimal as intended. It was decided not to include this group in further analysis.

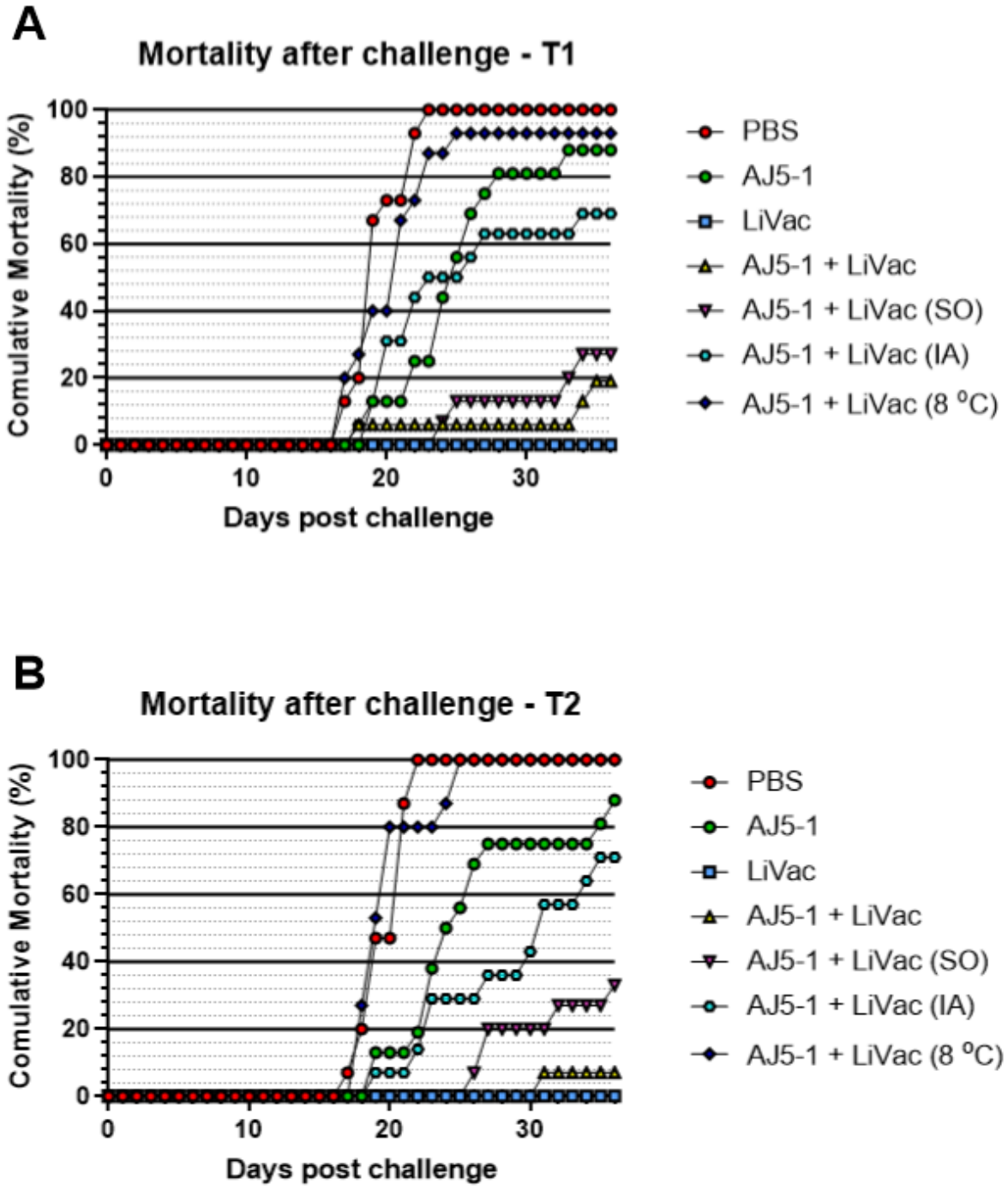


Figure 6 *– Cumulative mortality rate in percentage of vaccinated fish challenge with* P. salmonis *(n=15 / vaccine group/ tank) in duplicate tanks, A = Tank 1 (T1) and B = Tank 2 (T2). The vaccine groups and PBS control are divided according to colour and symbols. The PBS control group (Red circles), AJ5-1 group (green circles), LiVac group (blue squares), AJ5-1 + LiVac group (yellow triangle), AJ5-1 + LiVac (SO) group (Pink downward facing triangle), AJ5-1 + LiVac (IA) group (turquoise pentagon) and the AJ5-1 + LiVac (8°C) group (dark blue diamond).*

The relative percent survival value (RPS-end) was calculated at termination of the experiment, 36 dpc, for all vaccine groups and PBS control groups from both tanks (Table 10).

Table 10 *– An overview of the relative percent survival value (RPS-end) calculated at termination 36 days – post challenge for the vaccinated groups and control groups from tank 1 and tank 2.*

	Termination						
Tank	**PBS**	**AJ5-1**	**LiVac**	**AJ5-1 + LiVac**	**AJ5-1 + LiVac (SO)**	**AJ5-1 + LiVac (IA)**	**AJ5-1 + LiVac (8 ºC)**
1	0 %	12 %	100 %	81 %	73 %	31 %	7 %
2	0 %	12 %	100 %	93 %	67 %	29 %	0 %

For the PBS control group, the vaccinated AJ5-1 group and vaccinated LiVac group, the duplicate tanks provide good parallels the groups achieve the same RPS-value compared in both tanks. The RPS- value vary with 12 % between tanks for the vaccinated AJ5-1 + LiVac group, while the variation is only 3 % for the vaccinated AJ5-1 + LiVac (IA) group. For the vaccinated AJ5-1 + LiVac group and the AJ5-1 + Livac (8 ºC) group the RPS-value vary with 6 % and 7 %, respectively, between tanks.

Statistical analysis of differences in survival after experimental challenge of vaccinated fish and PBS controls were calculated at, cumulative mortality 60 % in the PBS groups and at termination of the experiment. The PBS control group were compared to the vaccinated groups (Table 11) and vaccine groups were compared (Table 12 - Statistical analyses of survival after experimental challenge of vaccinated fish, where only significant p-values are included in this table).

There is significantly higher mortality in the PBS control group compared to the vaccinated groups at 60 % mortality, except when compared to the AJ5-1 + LiVac (8 ºC) group (Table 11). This applies to both tanks. At termination, 36 dpc, the PBS control group has significantly higher moralities compared to the vaccinated groups LiVac, AJ5-1 + LiVac and AJ5-1 + LiVac (SO) in tank 1 and when compared to LiVac, AJ5-1 + LiVac, AJ5-1 + LiVac (SO) and AJ5-1 +

LiVac (IA) group in tank 2. The greatest significance level for both tanks and both timepoints is obtained between the PBS control group and the LiVac group (Table 11).

Table 11 *- Statistical analyses of survival after experimental challenge of vaccinated fish. Statistically significant differences in cumulative mortality (%) when comparing the PBS control group and the different vaccine groups calculated at the time of 60 % mortality in the PBS control group and at termination of the experiment. C.m* is the cumulative mortality in percentage (%) for the PBS control group, while C.m.** is the cumulative mortality in percentage (%) for the vaccine groups.* x^2 *is Yates´ continuity corrections in Pearson´s Chi-Squared test. Differences in cumulative mortality (%) was significant if p-values < significance level,* $\alpha < 0.05$*. All significant p-values are in bold. (NaN = Not applicable number, NA= not applicable, T = tank).*

		60 % mortality				**Termination**			
T	PBS control vs. Vaccine group	C.m.*	C.m.**	X^2	p-value	C.m.*	C.m.**	X^2	p-value
1	AJ5	87	13	6.81	**0.0091**	100	88	0	1
	LiVac	87	0	12.15	**0.0005**	100	0	26.13	**3.2×10^{-7}**
	AJ5+LiVac	87	6	9.19	**0.0024**	100	19	16.09	**4.1×10^{-5}**
	AJ5+LiVac (SO)	87	0	12.15	**0.0005**	100	27	14.35	**0.0002**
	AJ5+LiVac (IA)	87	13	6.81	**0.0091**	100	69	2.60	0.1071
	AJ51+LiVac (8°C)	87	40	1.21	0.2723	100	93	0	1
2	AJ5	67	13	13.33	**0.0003**	100	88	0	1
	LiVac	67	0	19.55	**9.8×10^{-6}**	100	0	26.13	**3.2×10^{-7}**
	AJ5+LiVac	67	0	19.55	**9.8×10^{-6}**	100	7	22.63	**2.0×10^{-6}**
	AJ5+LiVac (SO)	67	0	19.55	**9.8×10^{-6}**	100	33	12.15	**0.0005**
	AJ5+LiVac (IA)	67	7	16.21	**5.7×10^{-5}**	100	71	3.84	**0.0500**
	AJ51+LiVac (8°C)	67	80	0	1	100	100	NaN	NA

Statistical significance in difference in mortality across vaccine groups are best observed at termination of the experiment, while at 60 % mortality in the PBS control group only a few of the vaccine groups have statistically significant difference in mortality (Table 12 - Statistical analyses of survival after experimental challenge of vaccinated fish, where only significant p-values are included in this table). At 60 % mortality it is also clear that the differences are higher in tank 2 compared to tank 1. The greatest level of significance is at termination is between the LiVac group and AJ5-1 + LiVac (8 ºC) in tank 2.

Table 12 *- Statistical analyses of survival after experimental challenge of vaccinated fish, where only significant p-values are included in this table, the remaining values are presented in Appendix B (Table 18). Statistical differences in cumulative mortality (%) when comparing a vaccinated group against another vaccinated group is calculated at the time of 60 % mortality in the PBS control group and at termination of the experiment. C.m* is the cumulative mortality in percentage (%) for the vaccinated control group, while C.m.** is the cumulative mortality in percentage (%) for the other vaccine group (*/**).* x^2 *is Yates´ continuity corrections in Pearson´s Chi-Squared test. Differences in cumulative mortality (%) was significant if p-values < significance level,* $\alpha < 0.05$*. All significant p-values are in bold. (T = Tank).*

		60% mortality				**Termination**			
T	Vaccine group vs. Vaccine group	C.m.*	C.m.**	X^2	p-value	C.m.*	C.m.**	X^2	p-value
1	AJ5-1 vs LiVac	-	-	-	-	88	0	22.6	**$2x10^{-6}$**
	AJ5-1 vs AJ5-1+LiVac	-	-	-	-	88	19	13.6	**0.0002**
	AJ5-1 vs AJ5-1 +LiVac (SO)	-	-	-	-	88	27	11.3	**0.0008**
	LiVac vs AJ5-1+LiVac (IA)	-	-	-	-	0	69	14.4	**0.0002**
	LiVac vs AJ5-1+LiVac (8°C)	0	40	5.2	**0.02**	0	93	22.6	**$2x10^{-6}$**
	AJ51+LiVac vs AJ51 +LiVac (IA)	-	-	-	-	19	69	6.6	**0.0104**
	AJ51+LiVac vs AJ51+LiVac(8°C)	-	-	-	-	19	93	13.6	**0.0002**
	AJ51+LiVac (IA) vs AJ51+LiVac (SO)	-	-	-	-	69	27	4.80	**0.0285**
	AJ5-1+LiVac (SO) vs AJ51+LiVac (8 °C)	0	40	5.2	**0.0225**	27	93	11.3	**0.0008**
2	AJ5-1 vs LiVac	-	-	-	-	88	0	22.6	**$2x10^{-6}$**
	AJ5-1 vs AJ5-1+LiVac	-	-	-	-	88	7	19.2	**$1.8x10^{-5}$**
	AJ5-1 vs AJ5-1 +LiVac (SO)	-	-	-	-	88	33	9.2	**0.0024**
	AJ5-1 vs AJ5-1 +LiVac (8°C)	13	80	10.9	**0.0010**	-	-	-	-
	LiVac vs AJ5-1+LiVac (IA)	-	-	-	-	0	71	12.2	**0.0005**
	LiVac vs AJ5-1+LiVac (SO)	-	-	-	-	0	33	3.8	**0.0500**
	LiVac vs AJ5-1+LiVac (8°C)	0	80	16.8	**$4.1x10^{-5}$**	0	100	26.1	**$3.2x10^{-7}$**
	AJ5-1+LiVac vs AJ5-1 +LiVac (IA)	-	-	-	-	7	71	9.2	**0.0024**
	AJ5-1+LiVac vs AJ5-1+LiVac (8°C)	0	80	16.8	**$4.1x10^{-5}$**	7	100	22.6	**$2x10^{-6}$**
	AJ5-1+LiVac (IA) vs AJ5-1+LiVac (8°C)	7	80	13.6	**0.0002**	71	100	3.8	**0.0500**
	AJ5-1+LiVac (SO) vs AJ5-1+LiVac (8°C)	0	80	16.8	**$4.1x10^{-5}$**	33	100	12.2	**0.0005**

RT-qPCR analysis of up to 33 % of fish from each vaccine group/tank were randomly sampled over time to confirm the presence of *P. salmonis* in dead fish, results are presented in Appendix B (Table 17).

3.2 Gross pathology of fish diseased with *P. salmonis*

Gross pathology was observed by necroscopy of fish after challenge with *P. salmonis*. Multiple signs of sepsis were observed, such as bleedings on gill opercula (Figure 7A), abdomen (Figure 7C), on dorsal – and anal fins (Figure 7C), additionally exophthalmia (Figure 7C) and eye haemorrhage were observed from the freshly dead fish (Figure 7B). White nodules were observed in the liver, in addition to bleedings in the abdominal wall which is most likely from the spleen (Figure 8).

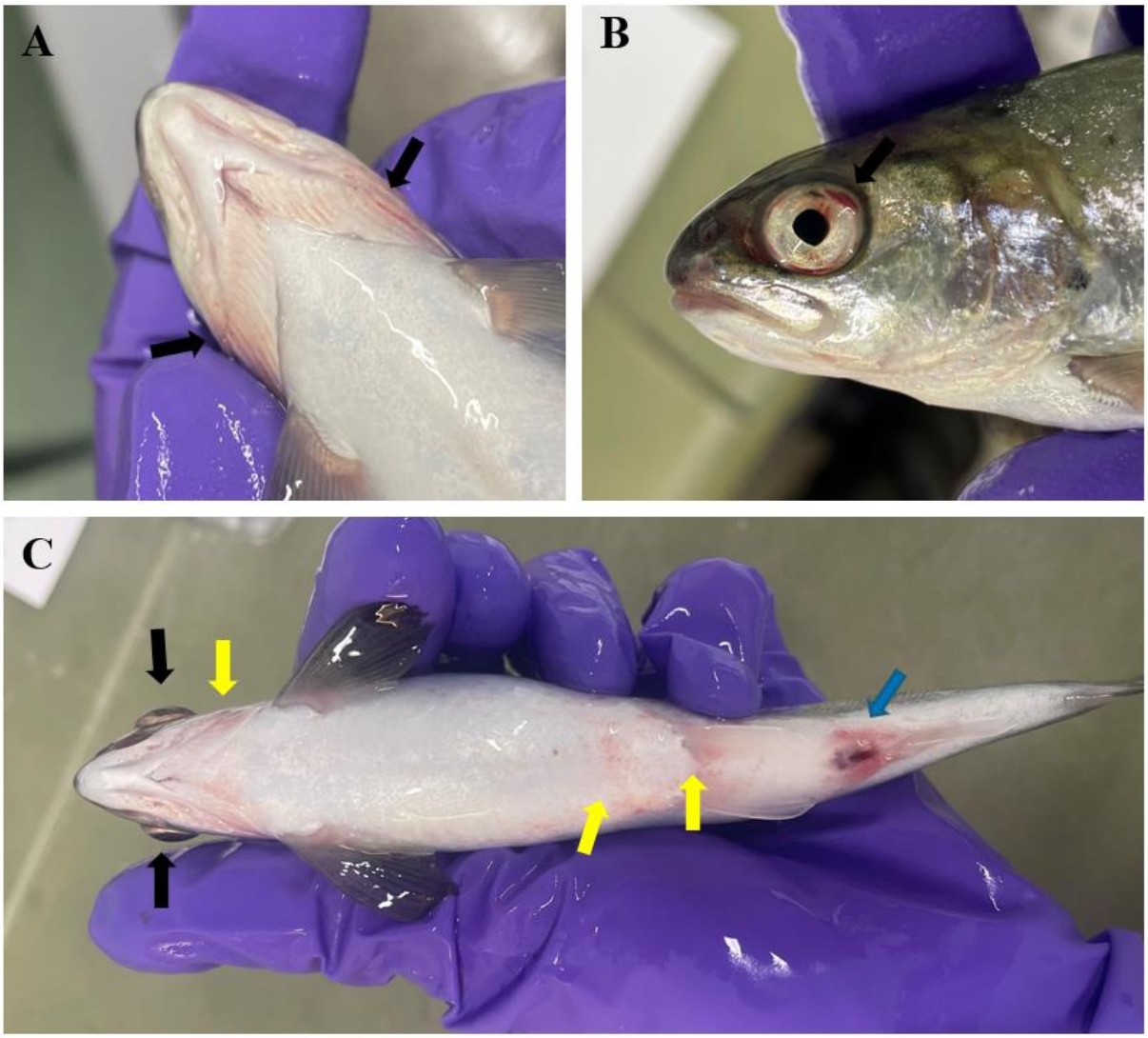


Figure 7 *– External examination of fresh dead fish diseased with* P. salmonis. *(A) displays petechial bleeding on both gill opercula (black arrows) and head region. (B) displays haemorrhage on the eye (black arrow). (C) displays exophthalmic on both eyes (black arrows), petechial bleeding on gill opercula, abdomen, and pectoral fins (yellow arrows) and skin haemorrhage around the anal fins (blue arrow).*

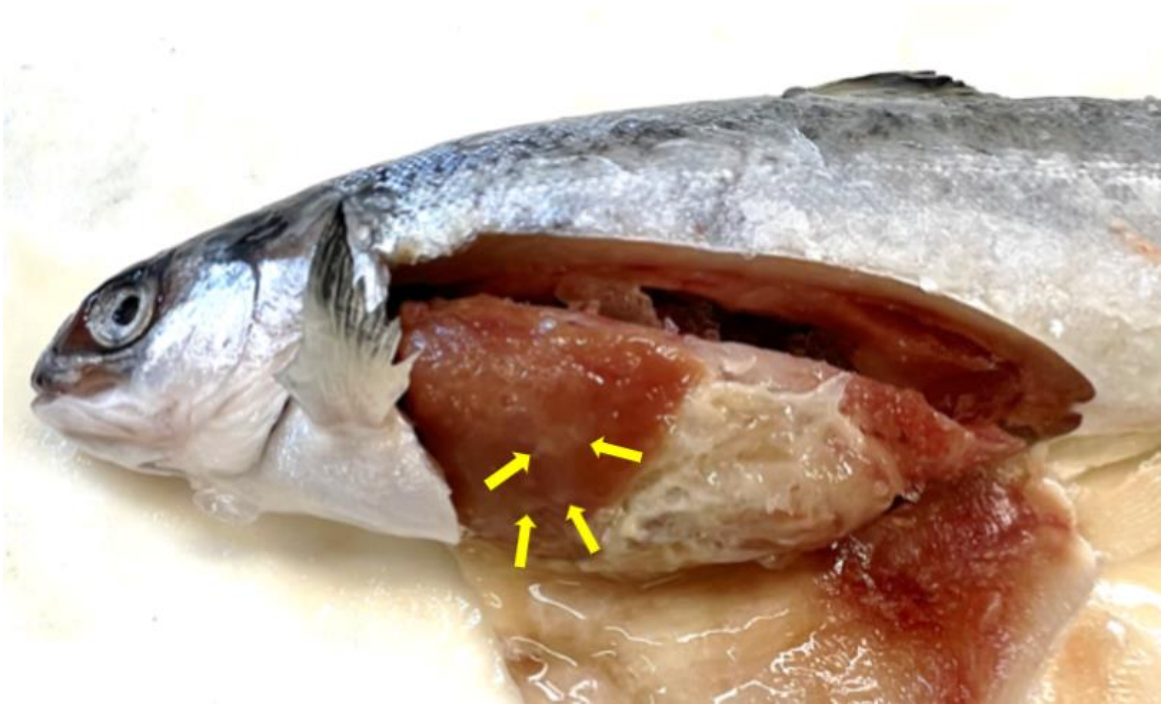


Figure 8 *–Internal examination of frozen and then thawed dead fish, diseased with* P. salmonis*. This fish was vaccinated with AJ5-1 + LiVac (IA) and died late in the experiment. White nodules were observed in the liver (yellow arrows) and bleeding on the abdominal wall, which likely is from the spleen when death occurred.*

3.3 ELISA

3.3.1 Ag concentration and plasma dilution

The results from optimizing Ag concentrations for coating of ELISA plates (5 µg/mL, 10 µg/mL, and 15 µg/mL) are presented as a floating box plot (Figure 9). Coating with Ag concentration of 5 µg/mL provided the lowest measured absorbance when measuring plasma in vaccinated fish while for the PBS control group absorbance was lowest when coating Ag was 10 µg/mL. When coating using 15 µg/mL Ag there was no increase in absorbance. Combined the results indicate lower grade of coating when using 5 µg/mL Ag, and loss of coating Ag (Ag clumping) in the first wash procedure before the blocking step when coating using 15 µg/mL Ag. Based on the results 10 µg/mL of Ag were chosen for coating the plates for analysis of plasma samples.

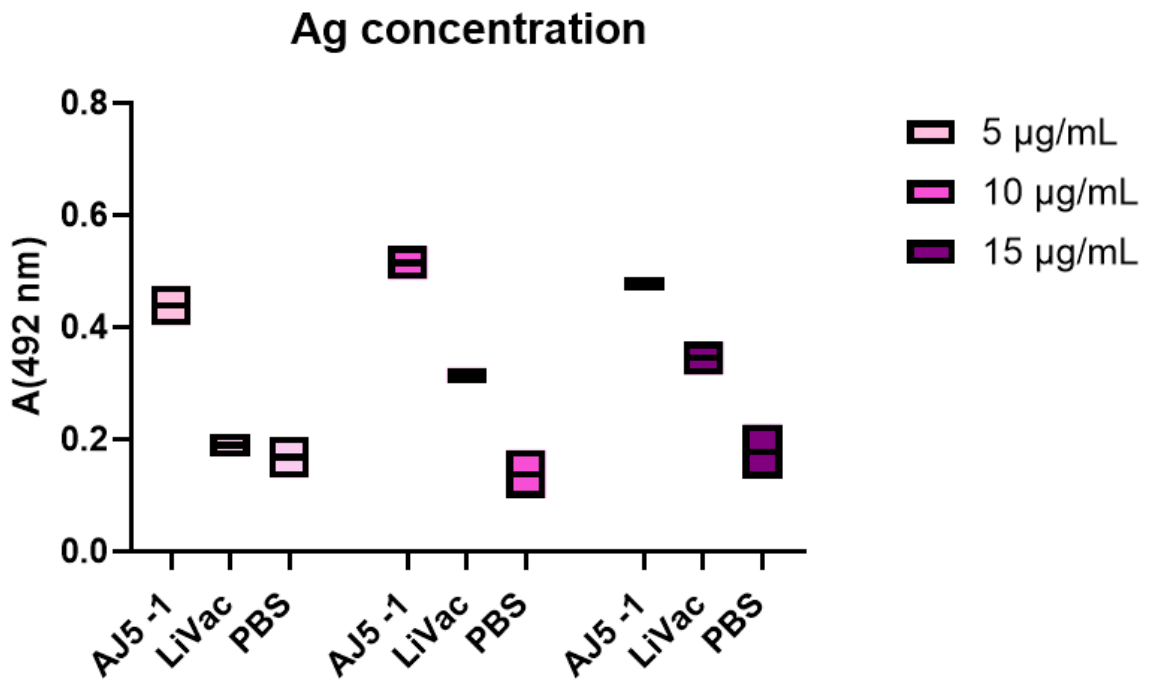


Figure 9 *– Floating box plot shows results from testing Ag concentration for coating of ELISA plates 5 µg/mL, 10 µg/mL and 15 µg/mL. Plasma collected at 48 dpv from two vaccinated groups AJ5-1 and LiVac, and a PBS control group was used. Ag concentration of 5 µg/mL (light pink boxes), 10 µg/mL (neon pink boxes) and 15 µg/mL (dark purple boxes).*

Two-fold dilution series of plasma measured by ELISA, same samples as used for the Ag concentration test, were used to compile dilution curves (Figure 10). Plasma from fish in the vaccinated AJ5-1 group (green curves) has the highest initial absorbance indicating highest antibody titres. This group also have the overall highest absorbance. Absorbance measured using plasma from the vaccinated LiVac group (blue curves) mainly lies just below the AJ5-1 group. Plasma from the PBS control group (red curves) has a low absorbance and less difference between individual fish. Most of the curves from both vaccinated groups, starts declining at a 1:100 dilution of plasma. The 1:100 dilution also had good separation of the measurements from vaccinate fish compared to PBS controls, this dilution was therefore selected for further ELISA analysis.

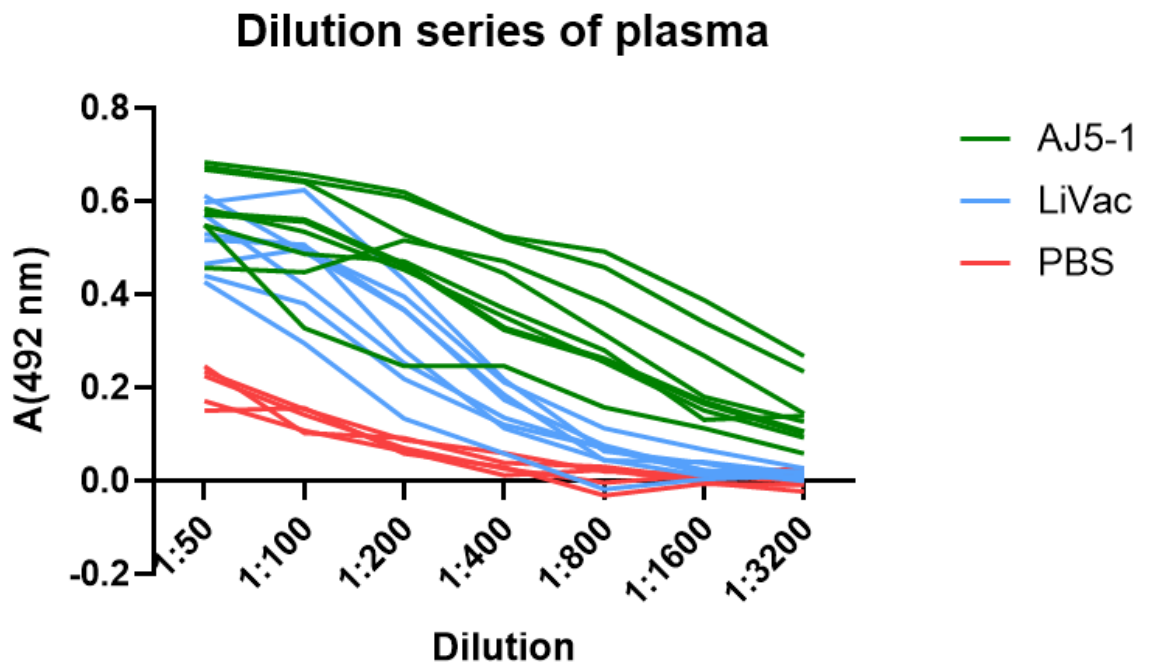


Figure 10 *– Dilution series of plasma collected 48 dpv (one curve/fish) from two vaccinated groups AJ 5-1 (green curves) and LiVac (blue curves), as well as a PBS control group (red curves) was analysed by ELISA, coated with* P. salmonis.

3.3.2 Antibody levels

Figure 11 and Figure 12 illustrates the absorbance measurements of antibodies in plasma from all vaccinated groups and the PBS control group from sampling, 14 -, 24 – and 48 dpv, when analysed using coating Ag concentration of 10 µg/mL and plasma dilution of 1:100.

There is a clear specific antibody response in all vaccinated groups, and the levels of antibodies increases by each sampling over increasing day-degrees(dd) (Figure 11 and Figure 12). The vaccinated groups AJ5-1 and AJ5-1 + LiVac both have significantly higher antibody levels compared to the PBS control group for all samplings. This also include comparing the vaccinated group with the baseline group (Figure 11, A and C). Furthermore, the vaccinated groups LiVac and AJ5-1 + LiVac (IA) both have significantly higher antibody levels compared to the PBS control group at sampling timepoint 24 – and 48 dpv. Compared to the baseline group there are higher antibody levels in these vaccinated groups at all sampling timepoint (Figure 11, B and D). The vaccinated group AJ5-1 + LiVac (8 ºC) have significantly higher antibody levels compared to the PBS control at sampling timepoint 24 - and 48 dpv (Figure 11E).

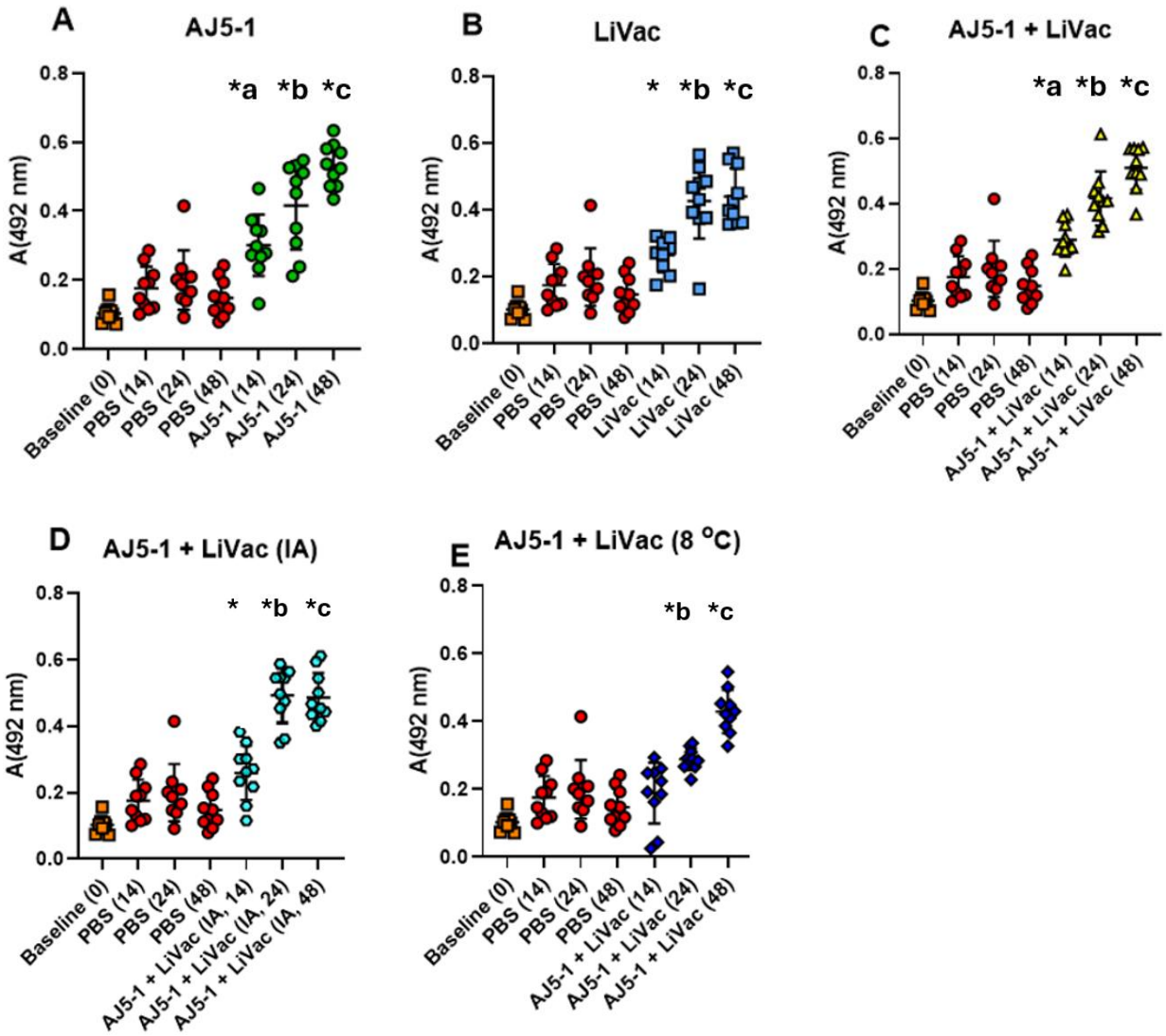


Figure 11 *– Scatter plots showing antibody levels in plasma collected before vaccination (baseline), 14-, 24 and 48 dpv of vaccinated groups and PBS control groups, analysed by ELISA. Scatter plots (Figure 11A-E) are divided according to vaccine group; however, baseline (orange squares) and PBS groups(red circles) are included in all plots as controls The vaccine groups are marked with the following colours and symbols: AJ5-1 group (green circles), LiVac group (blue squares), AJ5-1 + LiVac group (yellow triangle), AJ5-1 + LiVac (IA) group (turquoise pentagon), and the AJ5-1 + LiVac (8 °C) group (dark blue diamond). (*=p<0.05 between vaccinated group and baseline, a =p<0.05 between vaccinated group and PBS control (14 dpv) b =p<0.05 between vaccinated group and PBS control (24 dpv), * =p<0.05 between vaccinated group and PBS control (48 dpv)). The P-value are summarised in Appendix B (Table 19).*

The vaccinated groups are illustrated with regards to sampling timepoint (Figure 12). There is a clear increase in antibody levels in vaccinated groups over time, and there are significant differences from the PBS control groups. At sampling timepoint 14 dpv (Figure 12A), all the vaccinated groups have a significantly higher antibody response compared to the AJ5-1 + LiVac (8 °C) giving an adjusting p-value ($p<0.0001$). At sampling timepoint 24 dpv (Figure 12B), all vaccinated groups are significantly higher to the AJ5-1 + LiVac (8 °C). The adjusted p-value for each comparison is AJ5-1 ($p<0.0119$), LiVac ($p<0.0056$), AJ5-1 + LiVac ($p<0.0122$) and AJ5-1 + LiVac (IA) ($p<0.0001$). At sampling timepoint 48 dpv (Figure 12C), the vaccinated AJ5-1 group have significantly higher antibody levels than the LiVac group ($p<0.476$), in addition antibody levels in the AJ5-1 group are also significantly higher to the AJ5-1 + LiVac (8 °C) ($p<0.017$). The levels of significance and the P-values are summarised in Appendix B (Table 19 and Table 20).

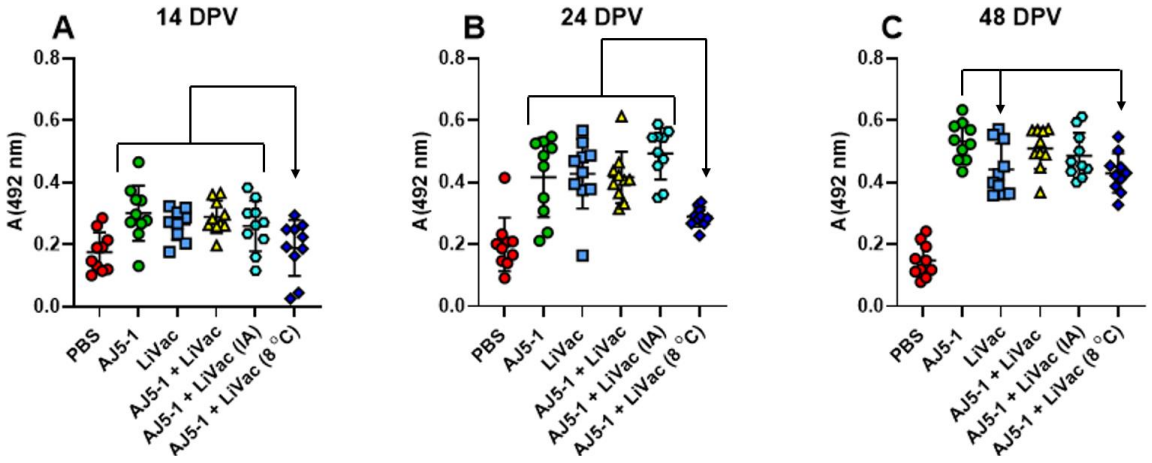
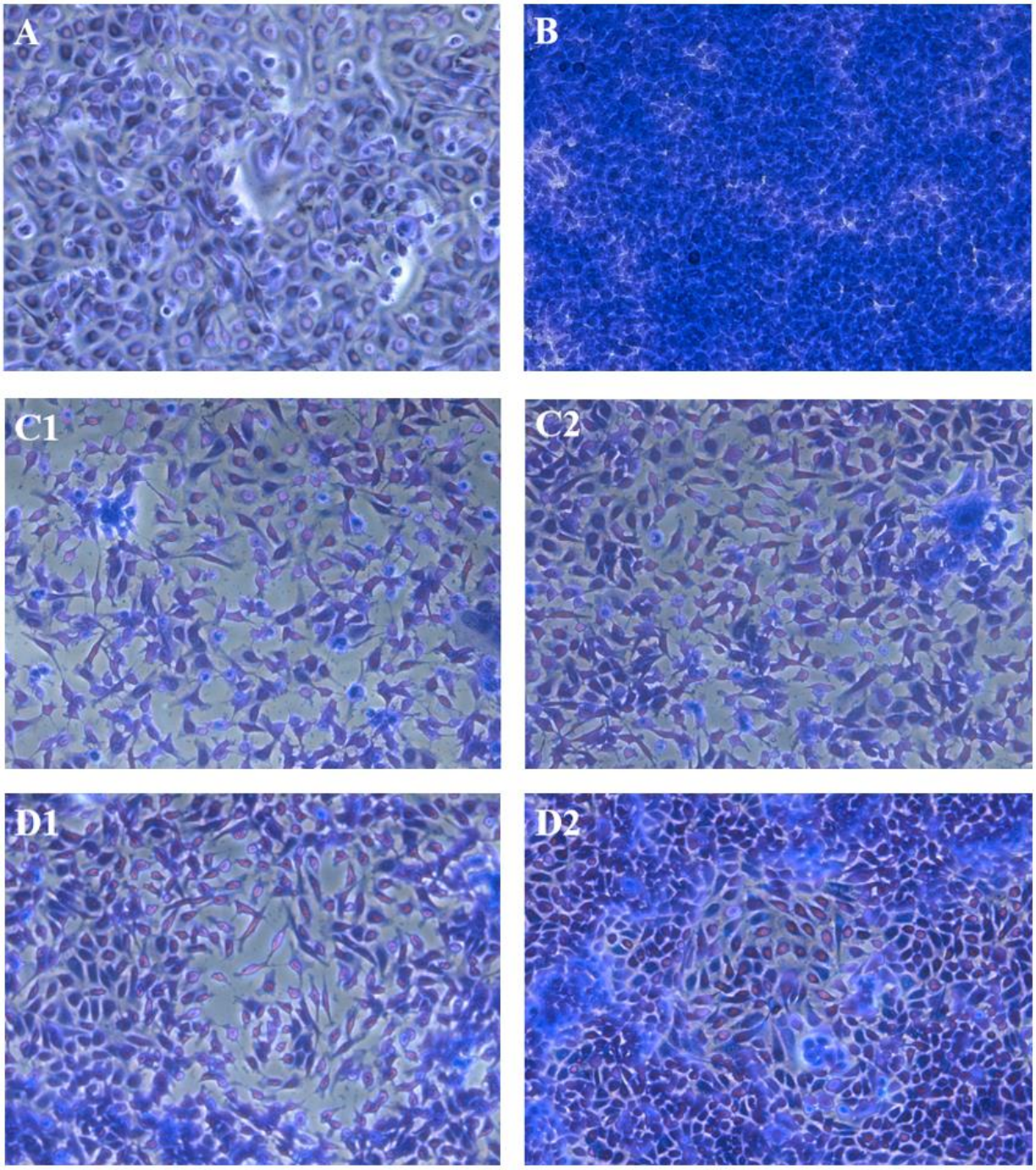


Figure 12 *- Scatter plots showing antibody levels in plasma of vaccine groups and PBS control, analysed by ELISA. A = 14 dpv, B = 24 dpv and C = 48 dpv. The PBS control group (red circles), AJ5-1 group (green circles), LiVac group (blue squares), AJ5-1 + LiVac group (yellow triangle), AJ5-1 + LiVac (IA) group (turquoise pentagon), and the AJ5-1 + LiVac (8 °C) group (dark blue diamond). Statistically significance is illustrated by clamps marking the groups which have statistically significance higher antibody levels to the group pointed at with arrows (Significant in difference to the PBS control group are not included in this figure). The P-value are summarised in Appendix B (Table 20).*

3.4 Analysis of presence of *P. salmonis* neutralizing antibodies

Plaques were observed in all wells where the cells had been exposed to *P. salmonis* combined with plasma from vaccinated fish, irrespective of vaccine groups. In general, the plaques were more frequently observed in the wells containing the lowest concentration of plasma. Plaques were observed as distinct craters in the cell monolayers with cell debris inside. A clear difference was observed comparing the positive control, CHSE-cells exposed to *P. salmonis* without supplement of plasma (Figure 13A), and the negative control, CHSE-cells non-exposed cells (Figure 13B). Cells exposed to *P. salmonis* in general had a lighter colour after staining and open space between cells were frequently observed. The cells in the negative control, were darker in colour after staining and contained thick confluent monolayers of cells. Monolayers of cells exposed to plasma from fish injected with PBS combined with *P. salmonis* (Figure 13C) displayed more frequent plaques compared to monolayers of CHSE-cells exposed to *P. salmonis* alone.


A
B
C1
C2
D1
D2

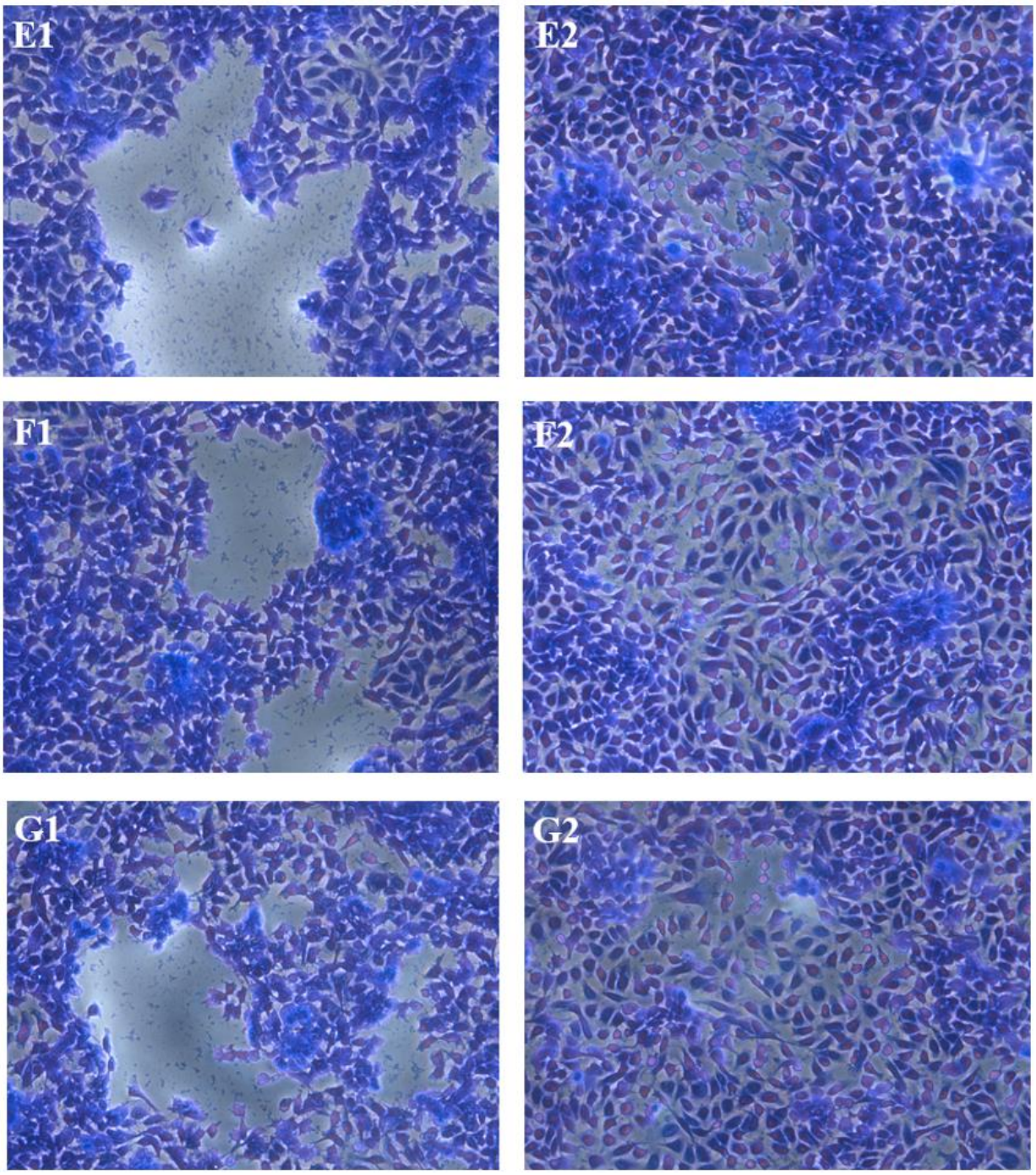


Figure 13 *– The findings of plaques formed in monolayers of CHSE-cells after incubation with* P. salmonis *(A) as positive control, CHSE-cells alone (B) as negative control and* P. salmonis *mixed with plasma from fish injected with PBS (C). Furthermore,* P. salmonis *mixed with plasma from the vaccinated groups, the AJ5-1 vaccine (D), the LiVac vaccine (E), the vaccine combination AJ5-1 + LiVac (F) and the vaccine combination AJ5-1 + LiVac (IA) (G). All vaccinated groups and PBS control group are marked with number 1 and 2, 2 indicating the highest and 1 lowest concentration of* plasma*, respectively, mixed with* P. salmonis*. All pictures were obtained using a magnification of 20x.*

3.4 Test of isolating leucocytes from liver

The CASY cell counter revealed a good yield of cells after sampling from liver both at day one and day two (Table 13). The aggregation factor, indicating on average how many cells are clumped together, was low on day two compared to day one. For three out of four samples the cell yields increased after incubating the liver overnight at 4 °C. Although when inspecting the cytospin preparations of the isolated cells in a microscope (Figure 14), it was revealed that the high number of cells at day two was mostly not leucocytes. The leucocyte yield on day two was thus lower compared to the leucocyte yield on day one when using freshly sampled liver.

Table 13 *– An overview of the results from isolating leucocytes from liver (L) from four unvaccinated fish (L1-L4) at day 1 and day 2. Showing the aggregation factor, viable cells/mL, and the cell viability in percentage.*

Day 1	Agg. Factor	Viable Cells/mL	Viability (%)	Day 2	Agg. Factor	Viable Cells/mL	Viability (%)
L1	4.2	$2.0x10^6$	83.4	**L1**	1.8	$2.0x10^6$	87.8
L2	3.8	$1.1x10^6$	81.5	**L2**	1.4	$2.9x10^6$	89.1
L3	4.8	$8.3x10^5$	82.7	**L3**	1.7	$1.2x10^6$	85.2
L4	1.8	$5.1x10^5$	65.0	**L4**	1.2	$3.0x10^6$	92.1

The leucocytes can be observed with a dark purple colour (Figure 14 A and B), while other cells from the liver can be observed having a light pink colour (Figure 14 C and D).

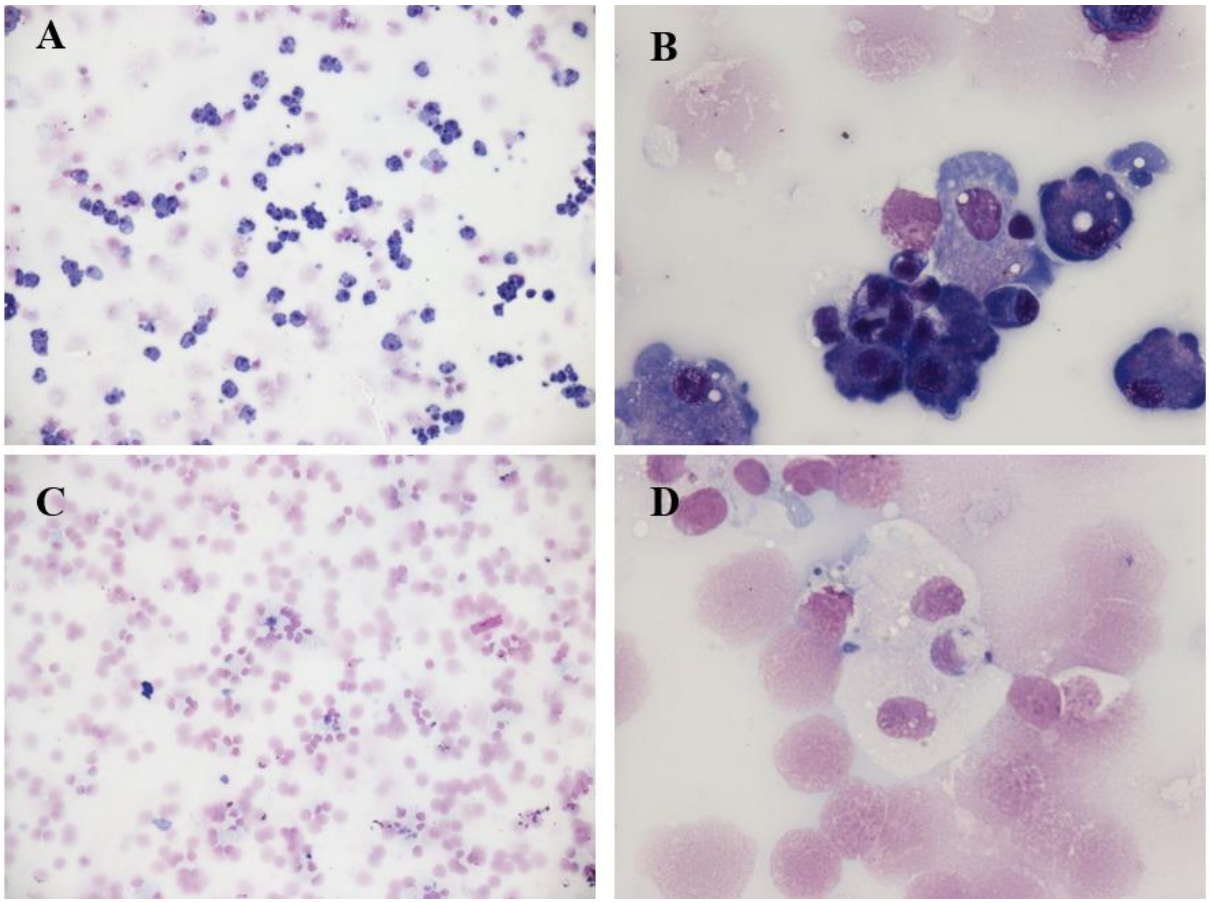


Figure 14 *– Pictures of cytospin preparations of isolated cells from liver at day 1 and day 2. (A and B) show leucocytes isolated from liver at day 1, using magnification 20x and 63x respectively. (C and D) show cells isolated from liver at day 2, when liver had been stored overnight at -4 °C, using magnification 20x and 63x respectively.*

3.5 RT-qPCR detection of *P. salmonis* post vaccination

The RT-qPCR for detection of *P. salmonis* pv, in vaccine groups that included the attenuated vaccine strain, was performed by PHARMAQ Analytic and covered by PHARMAQ AS. Baseline samples collected from HK and liver prior to vaccination were all negative for *P. salmonis* and are presented in Appendix B (Table 21). At 1 dpv *P. salmonis* was not detected in any samples from any of the vaccine groups. In the LiVac vaccinated fish *P. salmonis* was detected in 64 % of the samples at 7 dpv and in 44 % at 14 dpv, indicating a decrease over time. In the AJ5-1 + LiVac vaccinated fish, *P. salmonis* was detected in 50 % of the samples at 7 dpv and in 60 % at 14 dpv, indicating an increase over time. In the AJ5-1 + LiVac (SO) vaccinated fish *P. salmonis* was detected in 40 % of the samples at 7 dpv and in 45 % at 14 dpv, indicating

a slight increase over time. Average in numbers of positive liver samples and Cq-values from samples where *P. salmonis* were detected are presented in Table 14 and Table 15.

Table 14 *– An overview of RT-qPCR results of liver samples collected 7 days post vaccination and analysed for* P. salmonis*. The results are normalized relative to the Cq-value and the housekeeping gene.*

Vaccine group	Fish nr.	Days post vaccination	Results	Cq-value	Positive Cq
LiVac	F1	7	Not detected	39.98	64%
	F2		Detected	33.80	
	F3		Detected	35.81	
	F4		Detected	31.77	
	F5		Detected	36.12	
	F6		Not detected	37.20	
	F7		Detected	34.44	
	F8		Detected	30.82	
	F9		Not detected	-	
	F10		Detected	30.13	
	F11		Not detected	36.29	
AJ5-1 + LiVac	F1	7	Not detected	38.58	50%
	F2		Detected	32.93	
	F3		Not detected	39.18	
	F4		Not detected		
	F5		Detected	35.32	
	F6		Detected	30.50	
	F7		Not detected	-	
	F8		Detected	35.82	
	F9		Detected	32.93	
	F10		Not detected	37.62	
AJ5-1 + LiVac (SO)	F1	7	Not detected	-	40%
	F2		Not detected	-	
	F3		Not detected	-	
	F4		Detected	34.85	
	F5		Not detected	39.17	
	F6		Detected	36.13	
	F7		Not detected	-	
	F8		Detected	33.61	
	F9		Not detected	39.69	
	F10		Detected	32.79	

Table 15 *– An overview of RT-qPCR results of liver samples collected 14 days post vaccination and analysed for* P. salmonis*. The results are normalized relative to the Cq-value and the housekeeping gene.*

Vaccine group	Fish nr.	Days post vaccination	Results	Cq-value	Positive Cq
LiVac	F1	14	Not detected	37.51	44%
	F2		Not detected	-	
	F3		Not detected	-	
	F4		Detected	31.93	
	F5		Not detected	-	
	F6		Detected	34.20	
	F7		Detected	34.32	
	F8		Detected	35.65	
	F9		Not detected	-	
AJ5-1 + LiVac	F1	14	Not detected	-	60%
	F2		Detected	32.04	
	F3		Detected	35.10	
	F4		Detected	35.52	
	F5		Not detected	-	
	F6		Not detected	36.46	
	F7		Detected	31.40	
	F8		Not detected	37.06	
	F9		Detected	30.18	
	F10		Detected	34.69	
AJ5-1 + LiVac (SO)	F1	14	Detected	31.53	45%
	F2		Detected	32.29	
	F3		Not detected	-	
	F4		Not detected	-	
	F5		Not detected	-	
	F6		Not detected	-	
	F7		Detected	31.82	
	F8		Not detected	39.89	
	F9		Not detected	-	
	F10		Detected	33.46	
	F11		Detected	31.96	

3.6 RT-qPCR analysis

3.6.1 Test of assays

Agarose gel electrophoresis was performed on RT-qPCR products of the different assays to ensure that no genomic DNA were present in the samples (NRT) and no contamination of reagents (NTC) (Figure 15). Another agarose gel was performed on the same RT-qPCR products confirming all assays have the right base pairs size (Figure 16).

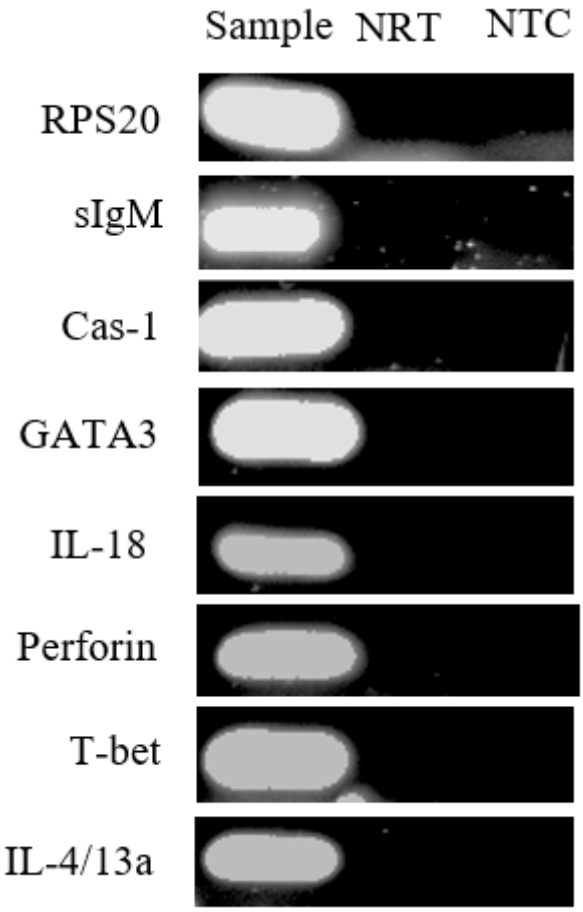


Figure 15 *– Agarose gel showing detected bonds in the samples. The Agarose gel shows negative NRT and NTC, indicating the samples were free off gDNA (NRT) and contamination of reagents (NTC).*

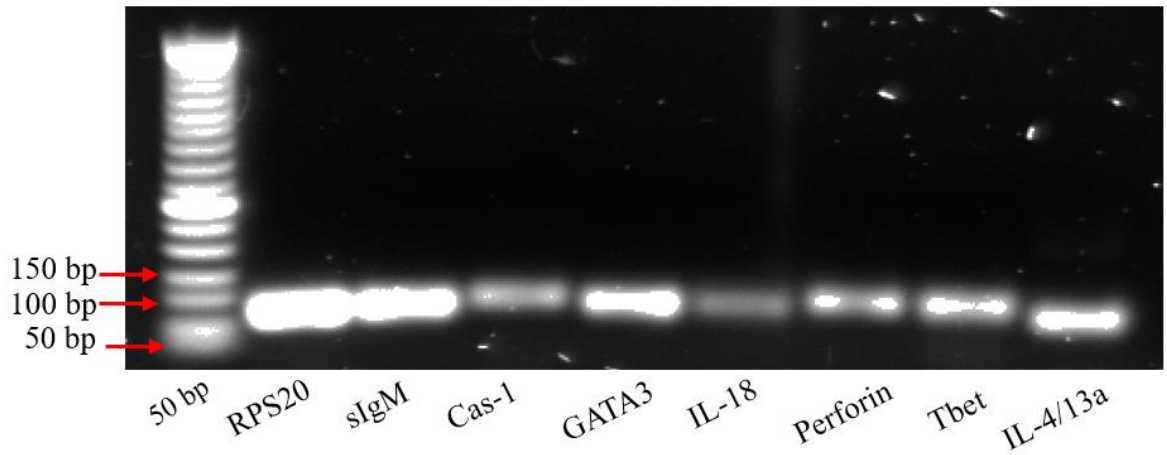
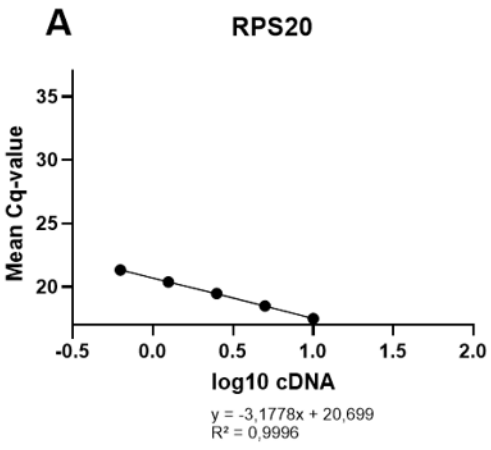
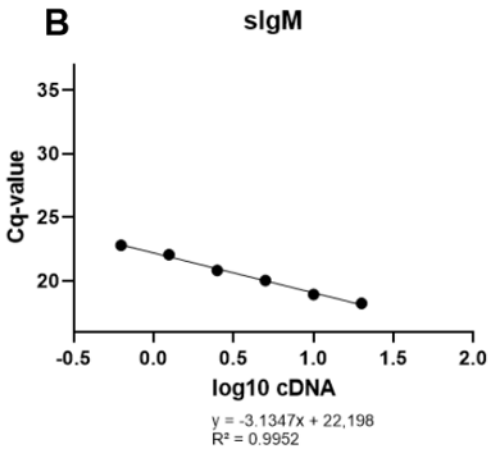
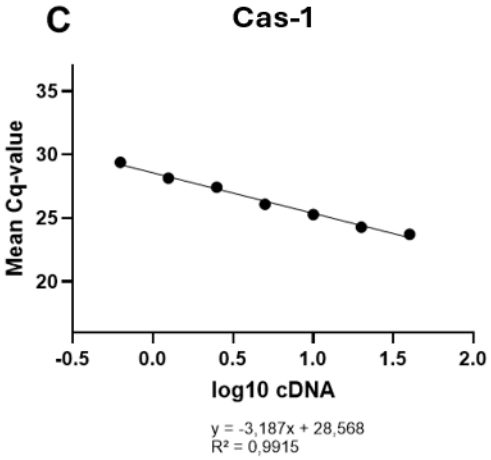


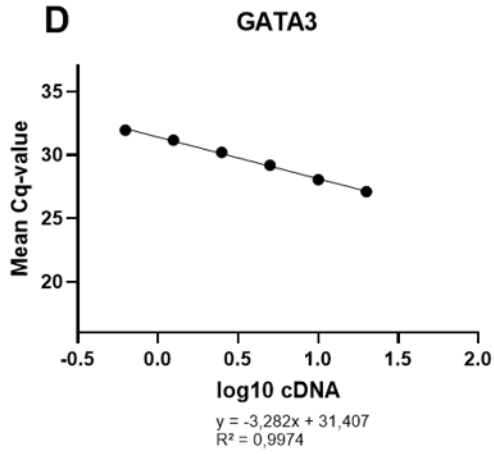
Figure 16 *– Agarose gel which shows the size of the RT-qPCR products, both bonds and right size for all products can be observed (see Table 7 for size for each assay).*

The Assays were tested by linear regression showing the mean Cq-value with log10 by amount of cDNA, slope and R-squared are also included (Figure 17). Standard curves were established to investigate the assays efficacy and specificity. Overall, the range show a decreasing Cq-value with increasing amount of template, indicating that the primers bind correctly and amplifies the genes. The housekeeping gene RPS20 show the lowest Cq-value of 22 by amount of template and decreasing (Figure 17A), hence the experimental assay targeted the IL-4/13a gene shows the highest Cq-value of 36 (Figure 17H).


A
RPS20
Mean Cq-value
35
30
25
20
-0.5
0.0
0.5
1.0
1.5
2.0
log10 cDNA
y = -3,1778x + 20,699
R² = 0,9996


B
sIgM
Cq-value
35
30
25
20
-0.5
0.0
0.5
1.0
1.5
2.0
log10 cDNA
y = -3.1347x + 22,198
R² = 0.9952


C
Cas-1
Mean Cq-value
35
30
25
20
-0.5
0.0
0.5
1.0
1.5
2.0
log10 cDNA
y = -3,187x + 28,568
R² = 0,9915


D
GATA3
Mean Cq-value
35
30
25
20
-0.5
0.0
0.5
1.0
1.5
2.0
log10 cDNA
y = -3,282x + 31,407
R² = 0,9974

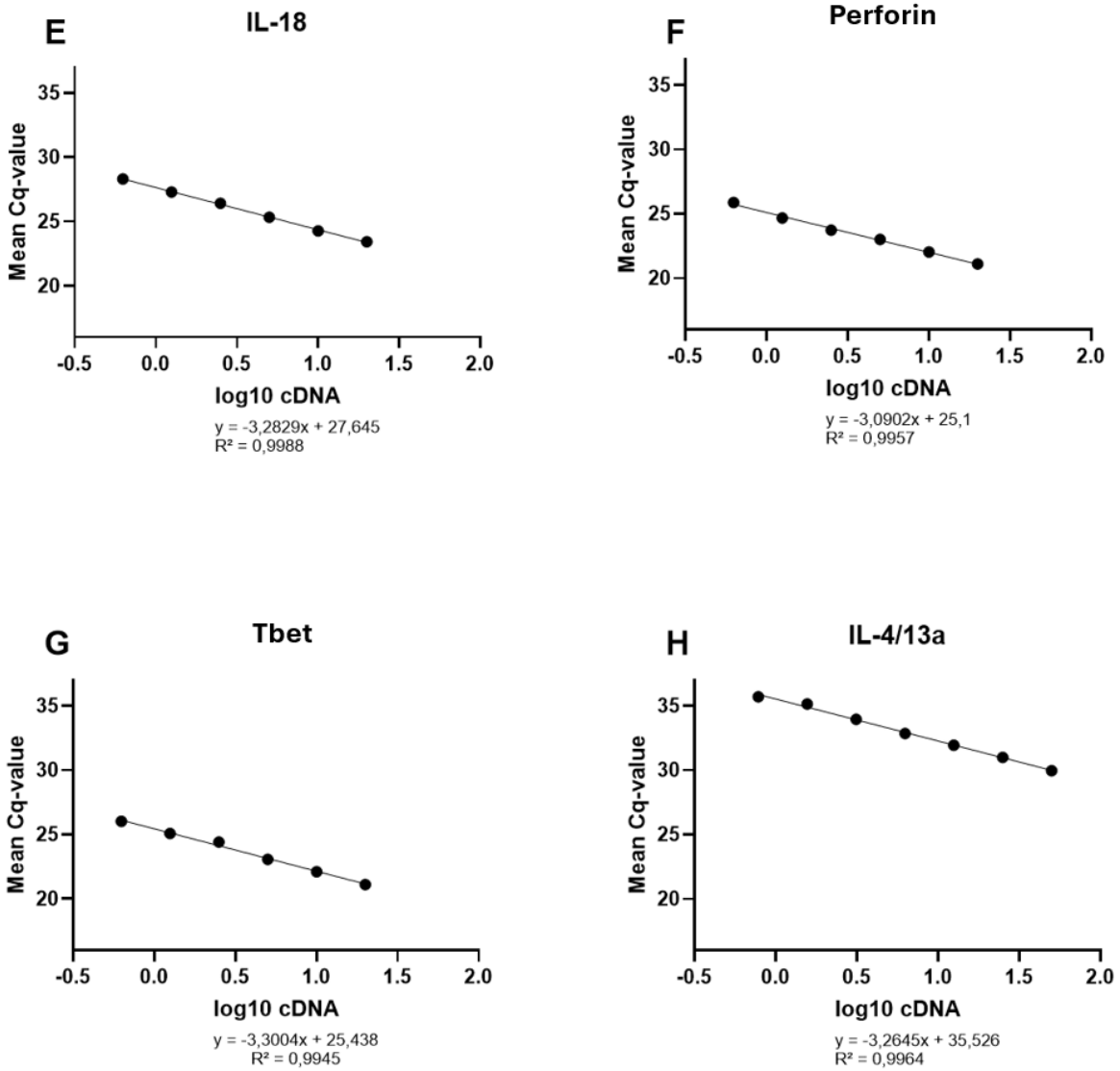


Figure 17 *– Linear regression of assays used for RT-qPCR, showing standard curves of Cq-values with log10 by amount of cDNA and are divided according to target gene. (A) reference assay RPS20, (B-H) show the experimental assays used to target (B) sIgM, (C) Cas-1, (D) GATA3, (E) IL-18, (F) Perforin, (G) Tbet and (H) IL-4/13a. also showing slope and R-squared for each assay.*

3.6.2 RT-qPCR of *in vitro* exposed leucocytes

Due to the large amounts of samples collected a preliminary test was first conducted to determine whether the samples harvested 6 hours post *in vitro* exposure or 24 hours post *in vitro* exposure should be prioritized for analysis. The test was conducted on leucocytes collected at 48 dpv after 6 and 24 h of *P. salmonis* exposure. For this test 24 samples were included, and they were analysed for the for the different target genes. Based on these results, it was decided to proceed with samples collected 6 hours post *in vitro* challenge.

RT-qPCR was performed on a total of 48 HK samples, in addition to NRT and NTC, from the vaccinated groups and control group. Due to the number of samples and the use of a pipette robot, the Cq- values obtained were organized in an excel sheet to be filtered. The 1.5 interquartile range (IQR) rule was used to filter outlying Cq-values between the triplicates for each sample. In addition, further filtration was performed by setting a strict limit of 0,5 value in difference between the triplicates for each sample.

Statistical analysis was conducted using a one-way ANOVA in Graphpad Prism and Sigma plot 15, however due to low number of replicates in sample size for some of the samples only a few statistical significances were detected. However, since ANOVA could not handle missing values to the lack of replicates, the data were analysed by fitting a mixed model. An overview of the adjusted P-value for each comparison between the different vaccinated groups for each target gene are presented in Appendix B (Table 23).

HKLs harvested from all vaccinated groups including the PBS control group had an upregulation for sIgM when exposed to *P. salmonis* (Figure 18A). The relative gene expressions for each group were quite similar, however, the LiVac group differs as it has the highest upregulation and the highest standard deviation.

There is almost no difference in regulation of Cas-1 post *P. salmonis* exposure between the vaccine group and when comparing the vaccine groups to the PBS control group (Figure 18B). Overall, there is a slight downregulation in expression for the vaccine group AJ5-1, as well as the PBS control group, the AJ5-1 + LiVac (IA) group show no regulation in gene expression compared to its respective unexposed group. A slight upregulation in HKLs harvested from AJ5-1 + LiVac and LiVac vaccinated fish, the latter has the highest upregulation in expression and the highest standard deviation together with the AJ5-1 + LiVac (IA) group.

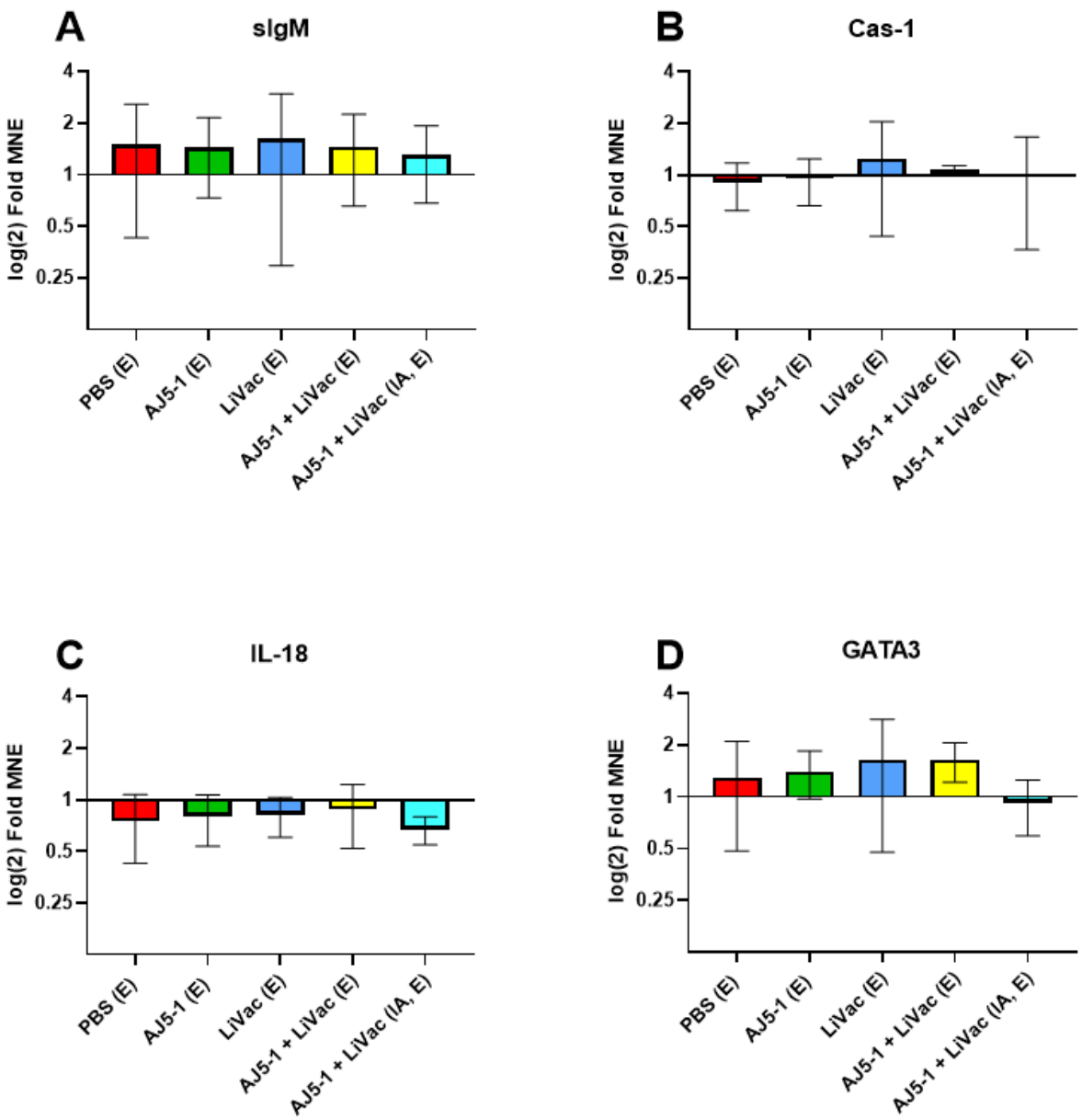
In all groups expression of IL-18 is down regulated when the leucocytes are exposed to *P. salmonis* (Figure 18D). The vaccinated group AJ5-1 + LiVac (IA) have the highest downregulation and the AJ5-1 + LiVac group have the lowest. The PBS control group have the highest standard deviation.

All vaccinated groups and the PBS control group have an upregulation in expression for GATA3 (indicating Th2 response) when exposed to *P. salmonis*, except for the HKLs harvested from the AJ5-1 + Livac (IA) vaccinated group where a slight downregulation in expression is measured (Figure 18C). The LiVac vaccinated group stands out with the highest upregulation.

The vaccinated groups AJ5-1 + LiVac and AJ5-1 + LiVac (IA) have a slight upregulation in expression for perforin gene (indicating activated Tc) when exposed to *P. salmonis*, this also applies to the PBS control group (Figure 18E). However, a downregulation in expression is measured for the vaccinated groups AJ5-1 and LiVac. The PBS control group have a high standard deviation.

All groups have an upregulation in expression of the Tbet gene (indicating Th1 response) when exposed to *P. salmonis,* whereas the PBS control group and the vaccinated groups AJ5-1 + LiVac and AJ5-1 + LiVac (IA) have a similar level of expression and standard deviation (Figure 18F). The vaccinated AJ5-1 group stand out with the highest gene expression for Tbet, the vaccinated LiVac group have the lowest gene expression.

All vaccinated groups and the PBS control group have a downregulation in expression for the IL-4/13a gene (indicating Th2 response) when exposed to *P. salmonis*, except for the AJ5-1 group which has an upregulation in expression compared to the PBS control and the other vaccine groups ($p<0.05$) (Figure 18G). The highest relative gene expression can be observed in the vaccinated AJ5-1 + Livac group, which also has the highest standard deviation.


A
sIgM
log(2) Fold MNE
4
2
1
0.5
0.25
PBS (E)
AJ5-1 (E)
LiVac (E)
AJ5-1 + LiVac (E)
AJ5-1 + LiVac (IA, E)
B
Cas-1
log(2) Fold MNE
4
2
1
0.5
0.25
PBS (E)
AJ5-1 (E)
LiVac (E)
AJ5-1 + LiVac (E)
AJ5-1 + LiVac (IA, E)
C
IL-18
log(2) Fold MNE
4
2
1
0.5
0.25
PBS (E)
AJ5-1 (E)
LiVac (E)
AJ5-1 + LiVac (E)
AJ5-1 + LiVac (IA, E)
D
GATA3
log(2) Fold MNE
4
2
1
0.5
0.25
PBS (E)
AJ5-1 (E)
LiVac (E)
AJ5-1 + LiVac (E)
AJ5-1 + LiVac (IA, E)

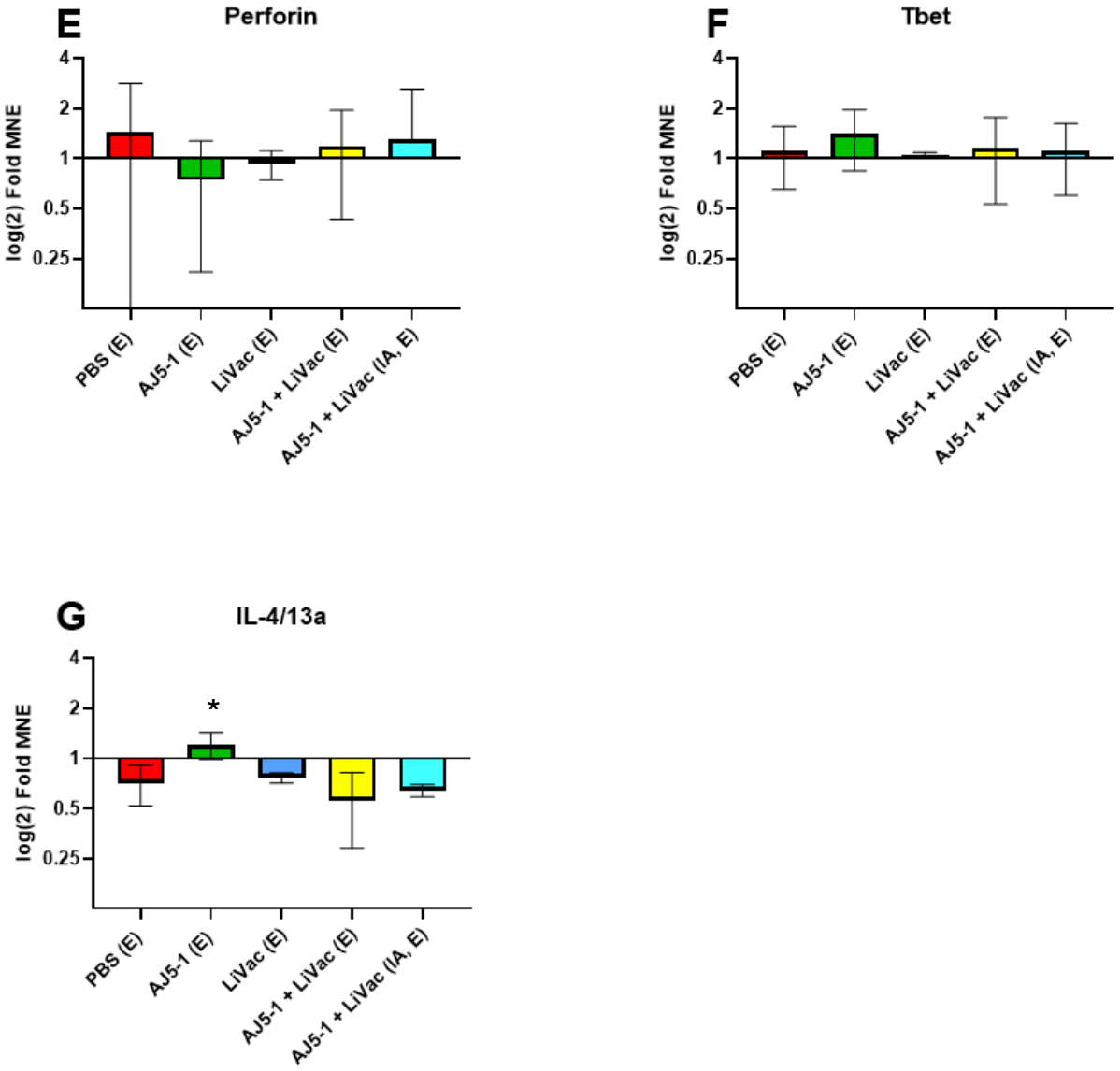


Figure 18 *– Bar charts with standard deviation showing the RT-qPCR results of gene regulation in in vitro exposed HKLs. The bar-charts are presented according to target gene (A-G). The groups exposed to* P. salmonis *is referred to in the figure as E=Exposed. The Cq-values were converted to MNE, where exposed group is folded against the unexposed control group, within each group and log2 transformed. Coloured bars are used to distinguish between the vaccine groups, the PBS control group (red), the AJ5-1 group (green), the LiVac group (blue), the AJ5-1 + LiVac group (yellow) and the AJ5-1 + LiVac (IA) group (turquoise). The standard deviation for the PBS (E) (Figure 18 E) reaches over the x-asis, as the log value cannot be negative. *p < 0.05.*

There is an upregulation in expression of the sIgM gene in exposed vaccinated groups including the PBS control group both when unexposed and exposed to *P. salmonis* (Figure 19A). There is an observable difference between the groups of unexposed HKLs and those exposed to the bacterium, whereas the exposed groups have an overall higher gene expression for sIgM and highest standard deviations.

Most of the groups have an upregulation in gene expression for Cas-1, except for the exposed PBS control group which are down regulated (Figure 19B). The HKLs from the LiVac vaccinated group and the AJ5-1 + LiVac (IA) have a higher gene expression and highest standard deviation in exposed HKLs compared to unexposed HKLs.

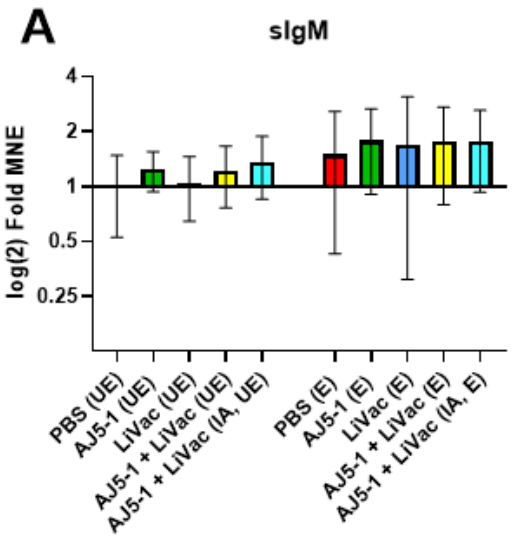
All vaccinated unexposed groups have a downregulation in expression for the GATA3 gene (Figure 19C). An upregulation in expression is observed in the exposed PBS control group and LiVac group and they have a relatively higher standard deviation compared to the other groups.

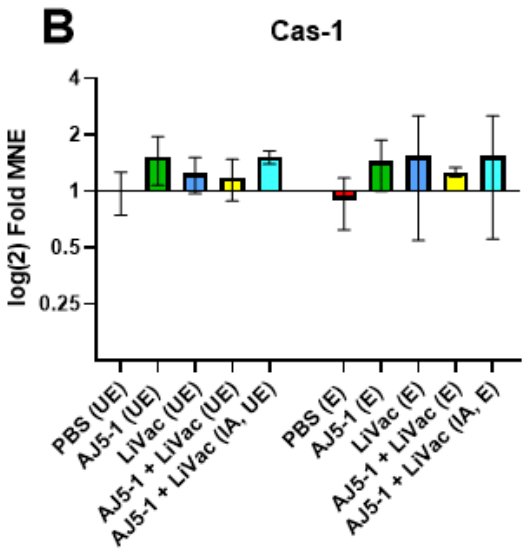
All groups exposed to *P. salmonis* has a downregulation in expression for the IL-18 gene, except for the vaccinated LiVac group which has an upregulation in expression (Figure 19D). Compared to the unexposed groups which has a slight downregulation in expression for the vaccinated groups AJ5-1 and AJ5-1 + LiVac, whereas the vaccinated LiVac group has an upregulation in expression, together with the AJ5-1 + LiVac (IA) group.

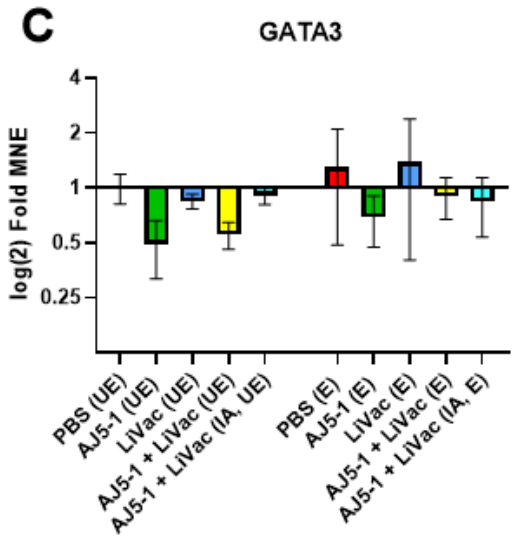
The unexposed and exposed groups have the same up-and downregulations for the perforin gene, with exception of the exposed PBS control group which has an upregulated expression after *P. salmonis* exposure (Figure 19E). There are relatively high standard deviations in both unexposed and exposed groups.

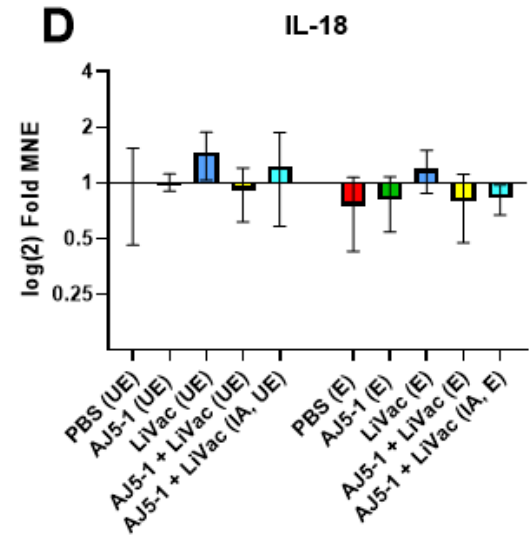
There is a relatively low gene expression for Tbet for groups both unexposed and exposed to *P. salmonis* (Figure 19F). The vaccinated groups AJ5-1 and AJ5-1 + LiVac are downregulated in expression for both exposed and unexposed HKLs. In contrast, the PBS control group, the vaccinated LiVac and vaccinated AJ5-1 + LiVac (IA) group are all slightly upregulated in expression.

All unexposed groups, as well as the vaccinated exposed AJ5-1 group and AJ5-1 + LiVac (IA) group are upregulated in expression of the IL-4/13a gene (Figure 19G). The exposed PBS control group and vaccinated LiVac and AJ5-1 + LiVac group have a downregulated gene expression for IL-4/13a. There is a clear difference between the upregulated unexposed AJ5-1 + LiVac compared with the corresponding exposed group.


A
sIgM
log(2) Fold MNE
4
2
1
0.5
0.25
PBS (UE)
AJ5-1 (UE)
LiVac (UE)
AJ5-1 + LiVac (UE)
AJ5-1 + LiVac (IA, UE)
PBS (E)
AJ5-1 (E)
LiVac (E)
AJ5-1 + LiVac (E)
AJ5-1 + LiVac (IA, E)


B
Cas-1
log(2) Fold MNE
4
2
1
0.5
0.25
PBS (UE)
AJ5-1 (UE)
LiVac (UE)
AJ5-1 + LiVac (UE)
AJ5-1 + LiVac (IA, UE)
PBS (E)
AJ5-1 (E)
LiVac (E)
AJ5-1 + LiVac (E)
AJ5-1 + LiVac (IA, E)


C
GATA3
log(2) Fold MNE
4
2
1
0.5
0.25
PBS (UE)
AJ5-1 (UE)
LiVac (UE)
AJ5-1 + LiVac (UE)
AJ5-1 + LiVac (IA, UE)
PBS (E)
AJ5-1 (E)
LiVac (E)
AJ5-1 + LiVac (E)
AJ5-1 + LiVac (IA, E)


D
IL-18
log(2) Fold MNE
4
2
1
0.5
0.25
PBS (UE)
AJ5-1 (UE)
LiVac (UE)
AJ5-1 + LiVac (UE)
AJ5-1 + LiVac (IA, UE)
PBS (E)
AJ5-1 (E)
LiVac (E)
AJ5-1 + LiVac (E)
AJ5-1 + LiVac (IA, E)

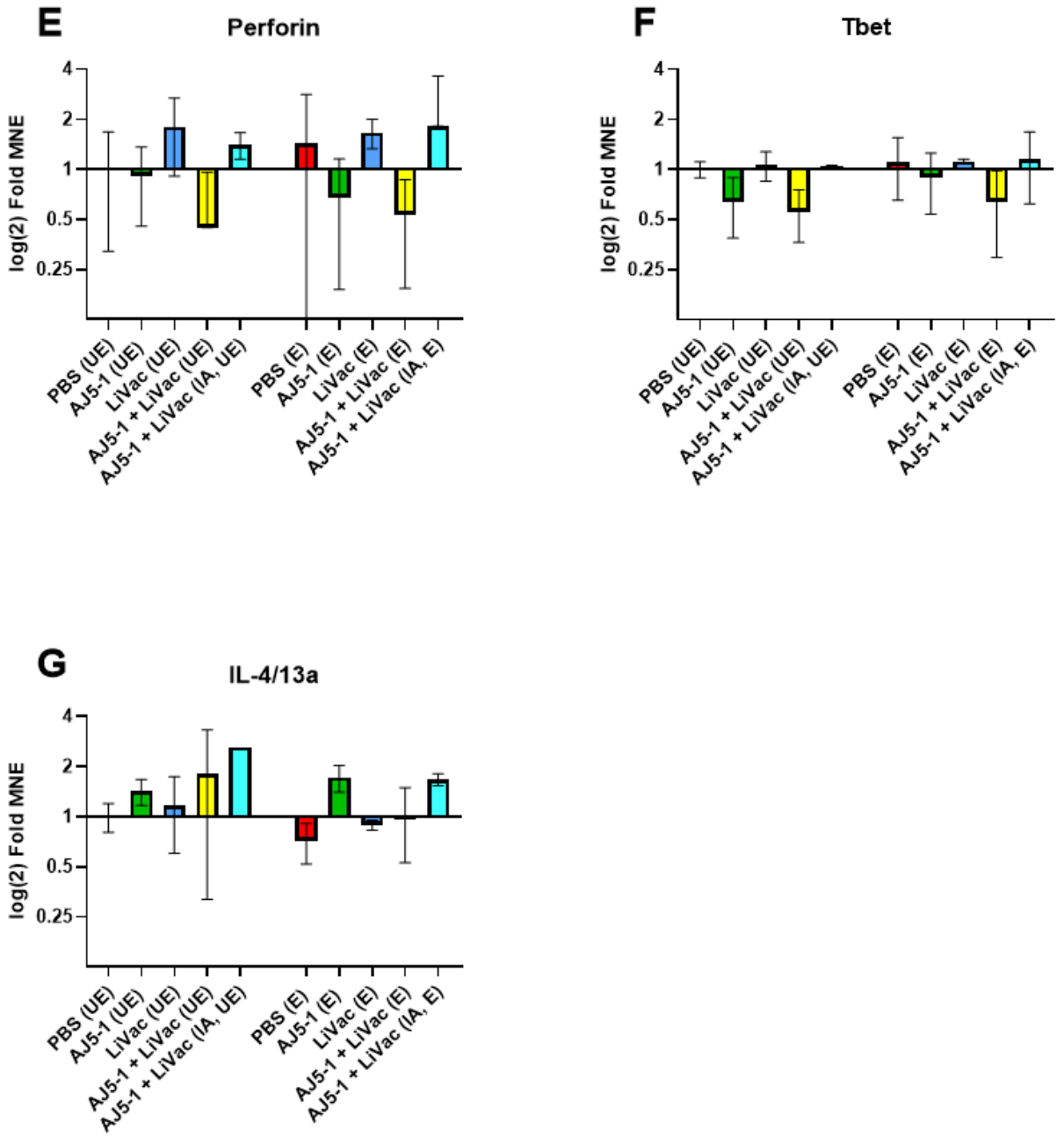


Figure 19 *- Bar charts with standard deviation showing the RT-qPCR results of gene regulation of in vitro exposed and unexposed HKLs. The bar charts are divided according to target gene (A-G). The groups unexposed and exposed to* P. salmonis *is referred to in the figure as UE=unexposed and E=exposed. The Cq-values were converted to MNE, where every group is folded against the unexposed PBS control group and log2 transformed. Coloured bars are used to distinguish between the groups, The PBS control group (red), the AJ5-1 group (green), the LiVac group (blue), the AJ5-1 + LiVac group (yellow) and the AJ5-1 + LiVac (IA) group (turquoise). The standard deviation for the PBS (E) (Figure 19 E) reaches over the x-asis, as the log value cannot be negative.*

4. Discussion

Vaccine efficacy

In the current study the efficacy of the live attenuated vaccine, ALPHA JECT LiVAc® SRS, was tested using an i.p. challenge model. The study shows that when following the SPC the vaccine was highly protective against SRS, providing 100 % survival in the vaccinated group 36 dpc. Whereas the PBS control group had an RPS-value of 0 %, indicating a relatively high infection pressure, as the ideal RPS-value of a control group should be around 20 %. The AJ5-1 + LiVac vaccinated group, which is the most used vaccine combination in Chile, were well protected and reached an RPS-value of above 80 %. The fish were challenged in duplicate tanks and there was no statistical difference in mortality within the vaccine groups across the duplicate tanks, indicating few variables between tanks.

No documentation of previous studies on the efficacy of the LiVac vaccine in field trials was found. However, due to environmental variables it is very difficult to measure effect during field trials. In accordance with the SPC, temperature was confirmed to be a crucial factor when vaccinating with LiVac, as the AJ5-1 + LiVac group vaccinated at 8 ºC had considerable higher mortality (RPS of 0-7 %) compared to the other groups which were vaccinated at 12 ºC. The LiVac vaccine is based on an EM90-like isolate, to this day there are no documentation of the extent to which the vaccine protects against the LF89-like strains, although PHARMAQ claims the LiVac vaccine can provide some cross protection (Karlsen, Pharmacademy, Puerto Montt, 20.03.2024). Chilean isolates are genetically distinct from *P. salmonis* isolates from Canada (Otterlei et al., 2016). The genetic variation among Chilean isolates is significantly higher than that observed in isolates from Canada and Northern European countries, including Ireland, Scotland and Norway (Reid et al., 2004).

Our study confirms the findings of Olsen et al. (2024), who found that salmon vaccinated with LiVac and AJ5-1 combination at temperatures of 10 ºC and higher experienced good protection against SRS, whereas fish vaccinated at 7-8 ºC were significantly less protected. Furthermore, according to Olsen et al (2024), the LiVac vaccine provided effective protection against SRS when challenged (i.p. injection and cohabitant challenge) 15 months after immunization. Other studies however claims that the LiVac vaccine provides no protection against SRS after experimental challenge (Figueroa et al., 2022). Figueroa et al. (2022) conducted two cohabitant challenge trials, the challenged fish were reared at a salinity of 32 ‰ and a temperature of 15

°C. In the first trial the fish were challenged using the LF89-like isolate (only vaccinated with AJ5-1), whereas in the second trial the fish were challenged with an EM90-like isolate (vaccinated with AJ5-1 + LiVac), in addition to exposure to *C. rogercresseyi* copepodites 7 dpc. The shedders were infected with medium lethal dose of *P. salmonis*, and results from both trials claims the vaccine did not protect against either of the genogroups, indicating no statistically significant difference in mortality rates between unvaccinated and vaccinated fish. The paper does, however, not mention which temperature the fish were immunized at. It is debatable whether vaccination was conducted according to the SPC.

Cohabitant and immersion challenge models are considered better alternatives compared to challenge by i.p. injection, as they better mimic the natural course of infection by *P. salmonis* (Meza et al., 2019; Rozas-Serri et al., 2017). It has been demonstrated in experimental challenge trials in both freshwater and seawater that *P. salmonis* have a horizontal route of transmission (Cvitanich et al., 1991), although the transmission route of the bacteria is not fully understood. In the current trial challenge was based on i.p. injection, the fish were challenged in freshwater at 15 °C. There are several advantages to using this model, each fish receives equal treatment with a known infection dose, a shorter period of infection and therefore less suffering of the fish and there is lower cost associated with the experiment. In addition, the model was chosen based on results and experience from previous trials.

The current challenge experiment resulted in 100 % mortality in the PBS injected control group, reflecting a high challenge pressure. The fish vaccinated under optimal conditions were however well protected, showing that the high infection pressure did not camouflage vaccine protection. A lower infectious dose could have resulted in lower mortality in the PBS injected control group but could also have made it more challenging to differentiate between protected and not protected groups.

If a cohabitant challenge model were used, the outcome in mortalities would likely be similar. Based on in house studies (PHARMAQ), shedder fish begin to die between 13-15 dpc, and two weeks later, the groups with lower levels of protection would likely start to die leading to an overall mortality rate as observed in the i.p. challenge model. Long et al. (2021), infected Atlantic salmon with *P. salmonis* using an immersion challenge model and showed that shedding was most efficient between 18- and 42 dpc and that the highest levels of the bacterium were shed to the water shortly before death (Long et al., 2021). Even if a natural course of infection is better simulated through the use of cohabitant or immersion challenge, there is also

a greater room for error. In addition, it is more difficult to find the correct infection dose, as there is a risk that too much or too few fish dies.

Antibody responses

The antibody response post vaccination measured by ELISA showed increased levels of specific antibodies over time for all vaccinated groups, indicating that the adaptive immune system has been stimulated to produce antibodies against *P. salmonis.* Although, when comparing the antibody responses to mortality after challenge, the results indicate that high antibody response does not necessarily equal good protection from disease. The measured antibody levels for the vaccinated groups are quite similar at both 14 – and 24 dpv, but the antibody levels in the AJ5-1 + LiVac (8 ºC) are lower compared to the levels in the other vaccinated groups. Indicating that vaccination at suboptimal temperatures stimulates the adaptive immune system at a lesser extent, leading to a longer response time and affecting the antibody production negatively. At sampling timepoint 48 dpv (dd pv is presented in Appendix B, Table 25), the antibody levels measured in the AJ5-1 vaccinated group are significantly higher compared to the LiVac group and the AJ5-1 + LiVac (8ºC). Reflecting that the inactivated multivalent vaccine stimulates only Th2 responses and production of plasma and memory cells, whereas the live attenuated vaccine stimulates both Th1- and Th2 responses. Previous studies have shown significant increase of *P. salmonis* specific IgM in serum after injecting outer membrane vesicles (OMV) derived from *P. salmonis* (Oliver et al., 2023). The fish were however not challenged with *P. salmonis,* and it is thus uncertain if the antibody levels correlate with protection.

P. salmonis has traditionally been cultivated on CHSE-cells and other fish cell lines (Fryer et al., 1992). The presence of plaques in monolayers of cells indicates that *P. salmonis* has infected the cells and replicated inside until the cell undergo lysis. When plaques cannot be observed, after incubation with *P. salmonis* mixed with plasma containing antibodies from vaccinated fish, it may indicate the presence of antibodies targeting important epitopes on the bacterium used during infection and may thus prohibits binding to host cell (Munang'andu et al., 2013). Lack of plaques may also indicate aggregation of the bacteria mediated by antibodies and in this way inhibition of binding to host cells. In the current study plaque were found in monolayers of CHSE-cells incubated with plasma from vaccinated fish, in all vaccinated groups. The CHSE-cells incubated with plasma from the PBS control group more plaques were observed. The current results are inconclusive in differentiating the efficiency of antibodies

produced in the different vaccine groups and in explaining why high antibody levels could not correlate to protection from disease.

It is unknown how and to what extent increased antibody levels prevent infection or combat undergoing infections of *P. salmonis* (Evensen, 2016). It is also unknown how *P. salmonis* spread from cell to cell if it is by using the extracellular space or if it uses actin-based motility in similar manner as *Listeria monocytogenes* (Dowd et al., 2020). In the extracellular space antibodies have the potential to fight the bacterium by using opsonization, promoting phagocytosis. Some protection against SRS in early stages after vaccination using inactivated vaccines has been shown in previous studies, this could indicate that the bacterium uses the extracellular space and is detected by antibodies (Evensen, 2016). Furthermore, there are indications that the levels of antibodies decline after infection with the pathogen (Tobar et al., 2015).

Regulation of immune genes

Many intracellular pathogens use Clathrin-mediated endocytosis as entry port to host cells (Latomanski and Newton, 2019). This includes *P. salmonis*, which can enter a phagocytic cell through clathrin-mediated endocytosis (Rozas-Serri et al., 2018). Clathrin coated vesicles containing *P. salmonis* (PCVs) are formed at entry by a major reorganization and new synthesis of the cytoskeleton in the host cell (McCarthy et al., 2008; Rozas-Serri et al., 2018). Within host cells *P. salmonis* undergo cycles between replication and stationary phases, triggering responses, such as global shutdown of translation in the host cell (Zuniga et al., 2020).
A sufficient and long-term protection against SRS through vaccination will require an effective vaccine that will activate the CMI responses. The genes measured in this study could potentially differentiate between a Th1- or a Th2 response skewed immune response. An upregulation in the gene expression of Tbet and Perforin, would indicate an Th1response, a Th2 response would be indicated by an upregulation in gene expression of sIgM, GATA3, and IL-4/13a, and upregulation for Cas-1 and IL-18 would indicate intracellular location and recognition of the vaccine strain and/or challenge strain. Unfortunately, the results presented from the present study could only clarify few statistically significant differences in regulations of the selected immune genes when comparing the different vaccine groups or vaccinated fish compared to unvaccinated fish.

Liver samples provided low amount of leucocytes when harvested on Percoll gradients, thus analysis of regulation of immune genes were preformed using HKLs pv. The RT-qPCR analysis was performed on isolated HKLs sampled 48 dpv. From each fish and vaccine group, leucocytes exposed to *P. salmonis* and unexposed leucocytes were included. To measure both primary and secondary immune responses in the vaccine groups, leucocytes from PBS injected fish was also included. The leucocytes were harvested at 6 and 24 hours post exposure (hpe). There were only few significant differences between the different vaccine groups or between vaccinated and unvaccinated fish detected using a one-way ANOVA for statistical analysis. However, trends in difference were observed between the vaccine groups comparing HKLs exposed to *P. salmonis* and unexposed HKLs, where the standard deviation in the groups exposed to the bacterium, were higher compared to their respective unexposed groups, indicating greater variation between individuals in the same group.

An upregulation in the expression of IL-4/13a was measured in *P. salmonis* exposed HKLs from the AJ5-1 vaccinated fish compared to exposed HKLs from the other vaccine groups and the PBS control. IL-4/13a is secreted by Th2- cells to stimulate proliferation and differentiation of B-cells and the result indicate that HKLs from AJ5-1 vaccinated fish are stimulated to a higher degree compared to the other vaccine groups. As there is no statistically significant difference in the expression of IL-4/13a between exposed and unexposed HKLs from AJ5-1 vaccinated fish it may indicate that the up regulation is caused by the vaccine and not the *in vitro* exposure to *P. salmonis*. AJ5-1 contain inactivated antigens and the vaccine is expected to induce Th2 responses, but surprisingly the increase in regulation is also higher compared to the AJ5-1 + LiVac (IA) group injected with two doses of inactivated antigens.

It is uncertain if the more interesting results lie with the isolated leucocytes harvested at 24 hpe. Furthermore, interesting results could also be found if analysing the leucocytes isolated at 24 dpv. The choice to harvest the leucocytes after 6- and 24-hours exposure with P. *salmonis*, was based on previous studies that have shown that these timepoints are suitable (Eggestøl et al., 2018). The choice to analyse the samples harvested after 6 hours as opposed to 24 hours were based on the RT-qPCR results from a pre-test. It is also possible that some of the gene regulations should have been measured at 24 hpe and some at 6 hpe. In addition, is it difficult to know whether the gene measured is about to be up/down regulated or whether it is fully expressed or not when the sampling is performed. It is not unlikely that the RT-qPCR results would have been more conclusive if the procedure had been carried out by a diagnostic facility eliminating human sources of error.

Further complicating the measurement and results, *P. salmonis* has mechanism to evade and modulate the host immune responses (Rozas-Serri et al., 2019). For example, to evade CD8+ T-cell responses *P. salmonis* inhibits the MHC class I pathway and activates the MHC class II pathway (Vargas et al., 2021). It has been showed that *P. salmonis* induces differentiation of T-reg cells (Morales-Lange et al., 2021). *P. salmonis* may also promote production of IFN-β and reduction of IL-12, leading to reduction in Tbet and perforin production (Rozas-Serri, 2022). It has been shown that upregulation of MHC class II and CD4+ T-cells by *P. salmonis* cannot correlate with the protection of vaccines and or reduction in mortality in field (Rozas-Serri, 2022).

It is many unknows linked to the amount of time the CD8+ T-cells remains active after vaccination and infection with *P. salmonis,* as well as the extent of protection the CD8+ T-cells provide. In addition, is it unknown how the live intracellular vaccine against SRS leads to protective CMI responses in the host (Rozas-Serri, 2022). The reason why there is little knowledge about this in fish, compared to mice and humans, are because we lack commercial cell specific antibody markers to measure subgroups of cells after an infection.

SRS in Norwegian farmed salmon

P. salmonis favour the water temperatures in Chile, and SRS are to this date under surveillance and have until recently not been a major problem in other fish farming countries. However, SRS is already becoming a rising problem in Ireland where *P. salmonis* isolates similar to the EM genogroup have been isolated (Feeks, 2023). Due to global warming and rising water temperatures, it is not unlikely that *P. salmonis* may become a problem also in Norway should more virulent genotypes arise in North-Europe. Norway follows the EU regulations when it comes to approving new vaccines for the Norwegian market. However, it would be challenging to get approval of a live attenuated vaccine for use in the Norwegian fish farming industry. This is based on the risk of secondary mutations, leading to reversion of virulent strain. Weakened individuals are more susceptible to these mutations (Yadav et al., 2014). Another risk is what the regulatory authorities call “shed and spread” when disease-causing microorganisms are spread from vaccinated individuals to the environment. This is an absolute requirement that the live attenuated vaccines must meet the requirements stated in the monograph *Vaccines for veterinary use* (0062) (EDQM, 2020) to be licensed. In 2017 a DNA-vaccine was licensed in EU by the European Medicines Agency (EMA) against Salmonid alphavirus (EMA, 2016). The use of nucleic acid vaccines is an alternative for intracellular infections, as it also can induce

both humoral- and CMI responses, in addition it normally poses a lower risk for the environment (Tonheim et al., 2008). However, to develop a DNA vaccine against *P. salmonis*, a protein with the ability to stimulate both CMI - and humoral responses must be identified, and coding gene inserted into a plasmid. To this date, no DNA vaccine against *P. salmonis* have been successfully developed.

Directions for future research

Measurements of gene expression from the collected samples of exposed and unexposed HKLs not analysed in the current study might reveal more clear results and indicate differences across the different vaccine groups and between vaccinated and unvaccinated fish.

Regulations of immune genes could also be measured using tissues from vaccinated fish at different timepoints post vaccination.

To gain more knowledge on host-pathogen interaction with emphasis in the immune responses there are several aspects that could be further pursued. *P. salmonis* use clathrin mediated endocytosis when invading a host cell. It would thus be interesting to examine if *P. salmonis* also infect B-cells. If that is the case it could influence on adaptive immune responses.

Furthermore, it would be interesting to analyse *P. salmonis* infection and induction or inhabitation of inflammation by using macrophages, M1(pro-inflammatory) and M2 (anti-inflammatory) as markers. This could be accomplished by measuring nitric oxidase synthase (iNOS)(M1) and arginase 2 (M2) (Wiegertjes et al., 2016). M1 can be activated through the classical pathway by by IFN-ɣ and TNF-α and M2 by IL-4/13, IL-10 and tgfβ (Grayfer et al., 2018; Italiani and Boraschi, 2014; Yang et al., 2017).

Additionally, it would be interesting to include a more suboptimal state of the LiVac vaccine to stimulate possible errors which could occur in the field, aiming to measure and identify the limitations of the vaccine.

5. Conclusions

The aims of this study were to identify possible immunological markers which can directly correlate to a successful vaccination using ALPHA JECT LiVac® SRS. This was investigated by a vaccine efficacy trial, measurements of antibody response and by analysis of regulation of immune genes.

The current study was not successful in identifying immunological markers correlating to a successful vaccination. Although in vaccinated fish the individual differences in gene expression in HKLs post exposure for *P. salmonis* is higher compared to unexposed HKLs.

Furthermore, groups vaccinated with the LiVac vaccine alone and in combination, immunized at 12 ^{o}C showed good protection after experimental challenge with *P. salmonis.* Indicating that the LiVac vaccine is highly protective against SRS when used correctly.

Increased antibody responses were detected over time post vaccination for all vaccinated groups, but the antibodies produced in the poor performing groups (high mortality) did not protect against SRS.

6. References

Bravo, S., Campos, M., 1989. Coho salmon syndrome in Chile. AFSFHS Newsl. 3.

Bravo, S., Dolz, H., Lagos, C., Millanao, A., Urbina, M., 2005. Diagnóstico del uso de fármacos y otros productos químicos en la acuicultura. Proy. FIP 2003-28 Parte I. https://www.subpesca.cl/fipa/613/w3-article-89033.html

Bravo, S., Midtlyng, P.J., 2007. The use of fish vaccines in the Chilean salmon industry 1999-2003. Aquaculture 270, 36–42. https://doi.org/10.1016/j.aquaculture.2007.06.017

Bruno, D.W., Noguera, P.A., Poppe, T.T., 2013. A Colour Atlas of Salmonid Diseases, 2nd ed. Springer.

Buchmann, K., Secombes, C.J., 2022. Principles of Fish Immunology. Springer.

Cvitanich, J.D., Garate N., O., Smith, C.E., 1991. The isolation of a rickettsia-like organism causing disease and mortality in Chilean salmonids and its confirmation by Koch's postulate. J. Fish Dis. 14, 121–145. https://doi.org/10.1111/j.1365-2761.1991.tb00584.x

Dowd, G.C., Mortuza, R., Bhalla, M., Van Ngo, H., Li, Y., Rigano, L.A., Ireton, K., 2020. *Listeria monocytogenes* exploits host exocytosis to promote cell-to-cell spread. Proc. Natl. Acad. Sci. U. S. A. 117, 3789–3796. https://doi.org/10.1073/pnas.1916676117

Duff, D.C.B., 1942. The Oral Immunization of Trout Against Bacterium Salmonicida. J. Immunol. 44, 87–94. https://doi.org/10.4049/jimmunol.44.1.87

Eggestøl, H.Ø., Lunde, H.S., Rønneseth, A., Fredman, D., Petersen, K., Mishra, C.K., Furmanek, T., Colquhoun, D.J., Wergeland, H.I., Haugland, G.T., 2018. Transcriptome-wide mapping of signaling pathways and early immune responses in lumpfish leukocytes upon *in vitro* bacterial exposure. Sci. Rep. 8, 5261. https://doi.org/10.1038/s41598-018-23667-x

EDQM, Technical Guide for the elaboration and use of monographs for vaccines and immunological veterinary medicinal products, European Directorate for the Quality of Medicines & HealthCare. 2. Revision, edition 2020. URL https://www.edqm.eu/documents/52006/66555/08-elaboration-use_monographs-vaccines-immunological-veterinary-products.pdf/e4b5fde2-958e-3ef6-0748-7a8992f3f4d2?t=1623146225237 (accessed: 05.28.24)

EMA, First DNA vaccine in the EU recommended for use in salmon | European Medicines Agency [WWW Document], n.d. URL https://www.ema.europa.eu/en/news/first-dna-vaccine-eu-recommended-use-salmon (accessed 05.22.24).

Evensen, Ø., 2016. Immunization Strategies against *Piscirickettsia salmonis* Infections: Review of Vaccination Approaches and Modalities and Their Associated Immune Response Profiles. Front. Immunol. 7. https://doi.org/10.3389/fimmu.2016.00482

Feeks, C., 2023. Race to control bacterial disease which threatens Irish salmon production [WWW Document]. Fish Health Forum. URL https://fishhealthforum.com/race-to-control-bacterial-disease-which-threatens-irish-salmon-production/ (accessed 05.22.24).

Fhi, Bruk av legemidler i fiskeoppdrett i 2023 [WWW Document], 2024. Folkehelseinstituttet. URL https://www.fhi.no/he/legemiddelbruk/fisk/bruk-av-legemidler-i-fiskeoppdrett/ (accessed 05.13.24).

Figueroa, C., Torrealba, D., Morales-Lange, B., Mercado, L., Dixon, B., Conejeros, P., Silva, G., Soto, C., Gallardo, J.A., 2022. Commercial Vaccines Do Not Confer Protection against Two Genogroups of *Piscirickettsia salmonis*, LF-89 and EM-90, in Atlantic Salmon. Biology 11, 993. https://doi.org/10.3390/biology11070993

Flores-Kossack, C., Montero, R., Köllner, B., Maisey, K., 2020. Chilean aquaculture and the new challenges: Pathogens, immune response, vaccination and fish diversification. Fish Shellfish Immunol. 98, 52–67. https://doi.org/10.1016/j.fsi.2019.12.093

Fryer, J. l., Lannan, C. n., Garces, L. h., Larenas, J. j., Smith, P. a., 1990. Isolation of a Rickettsiales-Like Organism from Diseased Coho Salmon (*Oncorhynchus kisutch*) in Chile. Fish Pathol. 25, 107–114. https://doi.org/10.3147/jsfp.25.107

Fryer, J.L., Hedrick, R.P., 2003. *Piscirickettsia salmonis*: a Gram-negative intracellular bacterial pathogen of fish. J. Fish Dis. 26, 251–262. https://doi.org/10.1046/j.1365-2761.2003.00460.x

Fryer, J.L., Lannan, C.N., Giovannoni, S.J., Wood, N.D., 1992. *Piscirickettsia salmonis* gen. nov., sp. nov., the Causative Agent of an Epizootic Disease in Salmonid Fishes†. Int. J. Syst. Evol. Microbiol. 42, 120–126. https://doi.org/10.1099/00207713-42-1-120

Gómez, F.A., Tobar, J.A., Henríquez, V., Sola, M., Altamirano, C., Marshall, S.H., 2013. Evidence of the presence of a functional Dot/Icm type IV-B secretion system in the fish bacterial pathogen *Piscirickettsia salmonis.* PloS One 8, e54934. https://doi.org/10.1371/journal.pone.0054934

Grayfer, L., Kerimoglu, B., Yaparla, A., Hodgkinson, J.W., Xie, J., Belosevic, M., 2018. Mechanisms of Fish Macrophage Antimicrobial Immunity. Front. Immunol. 9, 1105. https://doi.org/10.3389/fimmu.2018.01105

Gudding, R., Van Muiswinkel, W.B., 2013. A history of fish vaccination: science-based disease prevention in aquaculture. Fish Shellfish Immunol. 35, 1683–1688. https://doi.org/10.1016/j.fsi.2013.09.031

Haes, W.D., Pollard, C., Vanham, G., Rejman, J., Haes, W.D., Pollard, C., Vanham, G., Rejman, J., 2012. "Wrapped Up" Vaccines in the Context of HIV-1 Immunotherapy, in: Immunodeficiency. IntechOpen. https://doi.org/10.5772/51583

Hordvik, I., 2015. Immunoglobulin Isotypes in Atlantic Salmon, *Salmo Salar.* Biomolecules 5, 166–177. https://doi.org/10.3390/biom5010166

Hornung, V., Ablasser, A., Charrel-Dennis, M., Bauernfeind, F., Horvath, G., Caffrey, D.R., Latz, E., Fitzgerald, K.A., 2009. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature 458, 514–518. https://doi.org/10.1038/nature07725

Isla, A., Haussmann, D., Vera, T., Kausel, G., Figueroa, J., 2014. Identification of the clpB and bipA genes and an evaluation of their expression as related to intracellular survival for the bacterial pathogen *Piscirickettsia salmonis*. Vet. Microbiol. 173, 390–394. https://doi.org/10.1016/j.vetmic.2014.08.014

Italiani, P., Boraschi, D., 2014. From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. Front. Immunol. 5, 514. https://doi.org/10.3389/fimmu.2014.00514

Kanhere, A., Hertweck, A., Bhatia, U., Gökmen, M.R., Perucha, E., Jackson, I., Lord, G.M., Jenner, R.G., 2012. T-bet and GATA3 orchestrate Th1 and Th2 differentiation through lineage-specific targeting of distal regulatory elements. Nat. Commun. 3, 1268. https://doi.org/10.1038/ncomms2260

Karlsen, M., 2024. Oral presentation. Experiences with use of and recent advantages in the understanding of efficacy. Pharmaquademy. Puerto Montt, Chile. 03.20.2024.

Latomanski, E.A., Newton, H.J., 2019. Taming the Triskelion: Bacterial Manipulation of Clathrin. Microbiol. Mol. Biol. Rev. 83, 10.1128/mmbr.00058-18. https://doi.org/10.1128/mmbr.00058-18

Li, J., Barreda, D.R., Zhang, Y.-A., Boshra, H., Gelman, A.E., Lapatra, S., Tort, L., Sunyer, J.O., 2006. B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. Nat. Immunol. 7, 1116–1124. https://doi.org/10.1038/ni1389

Long, A., Goodall, A., Jones, S.R.M., 2021. Development of a *Piscirickettsia salmonis* immersion challenge model to investigate the comparative susceptibility of three salmon species. J. Fish Dis. 44, 1–9. https://doi.org/10.1111/jfd.13261

Ma, J., Bruce, T.J., Jones, E.M., Cain, K.D., 2019. A Review of Fish Vaccine Development Strategies: Conventional Methods and Modern Biotechnological Approaches. Microorganisms 7, 569. https://doi.org/10.3390/microorganisms7110569

Magnadóttir, B., 2006. Innate immunity of fish (overview). Fish Shellfish Immunol., Reviews in Fish Immunology 20, 137–151. https://doi.org/10.1016/j.fsi.2004.09.006

Mauel, M.J., Giovannoni, S.J., Fryer, J.L., 1999. Phylogenetic analysis of *Piscirickettsia salmonis* by 16S, internal transcribed spacer (ITS) and 23S ribosomal DNA sequencing. Dis. Aquat. Organ. 35, 115–123. https://doi.org/10.3354/dao035115

Mauel, M.J., Miller, D.L., 2002. Piscirickettsiosis and piscirickettsiosis-like infections in fish: a review. Vet. Microbiol. 87, 279–289. https://doi.org/10.1016/s0378-1135(02)00085-8

McCarthy, U.M., Bron, J.E., Brown, L., Pourahmad, F., Bricknell, I.R., Thompson, K.D., Adams, A., Ellis, A.E., 2008. Survival and replication of *Piscirickettsia salmonis* in rainbow trout head kidney macrophages. Fish Shellfish Immunol. 25, 477–484. https://doi.org/10.1016/j.fsi.2008.07.005

Meza, K., Inami, M., Dalum, A.S., Bjelland, A.M., Sørum, H., Løvoll, M., 2019. 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. 42, 1001–1011. https://doi.org/10.1111/jfd.13004

Miranda, C.D., Godoy, F.A., Lee, M.R., 2018. Current Status of the Use of Antibiotics and the Antimicrobial Resistance in the Chilean Salmon Farms. Front. Microbiol. 9.

Morales-Lange, B., Nombela, I., Ortega-Villaizán, M.D.M., Imarai, M., Schmitt, P., Mercado, L., 2021. Induction of foxp3 during the Crosstalk between Antigen Presenting Like-Cells MHCII+CD83+ and Splenocytes CD4+IgM− in Rainbow Trout. Biology 10, 324. https://doi.org/10.3390/biology10040324

Munang'andu, H.M., Fredriksen, B.N., Mutoloki, S., Dalmo, R.A., Evensen, Ø., 2013. Antigen dose and humoral immune response correspond with protection for inactivated infectious pancreatic necrosis virus vaccines in Atlantic salmon (*Salmo salar L*). Vet. Res. 44, 7. https://doi.org/10.1186/1297-9716-44-7

Murphy, K., Weaver, C., 2017. Janeway`s immunobiology, 9th ed. Garland Science.

Oliver, C., Coronado, J.L., Martínez, D., Kashulin-Bekkelund, A., Lagos, L.X., Ciani, E., Sanhueza-Oyarzún, C., Mancilla-Nova, A., Enríquez, R., Winther-Larsen, H.C., Romero, A., 2023. Outer membrane vesicles from *Piscirickettsia salmonis* induce the

expression of inflammatory genes and production of IgM in Atlantic salmon *Salmo salar*. Fish Shellfish Immunol. 139, 108887. https://doi.org/10.1016/j.fsi.2023.108887

Olsen, A.B., Melby, H.P., Speilberg, L., Evensen, Ø., Håstein, T., 1997. *Piscirickettsia salmonis* infection in Atlantic salmon *Salmo salar* in Norway-epidemiological, pathological and microbiological findings. Dis. Pf Aquat. Org. 31, 35–48.

Olsen, R.H., Finne-Fridell, F., Bordevik, M., Nygaard, A., Rajan, B., Karlsen, M., 2024. The Effect of an Attenuated Live Vaccine against Salmonid Rickettsial Septicemia in Atlantic Salmon (*Salmo salar*) Is Highly Dependent on Water Temperature during Immunization. Vaccines 12, 416. https://doi.org/10.3390/vaccines12040416

Otterlei, A., Brevik, Ø.J., Jensen, D., Duesund, H., Sommerset, I., Frost, P., Mendoza, J., McKenzie, P., Nylund, A., Apablaza, P., 2016. Phenotypic and genetic characterization of *Piscirickettsia salmonis* from Chilean and Canadian salmonids. BMC Vet. Res. 12, 55. https://doi.org/10.1186/s12917-016-0681-0

Pérez-Stuardo, D., Morales-Reyes, J., Tapia, S., Ahumada, D.E., Espinoza, A., Soto-Herrera, V., Brianson, B., Ibaceta, V., Sandino, A.M., Spencer, E., Vallejos-Vidal, E., Reyes-López, F.E., Valdés, J., Reyes-Cerpa, S., 2019. Non-lysosomal Activation in Macrophages of Atlantic Salmon (*Salmo salar*) After Infection With *Piscirickettsia salmonis.* Front. Immunol. 10, 434. https://doi.org/10.3389/fimmu.2019.00434

Pietretti, D., Wiegertjes, G.F., 2014. Ligand specificities of Toll-like receptors in fish: Indications from infection studies. Dev. Comp. Immunol., Immunity to infectious diseases of fish 43, 205–222. https://doi.org/10.1016/j.dci.2013.08.010

Price, D., Stryhn, H., Sánchez, J., Ibarra, R., Tello, A., St-Hilaire, S., 2016. Retrospective analysis of antibiotic treatments against piscirickettsiosis in farmed Atlantic salmon *Salmo salar* in Chile. Dis. Aquat. Organ. 118, 227–235. https://doi.org/10.3354/dao02978

Reid, H.I., Griffen, A.A., Birkbeck, T.H., 2004. Isolates of *Piscirickettsia salmonis* from Scotland and Ireland Show Evidence of Clonal Diversity. Appl. Environ. Microbiol. 70, 4393–4397. https://doi.org/10.1128/AEM.70.7.4393-4397.2004

Rojas, V., Galanti, N., Bols, N.C., Marshall, S.H., 2009. Productive infection of *Piscirickettsia salmonis* in macrophages and monocyte-like cells from rainbow trout, a possible survival strategy. J. Cell. Biochem. 108, 631–637. https://doi.org/10.1002/jcb.22295

Rozas, M., Enríquez, R., 2014. Piscirickettsiosis and *Piscirickettsia salmonis* in fish: a review. J. Fish Dis. 37, 163–188. https://doi.org/10.1111/jfd.12211

Rozas-Serri, M., 2022. Why Does *Piscirickettsia salmonis* Break the Immunological Paradigm in Farmed Salmon? Biological Context to Understand the Relative Control of Piscirickettsiosis. Front. Immunol. 13, 856896. https://doi.org/10.3389/fimmu.2022.856896

Rozas-Serri, M., Ildefonso, R., Peña, A., Enríquez, R., Barrientos, S., Maldonado, L., 2017. Comparative pathogenesis of piscirickettsiosis in Atlantic salmon (*Salmo salar L.*) post-smolt experimentally challenged with LF-89-like and EM-90-like *Piscirickettsia salmonis* isolates. J. Fish Dis. 40, 1451–1472. https://doi.org/10.1111/jfd.12671

Rozas-Serri, M., Peña, A., Arriagada, G., Enríquez, R., Maldonado, L., 2018. Comparison of gene expression in post-smolt Atlantic salmon challenged by LF-89-like and EM-90-like *Piscirickettsia salmonis* isolates reveals differences in the immune response associated with pathogenicity. J. Fish Dis. 41, 539–552. https://doi.org/10.1111/jfd.12756

Rozas-Serri, M., Peña, A., Maldonado, L., 2019. Gene expression associated with immune response in Atlantic salmon head-kidney vaccinated with inactivated whole-cell bacterin of *Piscirickettsia salmonis* and pathogenic isolates. Fish Shellfish Immunol. 93, 789–795. https://doi.org/10.1016/j.fsi.2019.08.031

Rozas-Serri, Marco, Peña, A., Maldonado, L., 2018. Transcriptomic profiles of post-smolt Atlantic salmon challenged with *Piscirickettsia salmonis* reveal a strategy to evade the adaptive immune response and modify cell-autonomous immunity. Dev. Comp. Immunol. 81, 348–362. https://doi.org/10.1016/j.dci.2017.12.023

Sag, 2024 Productos biologicos immunologicos con registro provisional uso en salmonidos, Ultima modificacion: 07 de mayo de 2024. Ministerio de Agricultura, Gobierno de Chile.URLhttps://www.sag.gob.cl/sites/default/files/lista_salmonidos_registro_provisional_07052024.pdf (accessed 05.24.24).

Schober, I., Bunk, B., Carril, G., Freese, H.M., Ojeda, N., Riedel, T., Meier-Kolthoff, J.P., Göker, M., Spröer, C., Flores-Herrera, P.A., Nourdin-Galindo, G., Gómez, F., Cárdenas, C., Vásquez-Ponce, F., Labra, A., Figueroa, J., Olivares-Pacheco, J., Nübel, U., Sikorski, J., Marshall, S.H., Overmann, J., 2023. Ongoing diversification of the global fish pathogen *Piscirickettsia salmonis* through genetic isolation and transposition bursts. ISME J. 17, 2247–2258. https://doi.org/10.1038/s41396-023-01531-9

Sernapesca, Informe sobre uso de antimicrobianos en la salmonicultura nacional Primer semestre - Año 2023*;* Subdireccion de Acuicultura y Departamento de Salud Animal: Valparaíso, Octubre 2023. URL https://www.sernapesca.cl/app/uploads/2023/12/informe_sobre_el_uso_am_en_la_salmonicultura_nacional_-_primer_semestre_-_2023.pdf (accessed 05.24.24).

Shoemaker, C.A., Klesius, P.H., Evans, J.J., Arias, C.R., 2009. Use of Modified Live Vaccines in Aquaculture. J. World Aquac. Soc. 40, 573–585. https://doi.org/10.1111/j.1749-7345.2009.00279.x

Smith Schuster, P., Rojas, M.E., Guajardo, A., Contreras, J., Morales, M.A., Larenas, J., 2004. Experimental infection of coho salmon *Oncorhynchus kisutch* by exposure of skin, gills and intestine with *Piscirickettsia salmonis*. Dis. Aquat. Organ. https://doi.org/10.3354/dao061053

Sommerset, I., Krossøy, B., Biering, E., Frost, P., 2005. Vaccines for fish aquaculture. Expert Rev. Vaccines 4, 89–101. https://doi.org/10.1586/14760584.4.1.89

Tobar, I., Arancibia, S., Torres, C., Vera, V., Soto, P., Carrasco, C., Alvarado, M., Neira, E., Arcos, S., Tobar, J.A., 2015. Successive Oral Immunizations Against *Piscirickettsia Salmonis* and Infectious Salmon Anemia Virus are Required to Maintain a Long-Term Protection in Farmed Salmonids. Front. Immunol. 6, 244. https://doi.org/10.3389/fimmu.2015.00244

Tonheim, T.C., Bøgwald, J., Dalmo, R.A., 2008. What happens to the DNA vaccine in fish? A review of current knowledge. Fish Shellfish Immunol. 25, 1–18. https://doi.org/10.1016/j.fsi.2008.03.007

Tsoi, S., Park, K.C., Kay, H.H., O'Brien, T.J., Podor, E., Sun, G., Douglas, S.E., Brown, L.L., Johnson, S.C., 2006. Identification of a transcript encoding a soluble form of toll-like receptor 5 (TLR5) in Atlantic salmon during *Aeromonas salmonicida* infection. Vet. Immunol. Immunopathol. 109, 183–187. https://doi.org/10.1016/j.vetimm.2005.05.013

Uribe, C., Folch, H., Enriquez, R., Moran, G., 2011. Innate and adaptive immunity in teleost fish: a review. Veterinární Medicína 56, 486–503. https://doi.org/10.17221/3294-VETMED

Vargas, D., Vallejos-Vidal, E., Reyes-Cerpa, S., Oyarzún-Arrau, A., Acuña-Castillo, C., Imarai, M., Reyes-López, F.E., Sandino, A.M., 2021. The Analysis of Live-Attenuated *Piscirickettsia salmonis* Vaccine Reveals the Short-Term Upregulation of Innate and Adaptive Immune Genes in Atlantic Salmon (*Salmo salar*): An In Situ Open-Sea Cages Study. Microorganisms 9, 703. https://doi.org/10.3390/microorganisms9040703

Wiegertjes, G.F., Wentzel, A.S., Spaink, H.P., Elks, P.M., Fink, I.R., 2016. Polarization of immune responses in fish: The "macrophages first" point of view. Mol. Immunol. 69, 146–156. https://doi.org/10.1016/j.molimm.2015.09.026

Yadav, D.K., Yadav, N., Khurana, S.M.P., 2014. Chapter 26 - Vaccines: Present Status and Applications, in: Verma, A.S., Singh, A. (Eds.), Animal Biotechnology. Academic Press, San Diego, pp. 491–508. https://doi.org/10.1016/B978-0-12-416002-6.00026-2

Yang, S., Li, Q., Mu, Y., Ao, J., Chen, X., 2017. Functional activities of interferon gamma in large yellow croaker *Larimichthys crocea*. Fish Shellfish Immunol. 70, 545–552. https://doi.org/10.1016/j.fsi.2017.09.051

Zuniga, A., Aravena, P., Pulgar, R., Travisany, D., Ortiz-Severin, J., 2020. Transcriptomic Changes of *Piscirickettsia salmonis* During Intracellular Growth in a Salmon Macrophage-Like Cell Line. Front. Cell. Infect. Microbiol. 9. https://doi.org/10.3389/fcimb.2019.00426.

7. Appendix A

Recipes of solutions used in this study.

7.1 – Standard solutions

10 x PBS solution, pH 7.3, 1000 mL:

Na_2HPO_4 x $2H_2O$	7.20 g
KH_2PO_4	2.70 g
NaCl	85 g
Milli-Q (Adjust pH to 7,3 before adding all the water)	Until 1000 mL

1 x PBS solution, pH 7.3, 1000 mL:

Na_2HPO_4 x $2H_2O$	0.72 g
KH_2PO_4	0.27 g
NaCl	8.5 g
Milli-Q (adjust pH to 7.3 before adding all the water)	Until 1000 mL

1 x PBS (380 mOsm), pH 7.3 1000mL:

Na_2HPO_4 x $2H_2O$	0,854 g
KH_2PO_4	0,254 g
NaCl	11.104 g
Milli-Q (Adjust pH to 7,3 before adding all the water)	Until 1000 mL

0.2M Na_2HPO_4 x $2H_2O$ solution,1000 mL:

Na_2HPO_4 x $2H_2O$	35.6 g
Milli-Q	Until 1000 mL

0.36M NaCl 250 mL:

NaCl	5,26 g
Milli-Q	Until 250 mL

0.45M NaCl 250 mL:

NaCl	6,72 g
Milli-Q	Until 250 mL

7.2 – Solutions for ELISA

PBS-Tween (PBS-T) 100 mL:

0.,05 % Tween 20	50 µl
1 x PBS – Appendix A, 7.1	100 mL

Blocking solution 100 mL:

Skim milk powder 3 %	3 g
PBS-Tween	100 mL

PBS-E (1 % BSA, 0,1 % Azid, 25mM EDTA)

BSA	5,0 g
Na-azid	0,5 g
EDTA (Tittriplex)	4,65 g
PBS (380 mOSM) – Appendix A, 7.1	Until 500 mL
(Adjust pH to 7,3 before adding all the PBS)	

0.1M Citric acid solution 1000 mL:

Citric acid monohydrate	21.0 g
Milli-Q	Until 1000 mL

Phosphate – citrate buffer, pH 5.0, 100 mL:

0.1M Citric acid solution	24.3 mL
0.2M Na_2HPO_4 x $2H_2O$ solution	25.7 mL
Milli-Q	50.0 mL

Peroxidase substrate solution:

O-Phenyleneidamine (P-4664, Sigma)	1 Tablet
Phosphate – citrate buffer, pH 5.0	37.5 mL
30 % H_2O_2 (added immediately before use)	15 µl

2.5M H_2SO_4 solution 100 mL:

H_2SO_4	6.94 mL
Milli-Q	93.06 mL

7.3 – CHSE-cells

Culture medium 200 mL:

EMEM with EBSS and HEPES (Bio Whittaker 12-136)	173,8 mL
FBS (Bio Whittaker 14-705F)	20 mL
NEAA, 10 mM, 100 x (Bio Whittaker 13-114E)	2 mL
L-glutamine, 200 mM (Bio Whittaker 17-605E)	4 mL
Gentamicin sulfate. 50 mg/mL (Bio Whittaker 17-518L)	200 µl

Challenge medium 200 mL:

EMEM with EBSS and HEPES (Bio Whittaker 12-136)	173,8 mL
FBS (Bio Whittaker 14-705F)	20 mL
NEAA, 10 mM, 100 x (Bio Whittaker 13-114E)	2 mL
L-glutamine, 200 mM (Bio Whittaker 17-605E)	4 mL

Crystal violet staining (0.5 %), 100 mL:

Crystal violet	0.5 g
MeOH (100 %)	25 mL
MilliQ	75 mL

7.4 – Isolation of leucocytes

L-15 (370 mOsm):

Glucose	66 g
NaCl	2.5 g
$NaHCO_3$	2.8 g
Milli-Q	100 mL

L-15 + solution 100mL:

Gentamicin	100 µl
Heparin stock solution	200 µl
200mM glutamine	1 mL
1M HEPES	1,5 mL
Fetal calf serum (FCS)	5 mL
L-15	until 100 mL

Percoll solutions:

1,060 g/mL	Percoll	30 mL
	0,36M NaCl	33.9 mL
1,075 g/mL	Percoll	45 mL
	0,46M NaCl	31.9 mL

7.5 – *In vitro* exposure of bacterium

DTT 2 mL:

DTT	0.77 g
RNase-free water	2 mL

7.6 – RT-qPCR analysis

50 x TAE buffer 1000 mL:

Tris base	242 g
0.5M EDTA	100 mL
Acetic acid	57.1 mL
Milli-Q	Until 1000 mL

1 x TAE buffer 1000 mL:

50 x TAE buffer	20 mL
Milli-Q	980 mL

1 % Agarose gel 25 mL:

SeaKem® LE Agarose	0.25 g
1 x TAE buffer	25 mL

5 x loading buffer 50 mL:

Bromophenol blue (Merck)	125 g
0.5M EDTA	10 mL
Glycerol (Sigma Aldrich)	17.24 mL
Milli-Q	22.75 mL

1kb+ ladder 180 µl:

1kb (Invitrogen)	30 µl
5 x loading buffer	30 µl
RNase- free water	120 µl

Example 1 of DNase treatment:

RNA volume	27.10 µl
10 x reaction buffer	3.39 µl
DNase I	3.39 µl
Master mix for DNase treatment	6.78 µl
STOP-solution	3.39 µl

Example 2 of DNase treatment:

RNA volume	44 µl
10 x reaction buffer	9.60 µl
DNase I	9.60 µl
RNase-free water	32.78 µl
Master mix added	19.19 µl
STOP-solution	9.60 µl

Example of master mix for RT-qPCR

SYBR green	16.67 µl
Forward primer	1.33 µl
Reverse primer	1.33 µl
RNase-free water	0.67 µl

8. Appendix B

Tables from the results of this study.

Table 16 *– An overview of weights collected of 30 random fish at the time of vaccination, resulting in an average weight of approximately 26 g.*

Weight in grams									
26	29	27	23	31	26	21	26	31	23
30	27	23	32	27	26	25	26	31	29
21	29	22	28	26	27	19	23	28	21

Table 17 *– An overview of detected positive Cp-values obtained by performing RT-qPCR of liver samples from fish dead from challenge with* P. salmonis. *Up to 33 % of fish from each group/tank were randomly sampled over time to confirm the presence of* P. salmonis *in dead fish. The analysis was performed by PHARMAQ Anlaytiq to confirm death by pathogen.*

T	Fish nr.	PBS	AJ5-1	AJ5-1 + LiVac	AJ5-1 + LiVac (IA)	AJ5-1 + LiVac (SO)	AJ5-1 + LiVac (8 °C)
1	F1	16.96	13.78	17.70	22.63	17.18	19.52
	F2	16.56	17.43	15.73	18.32	18.44	17.61
	F3	20.68	22.03	19.36	17.28	14.86	17.61
	F4	19.35	17.77	-	16.84	23.52	16.1
	F5	18.70	19.02	-	18.18	-	17.85
2	F1	17.81	19.58	18.41	15.70	20.97	22.61
	F2	17.73	16.64	-	17.67	18.43	19.83
	F3	17.07	20.55	-	24.06	18.70	17.63
	F4	19.15	20.25	-	17.93	16.68	18.83
	F5	16.40	15.91	-	19.81	17.08	19.32

Table 18 *- Statistical analyses of survival after experimental challenge of vaccinated fish. Statistical differences in cumulative mortality (%) when comparing a vaccinated group against another vaccinated group is calculated at the time of 60 % mortality in the PBS control group and at termination of the experiment. C.m* is the cumulative mortality in percentage (%) for the vaccinated control group, while C.m.** is the cumulative mortality in percentage (%) for the other vaccine group (*/**).* x^2 *is Yates' continuity corrections in Pearson's Chi-Squared test. Differences in cumulative mortality (%) was significant if p-values < significance level,* $\alpha < 0.05$*. All significant p-values are in bold. */** .*

		60 % mortality				**Termination**			
T	Vaccine group vs. Vaccine group	C.m.*	C.m.**	X^2	p-value	C.m.*	C.m.**	X^2	p-value
1	AJ5-1 / LiVac	13	0	0.5	0.4642	88	0	22.6	**$2x10^{-6}$**
	AJ5-1 / AJ5-1+LiVac	13	6	0	1	88	19	13.6	**0.0002**
	AJ5-1 / AJ5-1 +LiVac (IA)	13	13	0	1	88	69	0.96	0.327
	AJ5-1 / AJ5-1 +LiVac (SO)	13	0	0.5	0.4642	88	27	11.3	**0.0008**
	AJ5-1 / AJ5-1 +LiVac (8ºC)	13	40	1.5	0.2155	88	93	0	1
	LiVac / AJ5-1+LiVac	0	6	0	1	0	19	1.5	0.2235
	LiVac / AJ5-1+LiVac (IA)	0	13	0.5	0.4642	0	69	14.4	**0.0002**
	LiVac / AJ5-1+LiVac (SO)	0	0	NaN	NA	0	27	2.6	0.1071
	LiVac / AJ5-1+LiVac (8ºC)	0	40	5.2	**0.02**	0	93	22.6	**$2x10^{-6}$**
	AJ51+LiVac / AJ51+LiVac (IA)	6	13	0	1	19	69	6.6	**0.0104**
	AJ51+LiVac / AJ51+LiVac (SO)	6	0	0	1	19	27	0	1
	AJ51+LiVac / AJ51+LiVac(8ºC)	6	40	3.0	0.0842	19	93	13.6	**0.0002**
	AJ51+LiVac (IA) / AJ51+LiVac (SO)	13	0	0.5	0.4642	69	27	4.80	**0.0285**
	AJ51+LiVac (IA) / AJ51+LiVac (8ºC)	13	40	1.5	0.2155	69	93	1.0	0.3272
	AJ5-1+LiVac (SO)/ AJ51+LiVac(8ºC)	0	40	5.2	**0.0225**	27	93	11.3	**0.0008**
2	AJ5-1 / LiVac	13	0	0.5	0.4642	88	0	22.6	**$2x10^{-6}$**
	AJ5-1 / AJ5-1+LiVac	13	0	0.5	0.4642	88	7	19.2	**$1.8x10^{-5}$**
	AJ5-1 / AJ5-1 +LiVac (IA)	13	7	0	1	88	71	1.9	0.1709
	AJ5-1 / AJ5-1 +LiVac (SO)	13	0	0.5	0.4642	88	33	9.2	**0.0024**
	AJ5-1 / AJ5-1 +LiVac (8ºC)	13	80	10.9	**0.0010**	88	100	0	1
	LiVac / AJ5-1+LiVac	0	0	NaN	NA	0	7	0	1
	LiVac / AJ5-1+LiVac (IA)	0	7	0	1	0	71	12.2	**0.0005**
	LiVac / AJ5-1+LiVac (SO)	0	0	NaN	NA	0	33	3.8	**0.0500**
	LiVac / AJ5-1+LiVac (8ºC)	0	80	16.8	**$4.1x10^{-5}$**	0	100	26.1	**$3.2x10^{-7}$**
	AJ51+LiVac / AJ51+LiVac (IA)	0	7	0	1	7	71	9.2	**0.0024**
	AJ51+LiVac / AJ51+LiVac (SO)	0	0	NaN	NA	7	33	1.9	0.1709
	AJ51+LiVac / AJ51+LiVac(8ºC)	0	80	16.8	**$4.1x10^{-5}$**	7	100	22.6	**$2x10^{-6}$**
	AJ51+LiVac (IA) / AJ51+LiVac (SO)	7	0	0	1	71	33	2.1	0.1441
	AJ51+LiVac (IA) / AJ51+LiVac (8ºC)	7	80	13.6	**0.0002**	71	100	3.8	**0.0500**
	AJ5-1+LiVac (SO)/AJ51+LiVac(8ºC)	0	80	16.8	**$4.1x10^{-5}$**	33	100	12.2	**0.0005**

Table 19 *– A one-way ANOVA was performed in Graphpad using Tukey`s multiple comparisons test of ELISA results, comparing vaccinated groups with PBS control group and baseline with regards on sampling timepoint, Summary of significance and adjusted P-value. (14 = 14 days post vaccination, 24 = 24 dpv, 48 = 48dpv) *p<0.05, ****p<0.0001 and p>0.05 = n.s.*

Tukey`s multiple comparisons test	Summary of significance	Adjusted P-value
AJ5-1 (14) vs Baseline	****	<0.0001
AJ5-1 (14) vs PBS (14)	*	0.0144
AJ5-1 (24) vs Baseline	****	<0.0001
AJ5-1 (24) vs PBS (24)	****	<0.0001
AJ5-1 (48) vs Baseline	****	<0.0001
AJ5-1 (48) vs PBS (48)	****	<0.0001
LiVac (14) vs Baseline	***	0.0006
LiVac (14) vs PBS (14)	n.s.	0.4893
LiVac (24) vs Baseline	****	<0.0001
LiVac (24) vs PBS (24)	****	<0.0001
LiVac (48) vs Baseline	****	<0.0001
LiVac (48) vs PBS (48)	****	<0.0001
AJ5-1 + LiVac (14) vs Baseline	****	<0.0001
AJ5-1 + LiVac (14) vs PBS (14)	**	0.0046
AJ5-1 + LiVac (24) vs Baseline	****	<0.0001
AJ5-1 + LiVac (24) vs PBS (24)	****	<0.0001
AJ5-1 + LiVac (48) vs Baseline	****	<0.0001
AJ5-1 + LiVac (48) vs PBS (48)	****	<0.0001
AJ5-1 + LiVac (IA) (14) vs Baseline	***	0.0003
AJ5-1 + LiVac (IA) (14) vs PBS (14)	n.s.	0.1256
AJ5-1 + LiVac (IA) (24) vs Baseline	****	<0.0001
AJ5-1 + LiVac (IA) (24) vs PBS (24)	****	<0.0001
AJ5-1 + LiVac (IA) (48) vs Baseline	****	<0.0001
AJ5-1 + LiVac (IA) (48) vs PBS (48)	****	<0.0001
AJ5-1 + LiVac (8 °C) (14) vs Baseline	n.s.	0.0883
AJ5-1 + LiVac (8 °C) (14) vs PBS (14)	n.s.	0.9992
AJ5-1 + LiVac (8 °C) (24) vs Baseline	****	<0.0001
AJ5-1 + LiVac (8 °C) (24) vs PBS (24)	*	0.0467
AJ5-1 + LiVac (8 °C) (48) vs Baseline	****	<0.0001
AJ5-1 + LiVac (8 °C) (48) vs PBS (48)	****	<0.0001

Table 20 *– A one-way ANOVA was performed in Graphpad using Tukey`s multiple comparisons test comparing the different vaccine groups based on sampling timepoint after ELISA analysis. Showing the summary of significance and adjusted P-value. *p<0.05, ****p<0.001 and p>0.05 = n.s.*

Sampling timepoint	Tukey`s multiple comparisons test	Summary of significance	Adjusted P- value
14 dpv	AJ5-1 vs LiVac	n.s.	>0.9999
	AJ5-1 vs AJ5-1 + LiVac	n.s.	>0.9999
	AJ5-1 vs AJ5-1 + LiVac (IA)	n.s.	>0.9999
	AJ5-1 vs AJ5-1 + LiVac (8 ºC)	****	<0.0001
	AJ5-1 vs PBS	****	<0.0001
	LiVac vs AJ5-1 + LiVac	n.s.	>0.9999
	LiVac vs AJ5-1 + LiVac (IA)	n.s.	>0.9999
	LiVac vs AJ5-1 + LiVac (8 ºC)	****	<0.0001
	LiVac vs PBS	****	<0.0001
	AJ5-1 + LiVac vs AJ5-1 + LiVac (IA)	n.s.	>0.9999
	AJ5-1 + LiVac vs AJ5-1 + LiVac (8 ºC)	****	<0.0001
	AJ5-1 + LiVac vs PBS	****	<0.0001
	AJ5-1 + LiVac (IA) vs AJ5-1 + LiVac (8 ºC)	****	<0.0001
	AJ5-1 + LiVac (IA) vs PBS	****	<0.0001
	AJ5-1 + LiVac (8 ºC) vs PBS	n.s.	>0.9999
24 dpv	AJ5-1 vs LiVac	n.s.	>0.9999
	AJ5-1 vs AJ5-1 + LiVac	n.s.	>0.9999
	AJ5-1 vs AJ5-1 + LiVac (IA)	n.s.	0.5139
	AJ5-1 vs AJ5-1 + LiVac (8 ºC)	*	0.0119
	AJ5-1 vs PBS	***	0.0001
	LiVac vs AJ5-1 + LiVac	n.s.	0.9899
	LiVac vs AJ5-1 + LiVac (IA)	n.s.	0.6766
	LiVac vs AJ5-1 + LiVac (8 ºC)	**	0.0056
	LiVac vs PBS	****	<0.0001
	AJ5-1 + LiVac vs AJ5-1 + LiVac (IA)	n.s.	>0.9999
	AJ5-1 + LiVac vs AJ5-1 + LiVac (8 ºC)	*	0.0122
	AJ5-1 + LiVac vs PBS	***	0.0001
	AJ5-1 + LiVac (IA) vs AJ5-1 + LiVac (8 ºC)	****	<0.0001
	AJ5-1 + LiVac (IA) vs PBS	****	<0.0001
	AJ5-1 + LiVac (8 ºC) vs PBS	n.s.	0.7060
48 dpv	AJ5-1 vs LiVac	*	0.0476
	AJ5-1 vs AJ5-1 + LiVac	n.s.	0.9770
	AJ5-1 vs AJ5-1 + LiVac (IA)	n.s.	0.6675
	AJ5-1 vs AJ5-1 + LiVac (8 ºC)	*	0.0170
	AJ5-1 vs PBS	****	<0.0001
	LiVac vs AJ5-1 + LiVac	n.s.	0.2362
	LiVac vs AJ5-1 + LiVac (IA)	n.s.	0.6798
	LiVac vs AJ5-1 + LiVac (8 ºC)	n.s.	0.9988
	LiVac vs PBS	****	<0.0001
	AJ5-1 + LiVac vs AJ5-1 + LiVac (IA)	n.s.	0.9723
	AJ5-1 + LiVac vs AJ5-1 + LiVac (8 ºC)	n.s.	0.1078
	AJ5-1 + LiVac vs PBS	****	<0.0001
	AJ5-1 + LiVac (IA) vs AJ5-1 + LiVac (8 ºC)	n.s.	0.4349
	AJ5-1 + LiVac (IA) vs PBS	****	<0.0001
	AJ5-1 + LiVac (8 ºC) vs PBS	****	<0.0001

Table 21 *– An overview of baseline samples collected from liver and HK tissue prior to vaccination was all negative to* P. salmonis.

Fish nr.	Tissue 1.	Tissue 2.	Result	Cq-value
F1	Liver	HK	Not Detected	-
F2			Not Detected	-
F3			Not Detected	-
F4			Not Detected	-
F5			Not Detected	-
F6			Not Detected	-
F7			Not Detected	-
F8			Not Detected	-

Table 22 *- Overview of the filtered group size of each vaccine group and PBS control group after RT-qPCR analysed for the different assays. The groups (UE =unexposed, E = exposed to P. salmponis) have a filtered group size varying between 3-5 fish per group, exceptions are marked with a *.*

Groups	RPS20	sIgM	Cas-1	GATA3	IL-18	Perforin	Tbet	IL-4/13a
PBS (UE)	4	4	4	4	4	4	4	4
AJ5-1 (UE)	5	5	4	4	3	4	5	5
LiVac (UE)	5	5	5	5	5	5	5	4
AJ5-1 + LiVac (UE)	5	5	4	4	5	4	5	5
AJ5-1 + LiVac (IA, UE)	3	3	3	3	3	3	*2	* 1
PBS (E)	4	4	4	4	4	4	3	3
AJ5-1 (E)	5	5	5	4	3	5	4	*2
LiVac (E)	4	4	4	4	4	4	4	*2
AJ5-1 + LiVac (E)	4	4	3	3	3	3	3	3
AJ5-1 + LiVac (IA, E)	3	3	3	*2	*2	3	3	*2

Table 23 *– Statistical analysis using A one-way ANOVA was performed by Tukey`s multiple comparisons test in Graphpad Prism, showing the adjusted P value. * = p<0.05 (marked in bold), E = exposed to* P. salmonis*, where the MNE of the different vaccine groups exposed to* P. salmonis *are folded against their respective unexposed vaccine group.*

Comparing every group with each other:	sIgM	Cas-1	GATA3	IL-18	Perforin	Tbet	IL-4/13a
PBS (E) vs. AJ5-1 (E)	>0.999	0.9991	0.9995	0.9933	0.7250	0.8991	*** 0.0330**
PBS (E) vs. LiVac (E)	0.9934	0.6376	0.9576	0.9716	0.9068	0.9924	0.7958
PBS (E) vs. AJ5-1 + LiVac (E)	>0.9999	0.8473	0.9680	0.9141	0.9851	0.9596	>0.9999
PBS (E) vs. AJ5-1 + LiVac (IA, E)	0.9983	0.9764	0.9829	>0.9999	0.9939	>0.9999	0.3584
AJ5-1 (E) vs. LiVac (E)	0.9877	0.7171	0.9890	0.9999	0.9967	0.9423	*** 0.0382**
AJ5-1 (E) vs. AJ5-1 + LiVac (E)	0.9997	0.9073	0.9919	0.9930	0.9718	0.9961	*** 0.0223**
AJ5-1 (E) vs. AJ5-1 + LiVac (IA, E)	0.9990	0.9952	0.9531	0.9954	0.9460	0.8398	*** 0.0161**
LiVac (E) vs. AJ5-1 + LiVac (E)	0.9984	0.9983	>0.9999	0.9979	0.9983	0.9971	0.7584
LiVac (E) vs. AJ5-1 + LiVac (IA, E)	0.9627	0.9457	0.8209	0-9834	0.9941	0.9960	0.2043
AJ5-1 + LiVac (E) vs. AJ5-1 + LiVac (IA, E)	0.9939	0.9921	0.8457	0.9446	>0.9999	0.9661	0.4288

Table 24 *– Statistical analysis using A one-way ANOVA was performed by Tukey`s multiple comparisons test in Graphpad Prism, showing the adjusted P value. * = p<0.05 (marked in bold), UE= unexposed, E = exposed to* P. salmonis*. The MNE of all exposed/unexposed vaccine groups and exposed PBS control group are foldet against the unexposed PBS control group.*

Comparing every group with each other:	sIgM	Cas-1	GATA3	IL-18	Perforin	Tbet	IL4/13a
PBS (UE) vs. PBS (E)	0.9875	>0.9999	0.9942	0.9904	0.9979	>0.9999	>0.9999
PBS (UE) vs. AJ5-1 (UE)	0.9996	0.7340	0.8355	>0.9999	>0.9999	0.4159	0.9962
PBS (UE) vs. AJ5-1 (E)	0.7102	0.9011	0.9898	0.9989	0.9996	0.9982	0.9735
PBS (UE) vs. LiVac (UE)	>0.9999	0.9984	>0.9999	0.6481	0.8706	>0.9999	>0.9999
PBS (UE) vs. LiVac (E)	0.6778	0.7342	0.9596	0.9990	0.9661	0.9983	>0.9999
PBS (UE) vs. AJ5-1 + LiVac (UE)	0.9998	0.9998	0.9108	>0.9999	0.9878	0.1805	0.7920
PBS (UE) vs. AJ5-1 + LiVac (E)	0.6300	0.9828	>0.9999	0.9992	0.9921	0.7267	>0.9999
PBS (UE) vs. AJ5-1 + LiVac (IA, UE)	0.9993	0.9152	>0.9999	0.9950	>0.9999	>0.9999	0.6207
PBS (UE) vs. AJ5-1 + LiVac (IA, E)	0.9053	0.6958	>0.9999	>0.9999	0.9613	0.9997	0.9827
PBS (E) vs. AJ5-1 (UE)	>0.0009	0.5281	0.3120	0.9902	0.9797	0.2497	0.9336
PBS (E) vs. AJ5-1 (E)	0.9988	0.7304	0.6636	>0.9999	0.8855	0.9597	0.8699
PBS (E) vs. LiVac (UE)	0.9972	0.9765	0.8851	0.1454	0.9994	>0.9999	0.9973
PBS (E) vs. LiVac (E)	0.9972	0.5280	>0.9999	0.7829	>0.9999	>0.9999	>0.9999
PBS (E) vs. AJ5-1 + LiVac (UE)	>0.9999	0.9938	0.4093	0.9990	0.7203	0.1042	0.5524
PBS (E) vs. AJ5-1 + LiVac (E)	0.9932	0.9217	0.9773	>0.9999	0.7919	0.5212	>0.9999
PBS (E) vs. AJ5-1 + LiVac (IA, UE)	>0.9999	0.7809	0.9766	0.7316	>0.9999	>0.9999	0.4507
PBS (E) vs. AJ5-1 + LiVac (IA, E)	>0.9999	0.5021	0.9740	>0.9999	>0.9999	>0.9999	0.8969
AJ5-1 (UE) vs. AJ5-1 (E)	0.9564	>0.9999	0.9998	0.9986	>0.9999	0.8860	>0.9999
AJ5-1 (UE) vs. LiVac (UE)	>0.9999	0.9764	0.9704	0.7917	0.6975	0.1704	>0.9999
AJ5-1 (UE) vs. LiVac (E)	0.9435	>0.9999	0.1847	0.9998	0.8741	0.0597	0.9958
AJ5-1 (UE) vs. AJ5-1 + LiVac (UE)	>0.999	0.9638	>0.9999	>0.9999	0.9992	0.9999	0.9958
AJ5-1 (UE) vs. AJ5-1 + LiVac (E)	0.9094	0.9998	0.9637	0.9989	0.9994	>0.9999	0.9983
AJ5-1 (UE) vs. AJ5-1 + LiVac (IA, UE)	>0.9999	>0.9999	0.9646	0.9984	0.9986	0.5898	0.8807
AJ5-1 (UE) vs. AJ5-1 + LiVac (IA, E)	0.9955	>0.9999	0.9946	>0.9999	0.8741	0.2064	>0.9999
AJ5-1 (E) vs. LiVac (UE)	0.7898	0.9991	>0.9999	0.2998	0.4167	0.9648	0.9956
AJ5-1 (E) vs. LiVac (E)	>0.9999	>0.9999	0.4716	0.9082	0.6599	0.7291	0.9737
AJ5-1 (E) vs. AJ5-1 + LiVac (UE)	0.9410	0.9974	>0.9999	>0.9999	>0.9999	0.6045	>0.9999
AJ5-1 (E) vs. AJ5-1 + LiVac (E)	>0.9999	>0.9999	0.9996	>0.9999	>0.9999	0.9811	0.9827
AJ5-1 (E) vs. AJ5-1 + LiVac (IA, UE)	0.9933	>0.9999	0.9996	0.8651	0.9829	0.9971	0.9891
AJ5-1 (E) vs. AJ5-1 + LiVac (IA, E)	>0.9999	0.9997	>0.9999	>0.9999	0.6911	0.9355	>0.9999
LiVac (UE) vs. LiVac (E)	0.7740	0.9767	0.7198	0.9707	>0.9999	0.9992	>0.9999
LiVac (UE) vs. AJ5-1 + LiVac (UE)	>0.9999	>0.9999	0.9913	0.3778	0.2652	0.0562	0.9317
LiVac (UE) vs. AJ5-1 + LiVac (E)	0.7070	>0.9999	>0.9999	0.3146	0.3653	0.4555	>0.9999
LiVac (UE) vs. AJ5-1 + LiVac (IA, UE)	>0.9999	0.9984	>0.9999	0.9972	0.9948	>0.9999	0.7433
LiVac (UE) vs. AJ5-1 + LiVac (IA, E)	0.9526	0.9612	>0.9999	0.6619	>0.9999	>0.9999	0.9977
LiVac (E) vs. AJ5-1 + LiVac (UE)	0.9265	0.9641	0.2526	0.9819	0.4560	* 0.0188	0.8642
LiVac (E) vs. AJ5-1 + LiVac (E)	>0.9999	0.9999	0.9109	0.9183	0.5538	0.1981	>0.9999
LiVac (E) vs. AJ5-1 + LiVac (IA, UE)	0.9891	>0.9999	0.9900	>0.9999	0.9996	0.9997	0.6502
LiVac (E) vs. AJ5-1 + LiVac (IA, E)	>0.9999	>0.9999	0.9160	0.9926	>0.9999	>0.9999	0.9817
AJ5-1 + LiVac (UE) vs. AJ5-1 + LiVac (E)	0.8867	>0.9999	0.9865	>0.9999	>0.9999	0.9994	0.8656
AJ5-1 + LiVac (UE) vs.AJ5-1 +LiVac (IA,UE)	>0.9999	0.9962	0.9871	0.9597	0.9153	0.3485	0.9898
AJ5-1 + LiVac (UE) vs. AJ5-1 + LiVac (IA, E)	0.9931	0.9411	0.9986	>0.9999	0.5129	0.0832	>0.9999
AJ5-1 + LiVac (E) vs. AJ5-1 + LiVac (IA, UE)	0.9795	>0.9999	>0.9999	0.8763	0.9352	0.7723	0.6661
AJ5-1 + LiVac (E) vs. AJ5-1 + LiVac (IA, E)	>0.9999	0.9992	>0.9999	>0.9999	0.5835	0.4388	0.9889
AJ5-1 + LiVac(IA, UE)vs.AJ5-1 +LiVac (IA,E)	0.9994	>0.9999	>0.9999	9807	0.9992	>0.9999	0.9847

Table 25 *– Fish were vaccinated and immunized at two temperatures, 8 °C and 12 °C, showing the day-degree (dd) at the different sampling timepoint post vaccination and at the day of challenge. After the 48 dpv sampling, fish were acclimatized to 15 °C prior to challenge.*

Sampling timepoint	Day-degree, 8°C	Day-degree, 12°C
0 (day of vaccination)	8	12
1 dpv	16	24
7 dpv	64	96
14 dpv	120	180
24 dpv	200	300
48 dpv	392	588
Challenge with *P. salmonis*	456	684