Report 32/2018 • Published November 2018

Validating a salmon lice vaccine candidate as a preventive measure against salmon lice at the lab-scale (Vaksine mot lakselus-laboratorietest)

Scientific final report

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Report

Title: **Validating a salmon lice vaccine candidate as a preventive measure against salmon lice at the lab-scale**	ISBN 978-82-8296-570-5 (pdf) ISSN 1890-579X *Report No.:* 32/2018
Tittel: **Vaksine mot lakselus – laboratorietest**	*Accessibility:* **Open**
Author(s)/Project manager: Jaya Kumari Swain, Lill-Heidi Johansen and Yamila Carpio González (CIGB, Cuba)	*Date:* 13.November 2018
Department: Produksjonsbiologi	*Number of pages and appendixes:* 36
Client: Fiskeri- og havbruksnæringens forskningsfond (FHF)	*Client`s ref.:* FHF 901461
Key words: TT-P0 vaccine, ecofriendly salmon lice control, reduced fecundity, Atlantic salmon, *Lepeophtheirus salmonis*	*Projectnr.:* 12211

English summary/recommendation:

Lepeophtheirus salmonis infections represents an important limitation to sustainable salmon farming today. Vaccines against these ectoparasites have the potential to be cost-effective means of controlling infestation and avoiding the disadvantages of chemical treatments. In this project the vaccine antigen TT-P0 potential against salmon lice was explored at lab-scale. Results showed good potential of TT-P0 vaccine in controlling these ectoparasites in 1st generation by affecting the adult female count and fecundity with greater presumptive potential effect in 2nd lice generation. This is a very important preliminary documentation of TT-P0 vaccine as preventive measure. Further validation is necessary to study long-term effectiveness.

Sammendrag/anbefalinger:

Infeksjoner med lakselus, *Lepeophtheirus salmonis,* representerer en av de viktigste begrensningene for bærekraftig lakseoppdrett i dag. Vaksiner mot disse ektoparasittene har potensiale til å være en kostnadseffektiv måte å kontrollere infeksjoner på og unngå mange av ulempene ved kjemiske behandlinger. Siden denne tilnærmingen fortsatt er i et tidlig stadium, fokuserte vi i dette prosjektet på å undersøke vaksineantigenet TT-P0 sitt potensiale mot lakselus i forsøk gjennomført i laboratorieskala. Resultatene fra vaksineforsøket med påfølgende smitteforsøk med lakselus, viste at TT-P0 vaksinen har et godt potensial for å kontrollere lakselus ved at den påvirker antall hunnlus og deres reproduksjonsevne i første generasjon med påfølgende større effekt på deres avkom, dvs andre generasjons lakselus. Undersøkelser av fisken ved bruk av Speilberg score viste at vaksinen ga lite bieffekter. Dette er en svært viktig foreløpig dokumentasjon av TT-P0 vaksinen som et forebyggende tiltak som vil kunne bidra til en fremtidig bærekraftig og lønnsom vekst i laksindustrien. Videre validering av vaksinen er imidlertid nødvendig for å undersøke den langsiktige effektiviteten i å kontrollere lakseluseinfeksjoner på en miljøvennlig og bærekraftig måte.

Preface

Kjell Maroni has been FHF's main contact person and observer.

We thank to FHF for the financing support of 1611 000 kroner. We also like to thank Kjell Maroni for formative and constructive discussions, cooperation and follow up.

The FHF appointed reference group for the project which consisted of:

Merete Bjørgan Schrøder (Norway Royal Salmon), merete.schroder@salmon.no

Michael Niesar (Sulefisk), michael.niesar@sulefisk.no

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We also thank reference group for their formative and constructive discussions and cooperation.

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1 Summary (both in english and Norwegian)/Sammendrag (både på norsk og engelsk)

English Summary

Infections with the salmon louse *Lepeophtheirus salmonis* (*L. salmonis*) represents one of the most important limitations to sustainable Atlantic salmon (*Salmo salar*) farming today. The parasite can exert direct and indirect negative impacts on health, growth and welfare of farmed fish and there are concerns of the impact on wild salmon populations. Current controls are mainly based on combination of prophylactic measures using cleaner fish, fallowing zones, non-medicinal controls using thermal and mechanical delousing and reduced use of chemical treatment due to the widespread evolved parasite resistance. However, the increased frequency of treatment methods and increased use of non-medicinal controls has led to considerable increase in production costs, injury, handling stress, mortality, risk of secondary infections and thus poor welfare. This has increased the necessity to develop sustainable non-medicinal therapies to combat the problem. Thus, our project addresses this issue in putting a step forward towards finding a non-medicinal, ecofriendly and cost-effective solution by validating a potential salmon lice vaccine candidate TT-P0 (Ribosomal protein P0 derived peptide fused to promiscuous T-cells epitopes) at the laboratory scale. To test the efficacy of the TT-P0 vaccine, the fish were vaccinated with TT-P0 + Montanide ISA50 V2 either intraperitoneally (i.p.) or both i.p. and by immersion. A group injected with oil adjuvant alone served as control. All the groups were challenged with about 35 copepodites per fish, 69 days after 1st vaccine administration. Lice count and sampling of blood, mucus and tissues (spleen, head kidney and skin) were done at chalimus, pre-adult (PA) and adult stage post infection with copepodites. The same experimental conditions, feeding and light regime were maintained throughout the experiment. The results from the challenge experiment showed that the vaccine mostly affected the numbers of females and female fecundity in the 1st generation, which resulted in delayed hatching pattern and reduced copepodite count in the 2nd generation. This indicates a larger impact on 2nd parasite generation by reduced re-infection ability and re-infection load, delayed production of parasite transmission stages via less females, delayed reproduction, and more decreased fecundity thus eventually leading to exponential decrease in lice re-infection load. On the other hand, vaccine showed minimal side effects with overall higher upregulation of IgM transcripts levels with corresponding higher specific IgM antibody titers against P0 antigen in serum and mucus of the vaccinated groups as compared to the control. Similarly, transcript levels of IgT was also significantly induced in lymphoid tissues and skin of the vaccinated group compared to control at all the sampling points post lice infestation. Moreover, the gene expression of IgM and IgT in all the tissues were significantly correlated. Thus, the result provided new insight into the crosstalk of mucosal and systemic immunty. In addition, expression of pro-inflammatory cytokines and T-cell related genes were also stimulated to some extent and were significantly correlated with each other, thus reflecting their important role in host-parasite interaction and mode of protection. To conclude, antibody levels, transcriptional levels and sampling results post challenge reveals the potential effectiveness of TT-P0 antigen as a good vaccine candidate against salmon lice (*L. salmonis*) with minimal side effects. This is a very important preliminary documentation of TT-P0 vaccine as preventive measure for future sustainable and profitable growth of salmon industry. However, further validation of this vaccine is neccessary to follow the potential of long term effectiveness in controlling salmon lice infestation in a eco-friendly and sustainable manner to be utilized by the salmon industry and vaccine companies nationally and internationally with good social acceptance.

Norsk sammendrag

Infeksjoner med lakselus *Lepeophtheirus salmonis* (*L. salmonis*) representerer en av de viktigste begrensningene for bærekraftig oppdrett av atlantisk laks (*Salmo salar*) i dag. Parasitten kan ha direkte og indirekte negative virkninger på helse, vekst og velferd hos oppdrettsfisk, og det er bekymringer for virkningen på villlakspopulasjoner. Nåværende kontroller er hovedsakelig basert på en kombinasjon av profylaktiske tiltak ved bruk av rensefisk, brakkleggingssoner, ikke-medikamentell behandling ved bruk av termisk og mekanisk avlusing og med redusert bruk av kjemisk behandling på grunn av den utbredte resistensutviklingen. Den økte frekvensen av behandlinger og økt bruk av ikke-medikamentelle kontroller har imidlertid ført til en betydelig økning i produksjonskostnader, skader, håndteringsstress, dødelighet, risiko for sekundære infeksjoner og dermed dårlig velferd. Dette har økt behovet for å utvikle bærekraftige ikke-medikamentelle produkter for å bekjempe luseproblemet. Dette prosjektet tar derfor tak i problemet ved å ta et skritt fremover for å finne en ikke-medikamentell miljøvennlig og kostnadseffektiv løsning, ved å validere en potensiell lakselus vaksinekandidat, TT-P0 (Ribosomal protein P0-avledet peptid fusjonert til promiskuøse T-celle epitoper), i laboratorieskala. For å teste effekten av TT-P0 vaksinen ble fisken vaksinert med TT-P0 + Montanide ISA50 V2 (oljeadjuvans) enten intraperitonealt (i.p) eller både i.p og bad. En gruppe injisert med oljeadjuvans alene fungerte som kontroll. Alle gruppene ble smittet med 35 kopepoditter per fisk 69 dager etter første vaksinering. Etter smitte ble det utført lusetellinger og prøvetaking av blod, mucus og vev (milt, nyre og hud) ved chalimus, pre-adult (PA) og adult stadium. De samme eksperimentelle forholdene, fôrings- og lysregime ble opprettholdt gjennom hele forsøket. Resultatene fra smitteforsøket viste at vaksinen hovedsakelig påvirket antall hunnlus og deres reproduksjonsevne i 1. generasjon, noe som resulterte i forsinket klekkemønster og redusert antall kopepoditter i 2. generasjon. Dette indikerer en større innvirkning på 2. parasittgenerasjon ved redusert re-infeksjonsevne og re-infeksjonsmengde, forsinket produksjon av ulike parasitt utviklingsstadier via mindre hunnlus, forsinket reproduksjon og mer nedsatt reproduksjonsevne, som dermed til slutt fører til en eksponensiell reduksjon i omfanget av lusens reinfeksjon. På den annen side gav vaksinen minimale bivirkninger og med gjennomgående høyere oppregulering av IgM-transkripsjonsnivåer med tilsvarende høyere spesifikke IgM antistoff titere mot P0 antigenet i serum og slim i de vaksinerte gruppene sammenlignet med kontrollen. På samme måte ble transkripsjonsnivåer av IgT også signifikant indusert i lymfoide vev og hud i den vaksinerte gruppen sammenlignet med kontrollen ved alle prøvetakingspunkter etter luseinfeksjon. Videre var genuttrykket av IgM og IgT i alle vevene signifikant korrelert. Resultatet ga således ny innsikt i samtidig aktivering av mukosal og systemisk immunitet. I tillegg ble uttrykket av proinflammatoriske cytokiner og T-cellerelaterte gener stimulert i en viss grad og var betydelig korrelert med hverandre, og reflekterer derved deres viktige rolle i verts-parasittinteraksjonen og beskyttelsesmodus. Oppsummert viser antistoffnivåer, transkripsjonsnivåer og analyseresultater etter lusesmitte TT-P0 antigenets potensielle effekt som en god vaksinekandidat mot lakselus med minimale bivirkninger. Dette er en svært viktig foreløpig dokumentasjon av TT-P0 vaksinen som forebyggende tiltak for fremtidig bærekraftig og lønnsom vekst i laksindustrien. Imidlertid er ytterligere validering av denne vaksinen nødvendig for å se på potensialet for langsiktig effektivitet i å kontrollere lakselusinfeksjoner på en miljøvennlig og bærekraftig måte, noe som kan benyttes av lakseindustri og vaksineprodusenter nasjonalt og internasjonalt med god sosial aksept.

2 Introduction

2.1 Background

Atlantic salmon (*Salmo salar*) is farmed in twelve countries around the world, with Norway, Chile and Scotland being the top three producers, accounting for 87 % of the total farmed Atlantic salmon production. Norway, by far the world's largest producer, currently accounts for 60 % of the world's total at 1.2 million metric tonnes. Global production of Atlantic salmon reached 2.07 million tons in 2014 (1), but losses due to salmon lice (*Lepeophtheirus salmonis*) are limiting industry growth and compromising its sustainability (2). Salmon lice control has cost salmon farmers more than €305 million per year during the last decade (3) and escalated to €430 million in Norway alone during 2015 (4), not including loss of productivity. Costs are set to increase as there is no effective vaccine against salmon lice at the moment. Only a small number of antiparasitic therapeutants are currently licensed for treatment and these are losing their efficacy due to evolved parasite resistance (5, 6). This has increased the necessity to develop new and alternative therapies to combat the problem and aquaculture companies and governments around the world are striving to address these issues. The search for non-medicinal solutions for salmon lice control, including commercial production of cleaner fish, is the current focus of research.

Research are prioritized based on the need to generate knowledge and solutions for socially, economically and environmentally sustainable growth and better health management in the Norwegian aquaculture industry to maintain and further develop Norway's position as the world's leading seafood nation. While all diseases are important, the salmon lice problem is foremost on the agenda of salmon producers because of cost and challenges and losses due to delousing and other handling stress involved in chemical and mechanical treatments, development of resistance to medications by the lice, possible transfer of lice to the wild populations and issues with social acceptance and fish welfare.

Several initiatives have been taken to encourage the development of new strategies, such as vaccines. Vaccines against the caligid copepod, *L. salmonis*, have the potential to be a cost-effective means of controlling the infection and avoid many of the disadvantages of medicine treatments including handling stress, development of resistance and environment pollution. Vaccination has been proposed as a means of controlling salmon lice following advances in the development of vaccines against ectoparasites of mammals, for example vaccines against ticks (7).

More recently, Carpio et al. (unpublished results) tested in salmon a vaccine candidate based on ribosomal protein, P0 fused to my32 in an immunization-challenge experiment and got between 28-35% reductions of parasites in the first parasite generation. With another variation of the vaccine candidate TT-P0, they got higher IgM antibody titers against P0 than with P0-my32 in tilapia. Based on this present knowledge, we collaborated with Dr. Carpio to introduce the new vaccine candidate TT-PO in this project proposal for its validation in salmon as an eco-friendly preventive measure against salmon lice at the lab-scale under Norwegian conditions.

Since, a good salmon lice vaccine is still not available in Norwegian aquaculture and validation of vaccine candidates is a demanding urge at present, our effort is the innovative approach of validating the new vaccine candidate TT-P0 (developed by Carpio group in Cuba) effectiveness in the Norwegian salmon against lice (*L. salmonis*).

2.2 Project scope

The main goals and sub-goals of the projects has been:

Main Goal: Validating the effectiveness of the vaccine candidate (based on a peptide derived from ribosomal protein P0) as a preventive measure against Norwegian salmon lice at the laboratory scale.

Sub-goal 1: To test the efficacy of the P0 recombinant subunit vaccine against salmon lice in Norwegian salmon in an immunization.

Sub-goal 2: To test the efficacy of the P0 recombinant subunit vaccine against salmon lice at different development stage in a challenge experiment.

Sub-goal 3: To find the important indicators or correlates of protection associated with protection against lice infestations.

Sub-goal 4: Evaluation and publishing of the results obtained and timely communication and reporting.

Based on each sub-goals, the project was divided into 4 work-packages.

WP1: Testing the efficacy of the vaccine during immunization trial by testing antibody titers against the antigen.

- Antibody titers of IgM and IgT in serum and mucus shall be measured to correlate protection.
- Side-effects of vaccine to be studied.

WP2: Testing the efficacy for the protection against salmon lice in the first generation cycle and also to follow its effect on the early next generation based on following parameters:

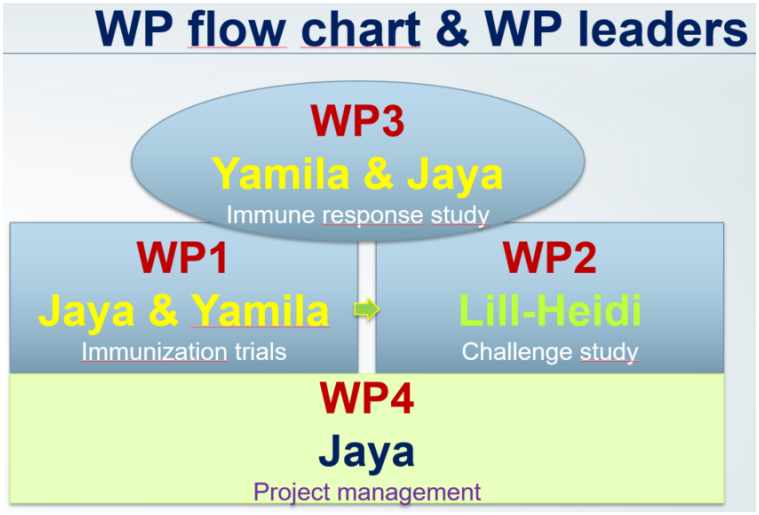
- Relative percent protection (RPP) with respect to different stages of the lice life-cycle,
- Effect on the number of adult males and female.
- Effect on female fecundity by measuring the number of adult females having the egg strings, number of egg strings per female and the length of the egg strings.
- 2^{nd} generation copepodite hatching effects and counts.

All the above parameters will add up to determine the efficacy of the vaccine.

WP3: To find out important markers associated with protection against salmon lice. Gene expression studies through quantitative real-time PCR (qPCR) will show specific clues about the mechanism of action of protection at the transcript level and the interaction of the genes for protection against sea lice when correlated with the antibody titers and lice count.

WP4: Communication and dissemination: Communication with the FHF contact person and the reference group will be done by the Project leader. Meetings will be done at several intervals. Minutes from the meeting, project status and reports will be timely delivered. Publication in open access journal and presentation of results in relevant conferences were planned be done whenever possible.

The fulfilled goals and results obtained from this proposed project will be verified in the next step at the small field scale in salmon farms under natural conditions in the follow-up project, which will be beneficial for the salmon industry for sustainable production and welfare and also for the next step commercialization of the vaccine candidate.



2.3 Project Group

1. Jaya Kumari Swain (Project Investigator) - Nofima, Tromsø, jaya.kumari@nofima.no; new email id: jaya.k.swain@uit.no
2. Lill-Heidi Johansen - Nofima, Tromsø, lill-heidi.johansen@nofima.no
3. Yamila Carpio González - Centre for Genetic Engineering & Biotechnology, Havana, Cuba, yamila.carpio@cigb.edu.cu

The reference group appointed by FHF as mentioned before for this project were:

1. Merete Bjørgan Schrøder (Norway Royal Salmon), merete.schroder@salmon.no
2. Michael Niesar (Sulefisk), michael.niesar@sulefisk.no
3. Solveig Gaasø (Gåsø Næringsutvikling AS), solveig.gaaso@froygruppen.no

The main objective of the reference group were to contribute to the project's relevance and benefit to the industry and assess a plan for utilization of the project results. They are responsible for the advisory role in the project and will assist the project professionally and be a natural discussion partner before major changes are decided and implemented. Moreover, they will also help to ensure that the project retains the relevance embodied in the project description.

3 Issue and purpose

The salmon louse *Lepeophtheirus salmonis* (Copepoda, Caligidae) is an important parasite in the salmon farming industry causing annual losses of hundreds of millions of dollars (US) worldwide. The parasite exerts negative impacts on health, growth and welfare of farmed fish and there are concerns of the impact on wild salmon populations. The main control measures have traditionally been pharmaceutically based, but increasing levels of resistance have led to a situation in which non-pharmaceutical methods now dominate. Although farmers now commonly use a combination of preventative measures including continual delousing (mainly cleaner fish) and both pharmaceutical and non-pharmaceutical methods, a sustainable and long lasting effect is on continuous demand for better welfare and sustainable growth of the aquaculture industry (8). Therefore, this research project will produce information directly relevant to the key issue that is faced by the salmon aquaculture industry i.e. salmon lice for long lasting eco-friendly protection potential and the better understanding of the mechanism of immune response.

Our results can be taken to the production level since the CIGB have the necessary facilities and experience to produce veterinary vaccines at large-scale. Additionally, it can establish a direct link with an enterprise selling veterinary vaccines in Norway in order to guarantee filling Norwegian regulations to market the vaccine more straightforward. This will help to add quality, marketing and increase slaughter volume and reputation to the Norwegian aquaculture nationally as well as internationally. Thus, the outcome from this project will be directly useful for salmon farmers or stakeholders, researchers and consumers.

The result will have future potential in controlling salmon lice and its commercial application in salmon farming will improve welfare of salmon and salmon industry: i) Gained knowledge about the efficacy of a salmon lice vaccine against different developmental stage of salmon lice infestation in Atlantic salmon. ii) The outcome from the work packages will be useful for the salmon farming industry and its application in the sea-cage trials in controlling salmon lice. iii) Increased knowledge will be gained about the immune responses of post vaccinated A. salmon against salmon lice. iv) Publication of results in peer reviewed journal, publications of reports, conference proceedings and v) Transfer of know-how to industries, farmers and benefit to consumers vi) Good reputation for Norwegian salmon industry nationally and internationally.

4 Project implementation

The project started from 15th of November 2017 and ended on 31st of July 2018. Several different tasks were performed to achieve project's main goal.

4.1 Production of vaccine (WP1) This task was performed by the Cuban partner

Cloning and expression in Escherichia coli of TT-P0 antigen: For the expression of *the recombinant* polypeptide, the pET28a-TT-P0 expression plasmid was transformed into E. coli BL21 (DE3) and were grown overnight at 37°C in Luria Bertani (LB) medium containing 50 µg/mL of kanamycin. Cultures were then diluted (1:20) in fresh LB medium and grown at 37°C until the OD600 reached approximately 0.5. The expression of recombinant proteins was initiated by the addition of isopropyl-β-D-thiogalactoside (IPTG) (Sigma) to a final concentration of 1 mM and incubation continued another 5 h for induction of recombinant protein expression. Then the bacterial cells were harvested by centrifugation at 10,000 x g for 10 min at 4°C. The cell pellet in resuspended in 300mM NaCl, 10mM Tris, pH 6 were disrupted in French Press (Ohtake, Japan) at 1 200 kgf/cm2. After centrifugation at 10,000 x g for 10 min at 4°C, the cell pellet containing the protein was resuspended in 1M NaCl, 1% Triton X-100 using politron Ultra-Turrax T25, IKA WERKE and centrifuge again at 10,000 x g for 10 min at 4°C. This step was repeated once again and purified inclusion bodies were resuspended in PBS (16 mM Na2HPO4, 4 mM NaH2PO4, 120 mM NaCl, pH 7.4). The purified cell pellet containing the protein was resuspended in 150 mL of Solubilization buffer (300 mM NaCl, 10 mM Tris, 10mM Imidazol, 6M urea, pH 8) and it was incubated for 2 h at room temperature with gentle agitation. Afterwards, the sample was centrifuged at 10,000 x g for 20 min at 4°C and the supernatant was used for further purification steps.
Affinity chromatography was performed under denaturing conditions employing IMAC SepharoseTM Fast Flow (GE Healthcare) according to the manufacturer's instructions. The clarified lysate with 10 mM Imidazole was loaded onto the previously equilibrated column with equilibration buffer (NaCl 300 mM, Tris-HCl 10 mM, Imidazol 10 mM, urea 1,5M, pH 8) at a flow rate of 1 mL/min. Then, wash was performed with the same buffer but 40 mM Imidazole. Protein elution was done with 200 mM Imidazole. For refolding, the fraction purified by affinity chromatography was dialyzed against NaCl 150 mM, Tris-HCl 10 mM, pH 8 buffer. Each fraction was checked by 15 % sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis under reducing conditions. Protein concentration was determined with a BCA protein assay kit (Pierce, USA) according to the manufacturer's instructions. The purity of recombinant protein was assayed by densitometry scanning of protein gels taking into account total protein concentration.

The Recombinant TT-P0 protein which is a patented vaccine candidate (Vaccine composition for controlling ectoparasite infestations PCT/CU2011/000005) was then emulsified in oil adjuvant Montanide ISA50 V2 (Seppic, France).

4.2 Fish immunization and challenge with *L. salmonis* - Experimental design: (WP1 + WP2)

To test TT-P0 vaccine efficacy under Norwegian conditions, the vaccination experiment was performed in Atlantic salmon, *Salmo salar* L. The fishes having an average weight (wt.) of 40 g were kept at the Aquaculture Research Station (Tromsø, Norway) in a circular 500 L tanks supplied with circulating fresh

water for 6 weeks at an ambient temperature of approximately 10 °C with 24 h illumination (summer stimuli). After 6 weeks the fish were transferred to sea water under same temperature and illumination until the experiment was terminated. Fish were fed with a commercial pellet diet (Nutra Olympic, Skretting). One hundred and twenty fish were placed in each tank per group. Prior to vaccination, fish were anaesthetized in 0.005 % benzocaine.

Fish were killed using 0.01 % benzocaine prior to collection of different tissues (skin, spleen and head kidney), blood and mucus samples. The experiment was approved by 'FDU' (http://www.mattilsynet.no/fdu/) to be in accordance with the animal welfare act as required by Norwegian law.

The experiment consisted of 3 groups. The time-line for vaccination and sampling of tissues (skin, spleen and head kidney), mucus and blood were as mentioned below. In the end, the adult sea lice with egg stings from different groups were incubated for second generation hatching of copepodites. The details of different experimental groups are mentioned below with different experimental parameters and sampling dates followed by schematic representation of the implemented experimental time-line, in figure 1.

3 experimental groups:

Group 1: PBS-Montanide ISA-50 V2 adjuvant (IP-injected), Control

Group 2: TT-P0-Montanide ISA-50 V2 adjuvant (IP-injected)

Group 3: TT-P0-Montanide ISA-50 V2 adjuvant (IP-injected) + plus immersion bath with TT-P0 inclusion bodies.

Adjuvant used is Montanide ISA50 V2 (Seppic, France)

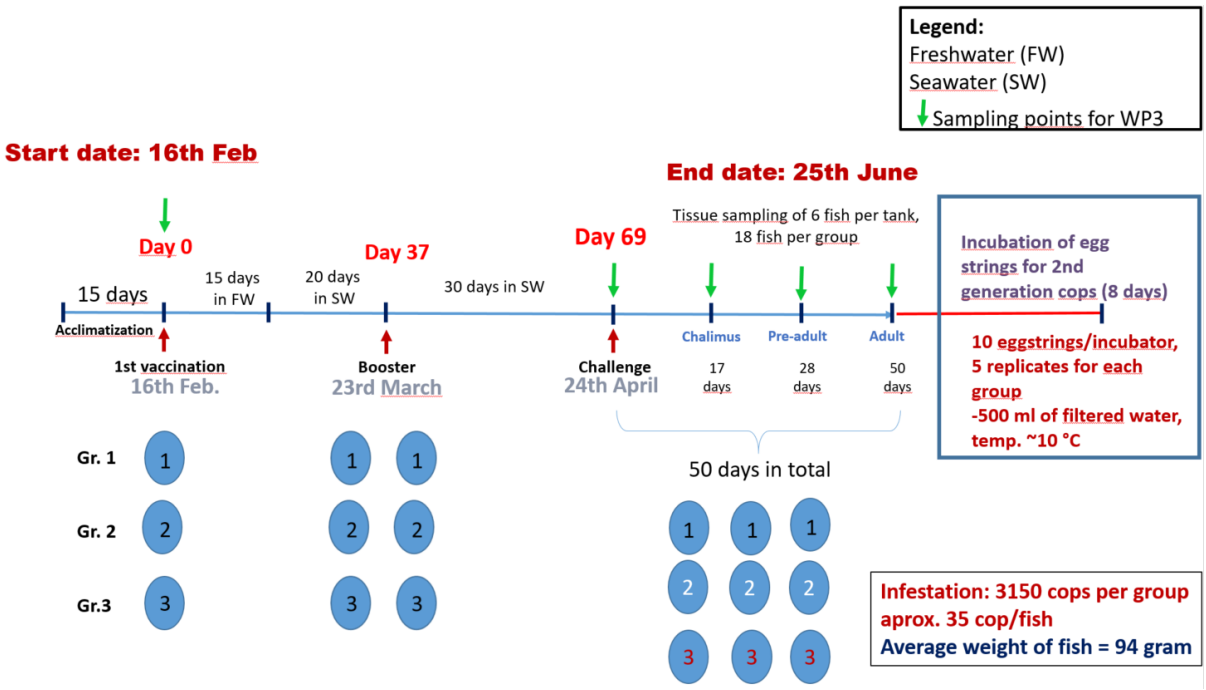


Figure 1 Schematic representation of the implemented experimental plan with time-line.

Vaccination and Sampling work outs

The fish were kept under the following conditions during the experiment:

Temperature: °10 C; *Light:* 24 hrs; *Oxygen level* : ~80-90 %; *Salinity*: 34-35 ppt

The start date of 1st vaccination was 19th of Feb. 2018. 120 fish per group were vaccinated having an average wt. of 43 g.

2nd vaccination in sea water was done on 23rd March 2018. Average wt. of the fish was ~ 60 g.

Dose: 1 µg/g body weight for injection and 200 µg/L for immersion.

Challenge: To check the protection efficiency of the candidate vaccine, fish were challenged with copepodites on 24th April 2018 i.e 69 days after first vaccination. All fishes in each group were challenged with about 35 copepodites/fish for 1 h bath treatment. Fish from each group were distributed into 3 replicate tanks on 26th April. Average size of the fish during challenge was 94 g. The lice was from IMR, Oslo/Gulen (same strain according to IMR). Lice count from 30 fish/group was done at the chalimus (17 days post challenge), pre-adult (28 days post challenge) and adult stage (50 days post challenge). The challenged fish were also kept at the similar conditions as before challenge.

Since this vaccine candidate has not been tested in salmon before, we did not know at which life stage of lice the vaccine will be effective. Therefore, counting of lice at chalimus, pre-adult and adult stage post challenge as well as counting of 2nd generation copepodites were planned and executed which will conclude the trend of protection due to the vaccine and its effects.

1st sampling post challenge: At chalimus stage II i.e. 17 days post challenge -11th of May 2018.

2nd sampling post challenge: At pre-adult stage (most were at PA-2 stage) i.e. 28 days post challenge - 22nd of May 2018.

3rd sampling post challenge: At adult stage when female had first batch of mature egg strings i.e. 50 days post challenge - 14th of June 2018.

Counting of the chalimus and pre-adults were done on the fish kept under water in a white tray. Fish were given an overdose of anesthesia before counting. Blood and mucus were collected from all the sampled fish and tissues were collected from 6 sampled fish.

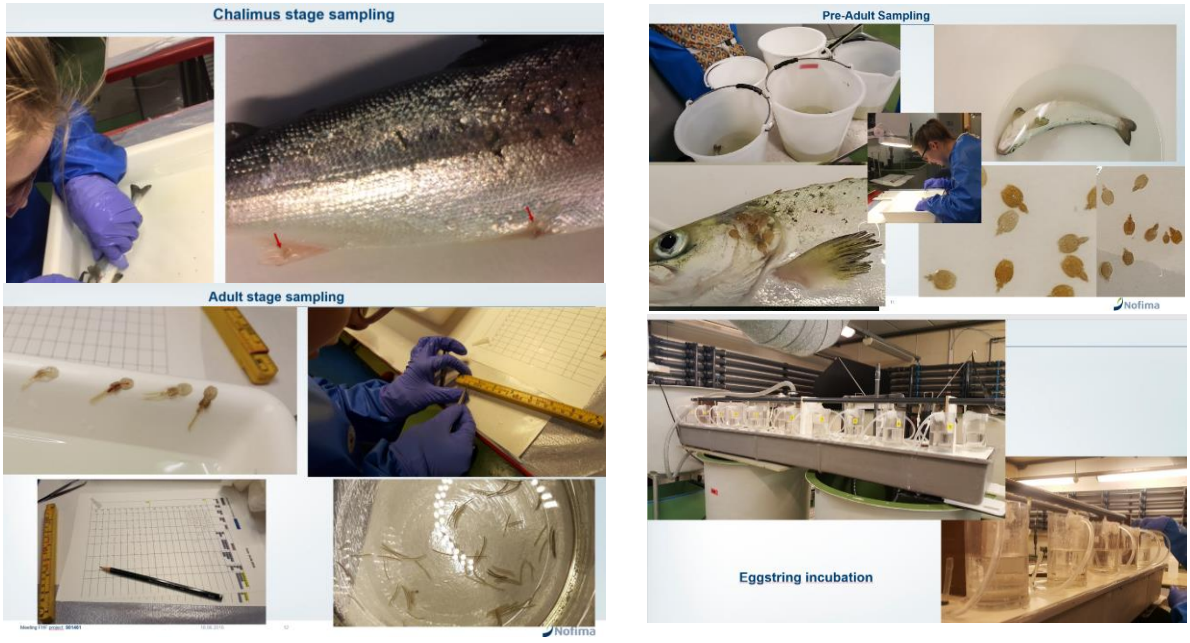


Figure 2 *The samplings performed at different stages post sea lice challenge and egg string incubation for 2nd generation copepodites.*

2nd generation copepodites incubation: To check if the effect of the vaccine is better in the 2nd generation of copepodites count reduction, we conducted the incubation of egg strings from the mature females for hatching of copepodites at the similar conditions as mentioned before for challenge.

Incubation of egg strings (1st batch) (50 egg strings in total from different groups, i.e 10 egg strings/replicate) for next generation copepodites were done on 14th of June 2018.

Incubation temperature ~10 °C; Salinity: 34-35 ppt

Volume of filtered water per incubator: 500 mL

1st observation noted in each incubator: 22nd June

The experiment was ended on 25th June after counting the total copepodites in each group.

After the 1st project meeting in January 2018, according to the suggestions from the reference group and the FHF contact person, the following workouts were included into the experimental plan for implementation:

Side effects of the vaccine using the Speilberg score and pigmentation.

Incubation of the egg string for counting next generation copepodites.

Checking the cross-reactivity of P0 from *Lepeophtheirus salmonis* with *Caligus rogercresseyi*

Evaluation of IgM titer in the mucus by ELISA.

Sampling for WP2 and WP3: Spleen, head kidney and skin tissues were sampled aseptically from fish of all groups at 0, 69, 80, 95 and 110 days post vaccination (dpv), and immediately transferred to RNA later (Ambion) and stored at -20 °C. Blood and mucus were collected from each fish (n=10) at the similar time-points as tissue sampling. Serum was collected from the blood using the standard protocol.

4.3 Side effects of TT-P0vaccine

To check the side effects of the TT-P0 vaccine having the Montanide ISA50 adjuvant, Speilberg scoring was performed according to the criteria detailed by Midtlyng et al. 1986 (9). A separate score for pigmentation for each fish was also performed.

4.4 Serum and mucus antibody levels (WP3)

This approach was used to detect the antibody level raised against the antigen, P0 in the vaccine and whether it is correlated to protection. Moreover, it was also to understand the mechanisms behind protection.

The samples for IgT detection were shipped to Prof. Oriol Sunyer's laboratory in Philadelphia, USA, where the analysis was performed. IgM antibody levels were performed at Nofima, Tromsø. Antigen-specific IgM and IgT antibodies in the serum and mucus of vaccinated fish were determined by indirect ELISA. High binding microtiter plates (Nunc, Denmark) were coated for 16 h at 4 °C with 10 µg/mL of 35 aa synthetic P0. After three washes with PBS-Tween 0.05 %, blocking was performed with 5 % skimmed milk for 2 h at room temperature (RT). Afterwards, two-fold serial dilutions or required dilution of sera or mucus to measure IgM or IgT, respectively were applied and incubated for 16 h at 4 °C. Bound antigen-specific antibodies were detected by sequential incubation with anti-rainbow trout/Atlantic salmon IgM monoclonal antibody (ADL Aquatic Diagnostics, UK) or anti-trout IgT monoclonal antibody (produced in Prof. Sunyer's lab) for 2 h at RT, followed by three wash steps and 1 h at 25 °C with an anti-mouse IgG conjugated to horseradish peroxidase. After washing, the chromogen TMB in substrate buffer was added and incubated for 10 min or until color development. After stop the reaction with 50 µL/well of 2.5 M H_2SO_4, the color intensity was measured at 450 nm in an ELISA reader.

4.5 Gene expression studies (WP3)

All organs kept in RNA-later (Ambion, Austin, TX, USA) was subsequently processed for RNA isolation. Total RNA was extracted by MagMAX™-96 Total RNA Isolation Kit including the turbo DNase treatment (Invitrogen) according to manufacturer's instruction. Analysis of Gene Expression by Real-time PCR (qPCR). qPCR was performed in duplicates with a Quant5 Real-Time PCR System (Applied Biosystem) using SYBER Green (Applied Biosystem) in 384 well plates. For each mRNA, gene expression was normalised to the geometric mean of the 3 house-keeping genes (EF-1a, 18S and beta-actin) in each sample and fold change was calculated according to Pfaffl method (10) using the primer efficiency (E).

4.6 Statistical analysis

All the statistical analysis were done in Graphpad Prism version 6.0. One-way ANOVA or Kruskall Wallis test were done depending on the normal distribution and equal variance of the data.

For qPCR analysis, Graphpad Prism software was used to find the outliers. Outlier cleaned data were used for further statistical analysis. One way ANOVA or Kruskall Wallis test was done depending on the results of normal distribution test followed by Tukey or Dunn's Multiple Comparison.

5 Achieved results, discussion and conclusion

The results obtained from the vaccine validation experiment are mentioned, discussed and concluded below:

5.1 Vaccine candidate production and purification

The figure below shows the purity of the TT-P0 batch produced to be used in vaccine for the validation experiment in this project.

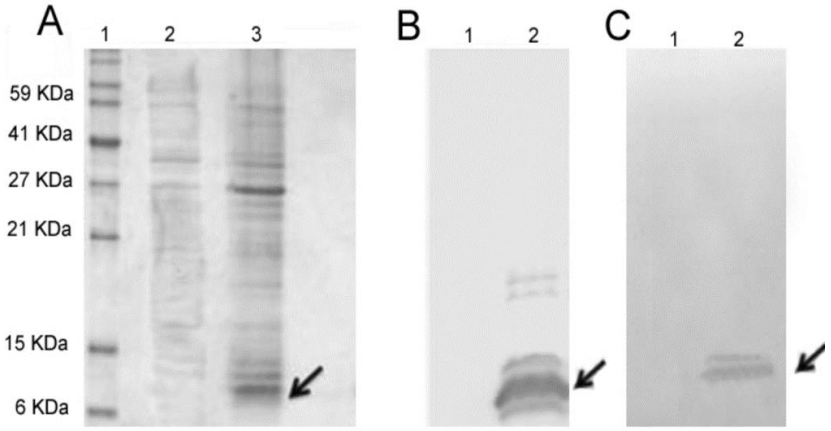


Figure 3 *Recombinant expression in Escherichia coli and immune identification of TT-P0. (A) SDS-PAGE 15 %: Lane 1: Molecular weight marker Prestained SDS-PAGE Standards Broad Range (BioRad, EE.UU), Lane 2: BL21(DE3), lane 3: BL21(DE3)-TT-P0 cell extract (B) Western blot using anti-His monoclonal antibody (C) Western blot using a polyclonal serum against P0 generated in rabbits. Lane1: BL21(DE3), lane 2: BL21(DE3)-TT-P0 cell extract.*

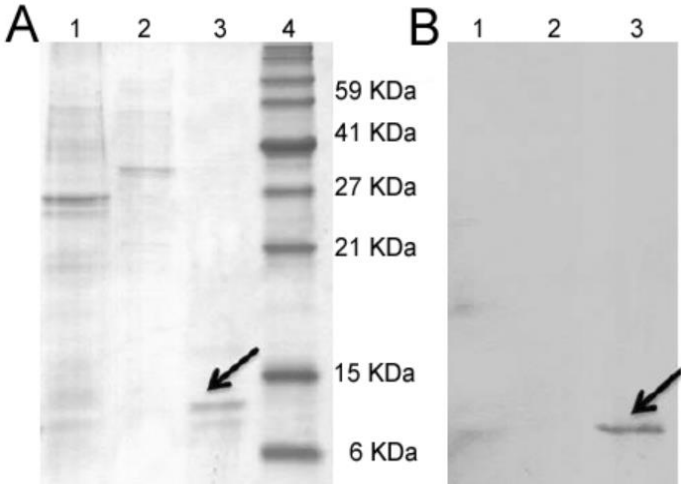


Figure 4 *TT-P0 purification by metal affinity chromatography. (A) SDS-PAGE 15% (B) Western blot using anti-His monoclonal antibody. Lane 1: Not-bound fraction, lane 2: fraction eluted with wash buffer, lane 3: Elution, lane 4: Molecular weight marker Prestained SDS-PAGE Standards Broad Range (BioRad, EE.UU). The arrow indicates the purified protein.*

5.2 Challenge study and protection efficiency

Counting of lice were performed at chalimus, pre-adult and adult stage post challenge. Length and weight of the fish were registered during sampling. We checked the number of males and females, number of egg strings (1 or 2 or none) and length of the egg strings. The collected 1st batch of egg string from each groups were incubated for the next generation copepodites in 5 replicates/group.

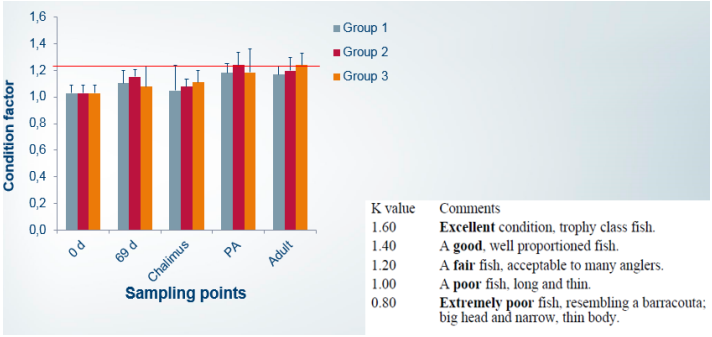
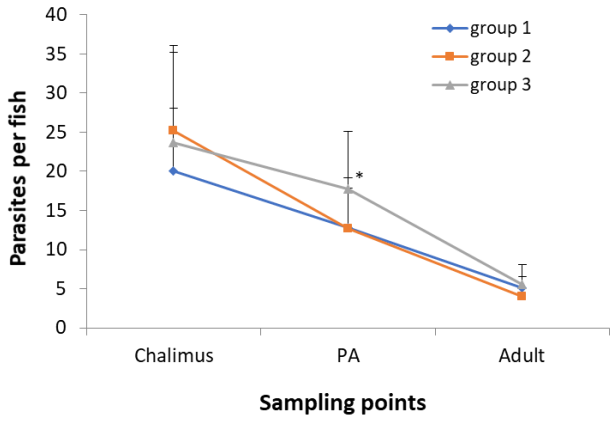


Figure 5 Graph showing condition factor in fish groups at different stages of sampling post vaccination and sea lice challenge.

We analysed the fish weight, length and condition factor (K) for all sampling points. Despite vaccinated fish had less weight and length, the condition factor is acceptable (1.2) and it is the same for all groups as shown in figure 5.

The lice count data at different stages were analysed for relative percent (%) protection, number of females and males, number of egg strings per female and egg string length as shown in figure 6 and 7.



		Group 1	Group 2	Group 3
% reduction in lice number compared to chalimus stage	PA	36	49	25
	Adult	74	84	77
			Group 2	Group 3
Relative % protection	PA		27	4
	Adult		12	4

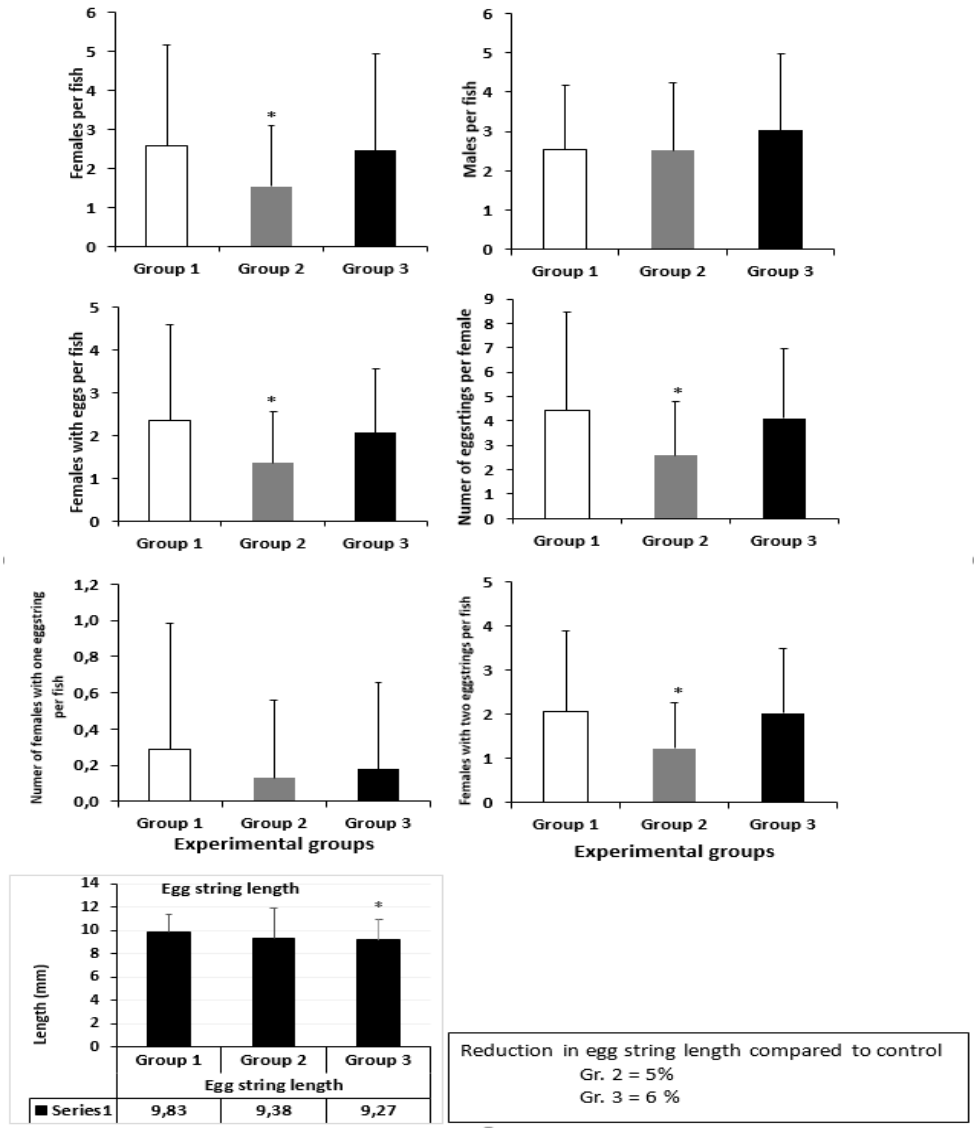
Figure 6 Line graph showing average lice count per fish for different groups. The error bars shows the mean value + SD (n=30). Asterisk (*) above the bar shows statistical significance (P<0.05). The right side table shows the relative percent protection in vaccinated group compared to control group.

The average lice count at different stages from each fish post copepodite challenge is shown in figure 6. The line graph shows that there was no reduction in average lice count per fish at the chalimus stage in both the TT-P0 vaccinated groups compared to the control group (Gr. 1). This might be the fact that we have not considered dead chalimus during counting and thus counted all the chalimus attached to salmon including the dead ones also. Moreover, it is difficult to distinguish dead chalimus from the live ones since they remain attached to its host after death and takes several days to detach from the host (according to research report from Havforskningsinstituttet) https://www.imr.no/nyhetsarkiv/2016/juli/ferskvatn_drep_best_unge_lakselus/nb-no).

Subsequently, there was reduction in average adult lice count per fish in both vccinated groups compared to control. Moreover, the average lice count analysis also indicates the relative percent protection of 27 at PA stage and 12 % relative protection at the adult stage in Gr. 2 compared to the control goup. On the other hand, Gr. 3 showed only 4 % relative protection for both PA and adult stage. Thus Gr. 2 vaccinated group performed better compared to Gr. 3 vaccinated group in terms of relative percentage protection.

In the adult stage counting, overall result showed (figure 7) that Gr. 2 had significantly lower number of females, lower number of total egg strings as well as lower number of paired egg string. Furthermore, Gr. 2 and 3 showed reduced eggsting length i.e reduction of 5 and 6 %, respectively compared to control, Gr. 1 (figure 7a). Gr. 3 with 6 % reduction in egg string length was statistically significant. This showed that the TT-P0 vaccine had affected by reduced adult female count and female fecundity in the 1st generation. Thus, indicating a bigger impact in the subsequent parasite generations and re-infection load.

a.



b.

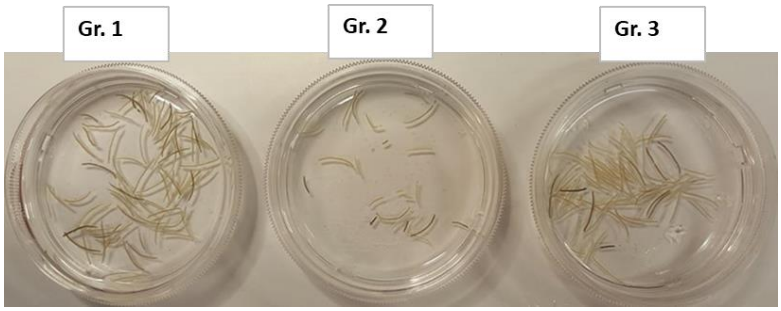


Figure 7 *a) Bar graph showing different parameters used in the lice counting for different groups per fish (n=30). Asterisk (*) above the bar shows statistical significance (P<0.05). The bar shows the mean value + SD (n=30). b) Egg strings collected from gravid females during adult stage sampling post challenge. The figure shows the remaining egg strings in each group after removal of 50 eggstrings for hatching experiment for 2nd generation copepodites count.*

Next generation copepodite count

To check if the reduced adult female fecundity in the vaccinated group as shown in figure 7 in the 1st generation has any consequences in the early 2nd generation, the next generation copepodites were counted on day 10 of incubation, and data were analysed.

The result showed the least number of copepodites in Gr. 2 with delayed hatching tendency compared to control, Gr. 1 (adjuvant only) as shown in figure 8b which correlates well with the reduced fecundity in 1st generation of the Gr. 2 adult females.

a.

Incubator no. Gr. No	Observation of copepodids made on 22.06.2018
1 - Gr.1	Little in the water column and some on the bottom
2 - Gr.1	Good numbers both in the water and bottom
3 - Gr. 1	Good numbers both in the water and bottom
4 - Gr. 1	Good numbers both in the water and bottom
5 - Gr. 1	Good numbers both in the water and bottom
6 - Gr.2	Little number on the bottom
7 - Gr. 2	Less on the bottom and in the water. Half of a string still to hatch.
8 - Gr. 2	Less numbers on the bottom. Nothing visually observed in the water. One egg sting to hatch still.
9 – Gr.2	Less numbers on the bottom. Nothing visually observed in the water
10 – Gr. 2	Less numbers on the bottom. Nothing visually observed in the water.
11 – Gr. 3	Many in the water. Less at the bottom
12 – Gr. 3	Many in the water. Less at the bottom
13 – Gr. 3	Few in the water. Less numbers at the bottom
14 – Gr. 3	Less in the water and bottom
15 – Gr. 3	Less numbers in the water and bottom

b.

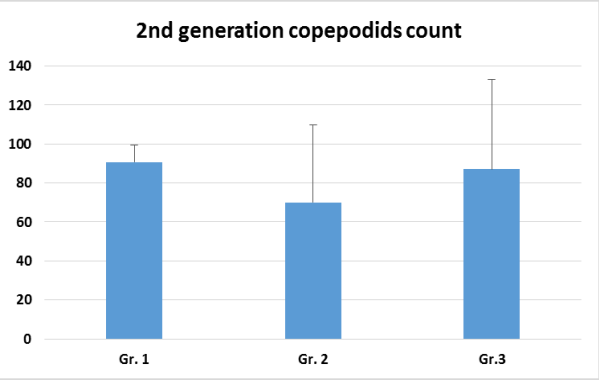


Figure 8 *a) Table showing observations noted on day 8 post incubation of eggstrings and b) graph showing the number of copepodites count on day 10 of incubation.*

In the above results, the number of copepodites counted on day 10 were not normal as expected based on observation made on day 8 (figure 8a). This was due to some unseen/technical problem occurred during the weekend, resulting in mortality of the copepodites before counting on day 10. However, cumulative result showed some impact on the reduction of copepodites count and the delayed hatching tendency in the vaccinated groups especially in Gr. 2. Further work is necessary to see a clear impact on the 2nd generation efficiency of survival and further infectivity potential at later developmental stages.

Concluding the sampling results of the salmon lice as mentioned above from the vaccinated and control group at different stages post challenge, the overall salmon lice vaccine efficacy for Gr. 2 showed the best having 86 % efficacy and Gr. 3 was negative compared to control since some parameters were worse than the control group, Gr. 1. The terminology 'vaccine efficacy' used here should not be interpreted as protection obtained. This is the overall vaccine effects on different parameters studied based on all sampling results in the section 5.2.

5.3 Side effects of the vaccine

To check the side effects of the TT-P0 vaccine having the Montanide ISA50 V2 adjuvant, Speilberg scoring was performed on the 3rd sampling post challenge, according to the criteria detailed by Midtlyng et al. 1986 (9). A separate score for pigmentation for each fish was also performed.

Figure 9 shows that control Gr. 1 with only adjuvant showed an average score of 2 compared to Gr. 2 and 3 which showed an average score of 2.8, i.e below 3 which is in acceptable range.

The pigmentation seemed to be less in the vaccinated group compared to control, Gr. 1 as shown in figure 9. In most of Gr. 2 and 3 fish, the pigment spots were found to be spread to anterior abdomen which was related to the spread of vaccine pockets. In Gr. 1 the pigmentation was localized near the vaccinated region. No pigmentation was found in the muscle or tissue, only the epithelial lining showed pigmentation.

We also checked the vaccine depots in all fish. The vaccine was encapsulated by connective tissue as small pockets. Vaccine residues were found in all fish. In Gr. 2 and 3 fish, more vaccine depots were spread within the peritoneal cavity compared to Gr. 1. The injections site was also checked for redness and lesions and it looked normal. Our result showed that the TT-P0 vaccine side effects in this experiment was under acceptable range and thus okay and acceptable for future application at field level.

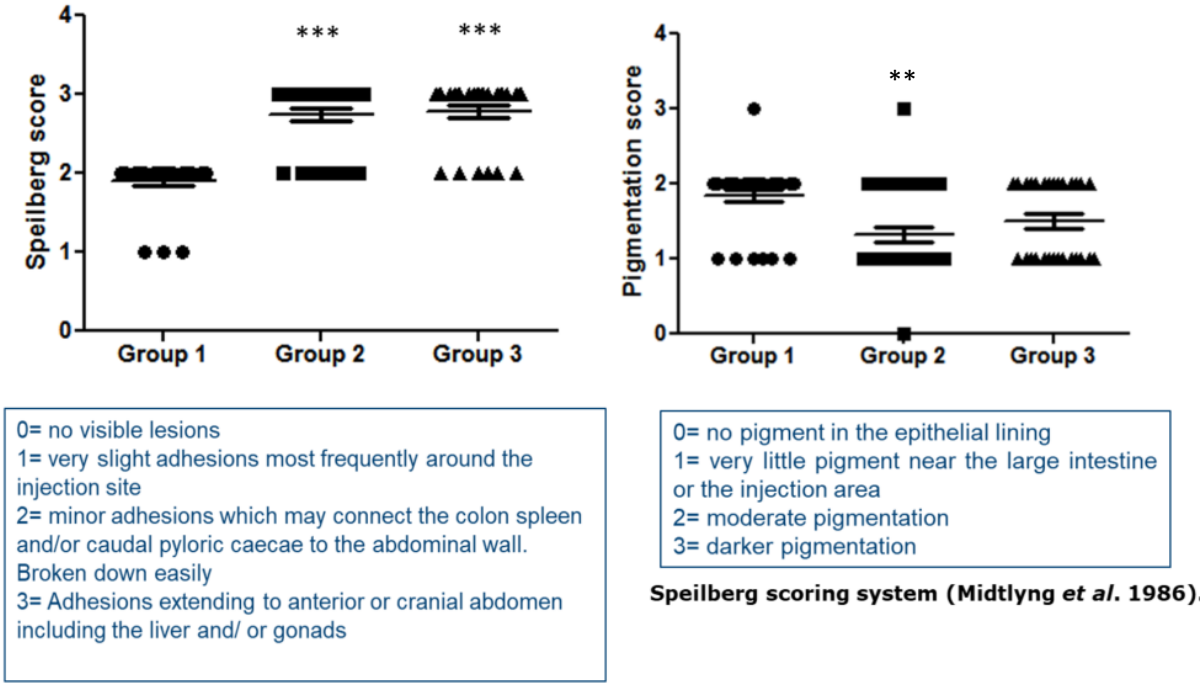


Figure 9 *Graphs showing the seconday effects of TT-P0 vaccine based on the Speilbergs score and the pigmentation score. Data represents mean +SD (n= 30). Asterisk (*) above the groups shows statistical significance (** P<0.001, ***P< 0.0001).*

5.4 Serum and mucus antibody levels

IgM titer in the serum: The IgM titers in the vaccinated groups (Gr. 1 and 2) were significantly higher at all sampling points studied with gradual increase in antibody titer with time post vaccination and lice infestation as shown in figure 10.

In contrast to the high IgM titers, no correlation was found between IgM titer and lice count of the individual vaccinated fish at any sampling points post copepodites infection (figure 11). This can be explained in chalimus stage with lice count having both live and dead lice because it was very difficult to distinguish between dead and live chalimus lice stage since both are attached to the skin. But in other free movable stages i.e PA and adult, this can also be explained by the fact that in 1st generation

the lice count is not correlated with the IgM antibody level but higher level of antibody at PA and adult stage has affected the number of male and female population, fecundity and quality of the egg production. Besides, this has also contributed to reduced hatching eficiency in the 2nd generation for the vaccinated group campared to control. But further research is necessary to investigate the long term efficacy of the vaccine with respect to protection in several lice generations.

In *in vitro* challenge conditions, the infestation load is usually very high i.e in this validation experiment, it was about 35 copepodites per fish, which is far higher than the natural conditions in the field. Consequently, the vaccine is expected to work more effectively under lower infestation load. Therefore, performing a long term challenge experiment to mimic lice infestation under natural conditions will be the next initiative for continuing the evaluation of TT-P0 vaccine efficacy.

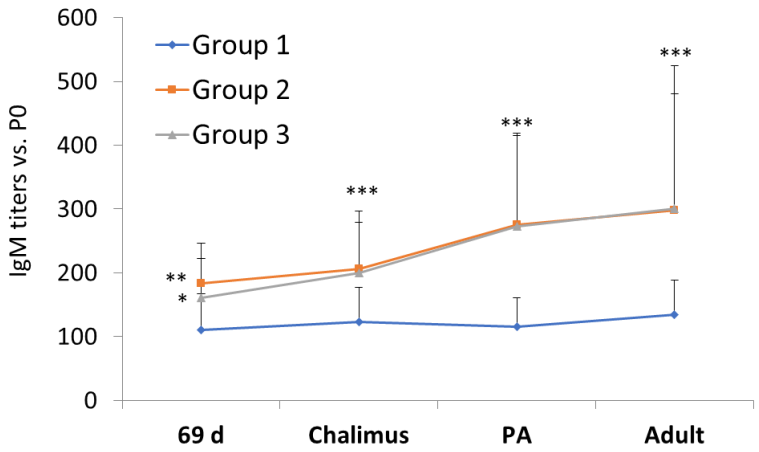
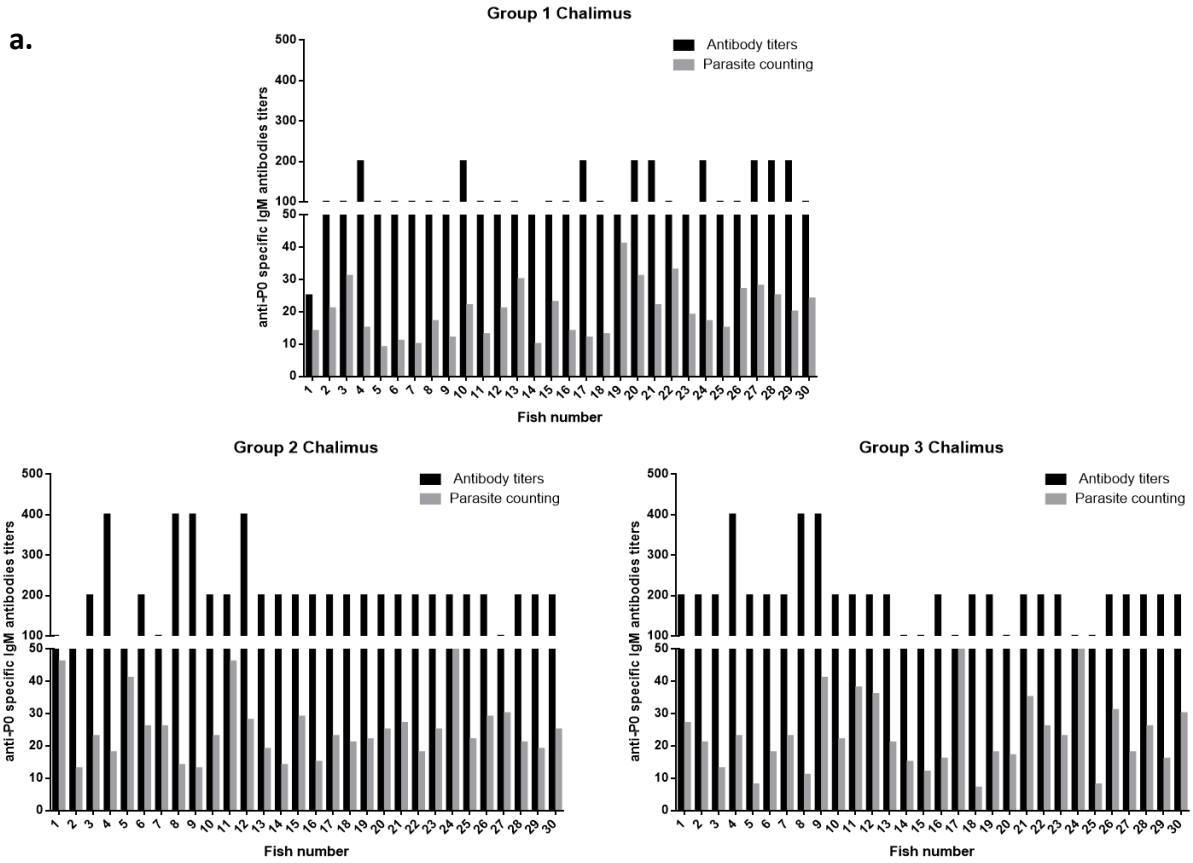
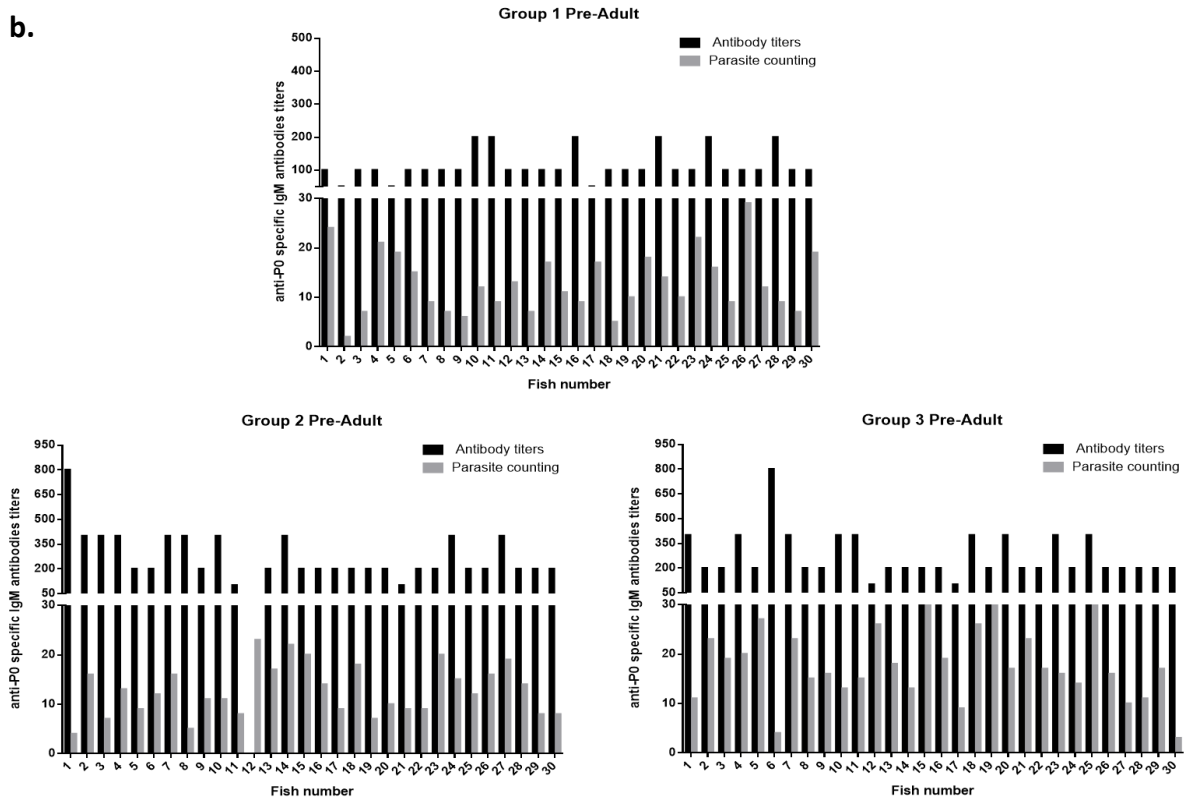


Figure 10 *Graphs showing the average IgM titer against the antigen P0 within different vaccinated groups and at different sampling time post vaccination and challenge. Data represents mean +SD (n= 30). * above the groups shows statistical significance (* P<0.05, ** P<0.001, ***P< 0.0001).*

a.


Group 1 Chalimus
Antibody titers
Parasite counting
anti-P0 specific IgM antibodies titers
Fish number
Group 2 Chalimus
Group 3 Chalimus

b.


Group 1 Pre-Adult
Antibody titers
Parasite counting
anti-P0 specific IgM antibodies titers
Fish number
Group 2 Pre-Adult
Group 3 Pre-Adult

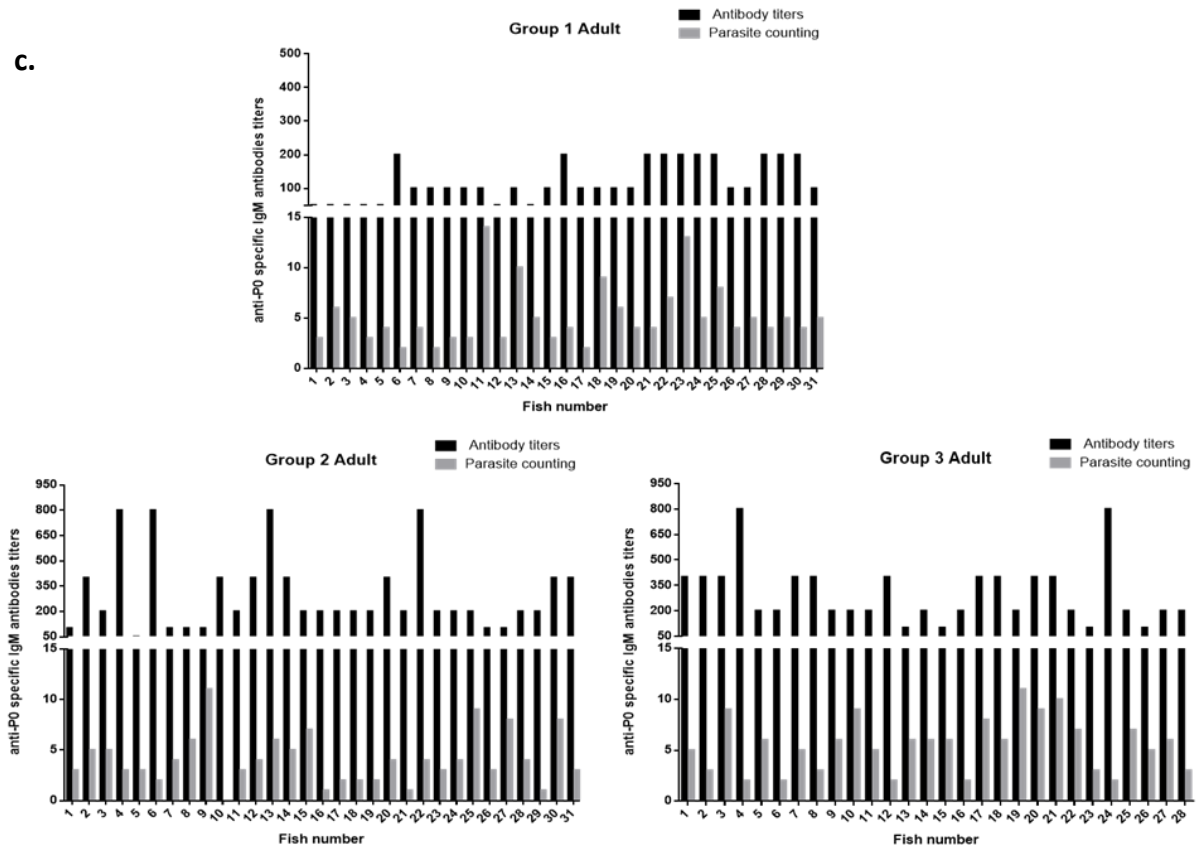


Figure 11 Graphs showing the parasite count and IgM titer from each individual within different vaccinated groups and at different sampling time post lice challenge. a) Chalimus, b) Pre-adult, and c) Adult.

Cross-reactivity ELISA test of L. salmonis TT-P0 serum antibody with Caligus rogercresseyi peptide P0 antigen: We selected the serum samples with highest IgM titers (~400) from Gr. 2 and Gr. 3 fish of PA stage sampling to check the cross-reactivity with Caligus peptide P0 antigen. The results showed some cross-reactivity of serum IgM with Caligus peptide P0 (titer ranging from 100-200). Similarly, test was also performed with the serum samples showing highest IgM titer from Gr. 2 and Gr. 3 fish of adult stage sampling. The cross-reactivity titer of serum IgM with Caligus P0 showed 50-100 with respect to 400 and 800 titer value with L. salmonis P0. Thus, the overall result showed low cross-reactivity of P0 antigen from *L. salmonis* with P0 antigen from *Caligus rogercresseyi*.

IgM titer in the mucus: It was interesting to see the IgM antibody titer in the mucus of the fish from TT-P0 vaccinated groups compared to only adjuvant injected control group. The result showed (figure 12) significantly high and consistant IgM titer in the mucus of Gr. 3 fish (vaccinated both by immersion and by ip injection) starting from day 69 post vaccination until the adult lice stage post lice infestation. In contrast, Gr. 2 which received only i.p. injected vaccine, showed gradual significant increase in IgM titer compared to control group post infestation with sea lice. This showed that the bath vaccination in Gr. 3 facilitated the presence of mucosal antigen specific IgM levels before sea-lice infestation compared to Gr. 2, where the infestation of the lice facilitated the influx of secretory IgM in the mucus.

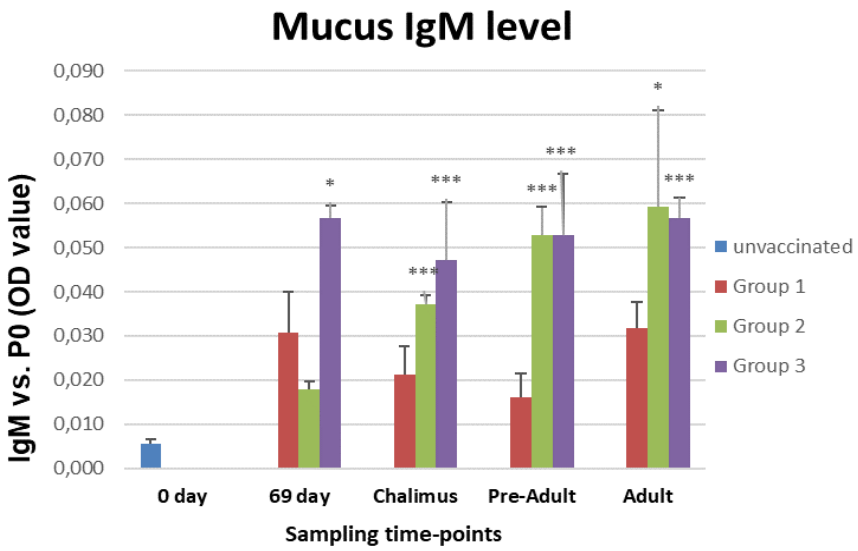


Figure 12 *Graphs showing the antibody levels (IgM) in the mucus of Atlantic salmon to P0 antigen at different sampling time-points post vaccination. Pooled mucus of 10 individual sera (pooled n = 3), diluted 1:25 were tested in ELISA. Data represents mean +SD (n= 3, pooled of 10). Asterisk (*) above the groups shows statistical significance (* P<0.05, ** P<0.001, ***P< 0.0001).*

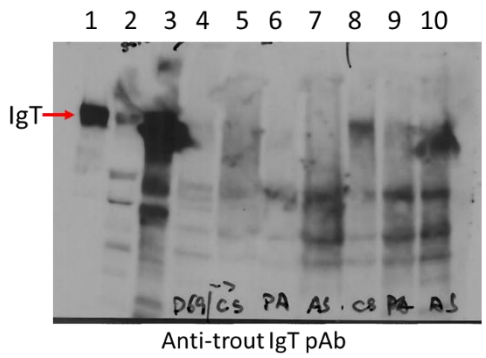
Since the PA and the adults are the active blood feeders in addition to tissue and mucus compared to other developmental stages and the IgM antibodies levels in the blood and mucus was high compared to control, the impact of ingesting antibodies and other immune effectors could be bigger and is well correlated with the sampling results in figure 6 and 7 with higher relative percent protection at PA and adult stage and reduced female count and fecundity at the adult stage in the vaccinated groups. Thus, the higher IgM antibody levels in blood and mucus post challenge supports the sampling data mentioned in section 5.2.

IgT titer in the mucus and serum: The IgT titer in the mucus and serum of A. salmon at different sampling points post vaccination to P0 antigen was dertermined by indirect ELISA. The result showed that there were no detectable titers of P0 antigen-specific IgT found in mucus and serum samples collected at different sampling points post vaccination.

To explain the undetectable titer in the indirect ELISA, we further performed western blot (WB) to check if A. salmon IgT of those samples is intact or had been degraded. Above that, to confirm that the anti-trout IgT can recognize the native salmon IgT molecule, sandwich ELISA was performed.

WB results showed (figure 13) that the trout IgT antibody can recognize the salmon IgT in both serum and mucus. The intact IgT with the correct molecular mass was found in a few samples but with some degradation. The results of sandwich ELISA showed that the anti-trout IgT could recognize the native salmon IgT by ELISA and the titer in serum is higher than in mucus. The explanation for this can be that there are a few IgT isoforms in salmon, so, it might be possible that the isoform expressed in salmon mucus is not as well recognized by the anti-trout IgT as the one expressed in salmon serum. In addition, for most mucus samples, the intact IgT was not observed and some degradation fragments were present. This strongly suggests that IgT in mucus was degraded in many or most samples and this in

turn made the P0 antigen specific IgT to be in an undetectable range in indirect ELISA, which might explain the lack of positive results as well.



1. Normal trout serum 1 µl
2. Salmon serum Day69 1 µl +Maker
3. Normal trout gill mucus 20 µl
4. Salmon mucus Day69 10 µl
5. Salmon mucus Chalimus Stage 10 µl
6. Salmon mucus Pre-Adult 10 µl
7. Salmon mucus Adult Stage 10 µl
8. Salmon mucus Chalimus Stage 10 µl
9. Salmon mucus Pre-Adult 10 µl
10. Salmon mucus Adult Stage 10 µl

Figure 13 *Western blot showing the detection of salmon IgT in serum and mucus samples by anti-trout IgT polyclonal antibody.The information of samples in well is given on the right hand side. Samples in lane 4-7 is the pooled sample from Gr. 1 and samples in lane 8-10 are pooled sample from Group 2 and 3.*

Also, IgT is very susceptible to proteases present in the mucus and in addition to that the handling of mucus and serum samples during sampling at 4 °C and freezing and thawing before shipping the samples to Prof. Oriol Sunyer's lab for IgT analysis, might have facilitated the degradation of the antigen specific IgT antibody.

5.5 Gene expression studies

We studied the transcription levels of markers of immune response for mucosal immunity (IgT, IL-22), humoral, cell-mediated immunity (IgM, CD4, CD8α, IL-4/13A, IFN-γ, IL10) and pro-inflammatory cytokines (IL-1β, TNF-α, IL-8, MMP-9) by qPCR. The result showed that the IgM and IgT transcript levels were significantly upregulated in the TT-P0 vaccinated groups (Gr. 2 and 3) compared to control group (Gr. 1) in all the tissue and sampling time-point studied (figure 14) and both the genes are significantly correlated (*P< 0.0001*) (table 1) thus, suggesting its important role in sea lice immunity. In A. salmon normally, IgM transcripts are most abundant followed by IgT, especially in spleen and head kidney (11). Similarly, the results in the present project showed stronger expression of IgT and secretory IgM (sIgM) in spleen and head kidney but the expression level of IgT in spleen was much higher than IgM in vaccinated groups post sea lice infestation, indicating its important role. This shows the important role of IgT together with IgM in systemic immune response. The result also showed that the transcript level of IgT was significantly higher in Gr. 3 (TT-P0 vaccinated i.p. + immersion) compared to control group and Gr. 2 (only i.p. vaccinated), 69 days post 1st vaccination (figure 14). This demonstrate that the immersion vaccination had contributed to the upregulation of IgT transcript compared to i.p. vaccination before sea lice infestation. After sea lice infestation, the IgT transcript level increased significantly in both the vaccinated groups with highest expression at PA stage post challenge, thus revealing the stimulation of IgT expression upon infestation and thus its important role in mucosal immunity. On the other hand, the highest sIgM gene expression compared to other tissues studied was observed in the skin of fish during PA stage sampling (figure 14). This high level of sIgM and IgT expression and high IgM antibody titers in the blood and mucus observed in PA and adult stage correlates well with the percentage relative protection shown in table of figure 6 and reduced adult

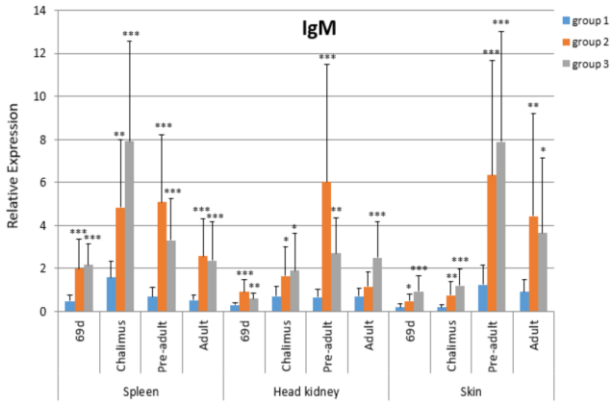
female count and fecundity discussed in section 5.2 and figure 7, thus indicating their interaction and contribution in sea-lice immunity. Moreover, the overall sIgM gene expression correlates well with the IgM antibody levels in the serum (figure 10). The sIgM gene expression pattern in the skin also shows maximum correlation with the IgM titer pattern in the mucus (figure 12). Therefore, following the trend of high IgM expression at the transcript level and its correlation with the high antibody level in the serum and mucus and more importantly strong correlation between IgM and IgT gene expression, also indicates the expectancy of the presence of IgT antibody level in the mucus and serum corresponding with upregulated IgT gene expression levels in the vaccinated groups. We were not able to detect the antigen specific IgT antibody levels in the mucus and serum due to the degradation of IgT, but we cannot rule out the existence of the antigen specific IgT levels in the mucus and serum of vaccinated groups post sea lice infestation.

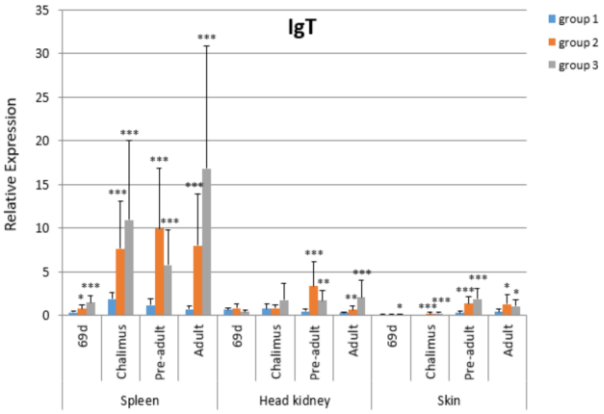
In addition, lice at the PA and adult stage feed more on blood along with mucus and tissue, the systemic higher levels of antigen specific IgM antibody, sIgM and IgT expression both in lymphoid tissue and skin/mucus documented here might have contributed to reduced female lice fecundity in the 1st generation and subsequently reduced hatching efficiency and copepodites counts in the 2nd generation as shown in figure 7 and 8 and also other effectors of the immune system. This shows that both IgM and IgT have contributed mainly to the observed protection in the vaccinated groups. Therefore, this vaccine candidate seems to act effectively on the fecundity parameters of the adult female lice. Concurrently, these results also revealed that IgT responses could be simultaneously induced in both mucosal and systemic tissues after vaccination via injection and immersion route followed by sea lice infection, but IgT might play a more important role in mucosal immunity than in systemic immunity. Further work is necessary to understand the crosstalk between the two immunoglobulin genes and their underlying mechanisms of protection.

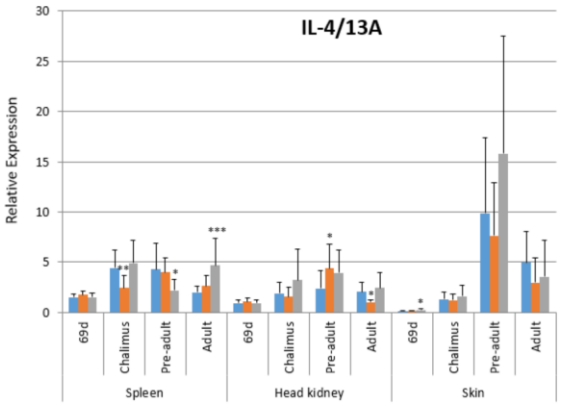
On the other hand, the activation of T-cell response genes - CD4, CD8α, IL-4/13A and IFN-γ showed significantly higher expression levels in the spleen of the adult stage sampled post lice infestation (figure 14). This trend was also seen in the PA stage in the head kidney showing the activation of T-cell mediated immunity and the involvement of Th1/Th2 response. Some significant decreasing trend was also found in these gene expression levels at other sampling points.

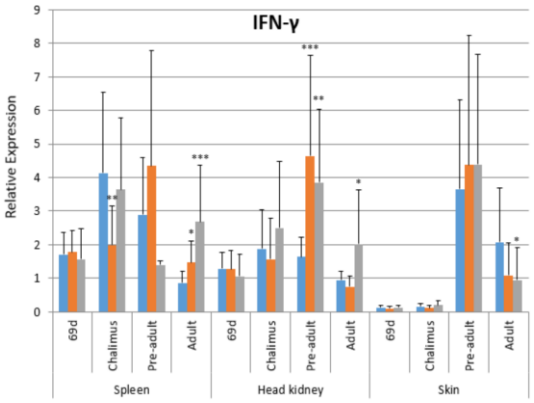
Chemokine IL-8 and MMP9 also showed higher expression in the vaccinated Gr. 2 and 3 compared to the control Gr. 1 in spleen and somewhat in the head kidney and skin tissue. This shows the possibility of continuous attraction and influx of neutrophils, macrophages and lymphocytes in the different tissues including skin to combat lice infection. The significantly higher IL-8 transcript level in skin during chalimus and PA stage sampling in vaccinated group and its significant correlation (*P< 0.0001*) with the IgT and IgM transcript pattern in the skin (table 1) might speculate the important role of IL-8 in recruiting neutrophils at the mucus for cumulative immune effect against lice-infection together with immunoglobulin molecules. An in-depth study is required to further reveal the underlying mechanisms.

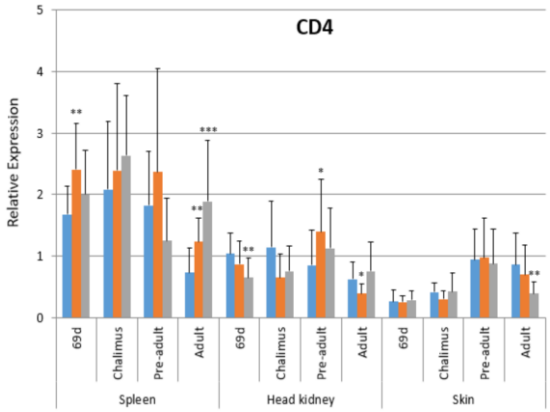
Pro-inflammatory cytokines TNF-α and IL-1β follows the similar pattern of expression with significant upregulation in the vaccinated groups (Gr. 2 and 3) compared to control, Gr. 1 in spleen tissue at all sampling time points and both the genes are strongly correlated (*P< 0.0001*) in all the tissues studied (table 1). Although, IL-22 and IL-10 shared a common pattern of gene expression and correlation but it did not show specific significant up-regulation.

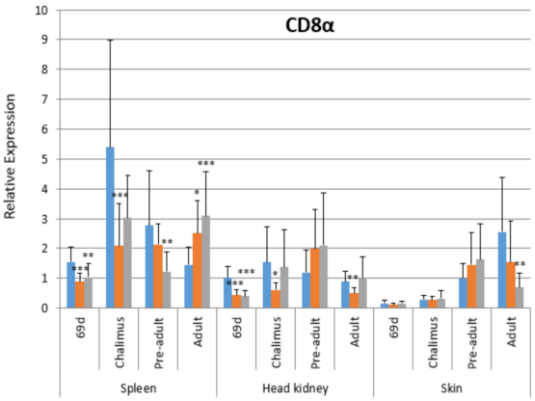

IgM
Relative Expression
group 1
group 2
group 3
69d
Chalimus
Pre-adult
Adult
Spleen
Head kidney
Skin


IgT
Relative Expression
group 1
group 2
group 3
69d
Chalimus
Pre-adult
Adult
Spleen
Head kidney
Skin


IL-4/13A
Relative Expression
69d
Chalimus
Pre-adult
Adult
Spleen
Head kidney
Skin


IFN-γ
Relative Expression
69d
Chalimus
Pre-adult
Adult
Spleen
Head kidney
Skin


CD4
Relative Expression
69d
Chalimus
Pre-adult
Adult
Spleen
Head kidney
Skin


CD8α
Relative Expression
69d
Chalimus
Pre-adult
Adult
Spleen
Head kidney
Skin

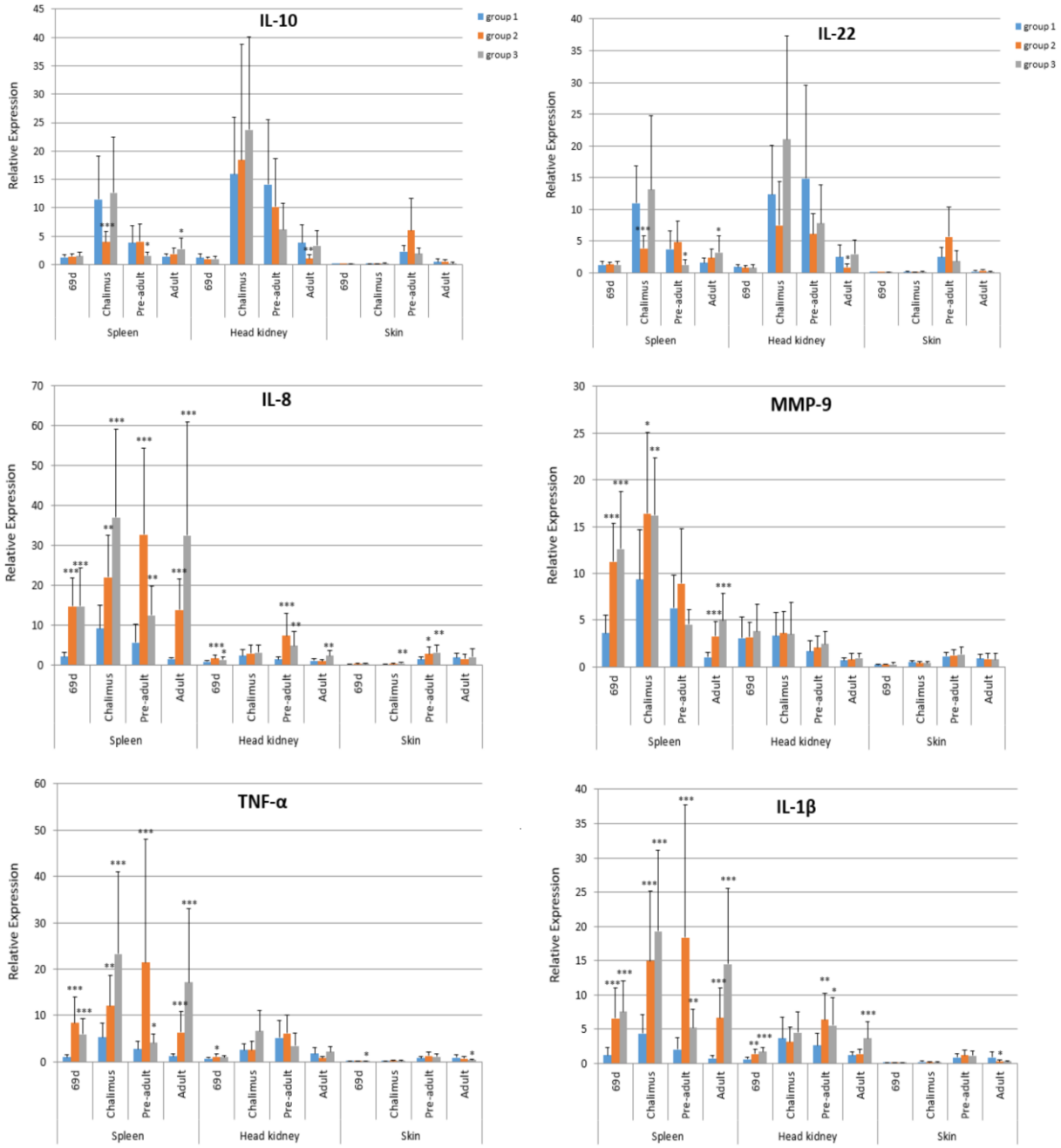


Figure 14 *mRNA level of selected immune genes in different tisues at different time-points after vaccination and infection with salmon lice. Bars represent the normalized relative expression levels compared to 0 day control. Each value represents the mean ± SD (n = 18). Statistical differences (P < 0.05, P < 0.001, and P < 0.0001) between different time-points compared to control are indicated by asterisk (*,**, and ***) respectively, above the bars.*

The common patterns found at the transcript level was that, all the genes were more or less significantly induced in the spleen tissue of the vaccinated group (Gr. 2 and 3) at the adult stage of lice post copepodite challenge suggesting the important role of spleen compared to head kidney in developing systemic immunity for this vaccine candidate and lice-infestation.

Table 1 *Correlation between different gene expression post vaccination and salmon lice infestation in different tissues a) skin, b) head kidney and c) spleen. Statistical differences (P < 0.05, P < 0.001, and P < 0.0001) between different gene expression are indicated by asterisk (*, **, and ***) respectively.*

a.

Spearman's rho Correlation coefficient (n = 213)

Skin Tissue	IgM	IgT	CD4	CD8α	IL-4/13A	IL-8	IL-10	IL-22	MMP9	IFN-γ	TNF-α	IL-1β
IgM	1,000	,893***	,448***	,572***	,594***	,656***	,516***	,523***	,561***	,637***	,646***	,546***
IgT	,893***	1,000	,502***	,664***	,653***	,716***	,569***	,566***	,574***	,721***	,710***	,586***
CD4	,448***	,502***	1,000	,820***	,741***	,714***	,645***	,649***	,710***	,757***	,815***	,685***
CD8α	,572***	,664***	,820***	1,000	,848***	,773***	,716***	,712***	,798***	,861***	,884***	,723***
IL-4/13A	,594***	,653***	,741***	,848***	1,000	,802***	,777***	,814***	,847***	,845***	,871***	,805***
IL-8	,656***	,716***	,714***	,773***	,802***	1,000	,652***	,701***	,723***	,801***	,842***	,875***
IL-10	,516***	,569***	,645***	,716***	,777***	,652***	1,000	,953***	,712***	,756***	,823***	,720***
IL-22	,523***	,566***	,649***	,712***	,814***	,701***	,953***	1,000	,749***	,765***	,822***	,768***
MMP9	,561***	,574***	,710***	,798***	,847***	,723***	,712***	,749***	1,000	,777***	,841***	,723***
IFN-γ	,637***	,721***	,757***	,861***	,845***	,801***	,756***	,765***	,777***	1,000	,885***	,739***
TNF-α	,646***	,710***	,815***	,884***	,871***	,842***	,823***	,822***	,841***	,885***	1,000	,810***
IL-1β	,546***	,586***	,685***	,723***	,805***	,875***	,720***	,768***	,723***	,739***	,810***	1,000

b.

Spearman's rho Correlation coefficient (n = 213)

HK	IgM	IgT	CD4	CD8α	IL-4/13A	IL-8	IL-10	IL-22	MMP9	IFN-γ	TNF-α	IL-1β
IgM	1,000	,686***	,340***	,472***	,622**	,667***	0,131	,176*	0,095	,449***	,488***	,562***
IgT	,686***	1,000	,632***	,564***	,522**	,649***	0,019	0,071	,331***	,531***	,461***	,489***
CD4	,340***	,632***	1,000	,641***	,495**	,587***	0,085	0,106	,467***	,685***	,464***	,411***
CD8α	,472***	,564***	,641***	1,000	,736**	,551***	,292***	,330***	,192**	,635***	,571***	,428***
IL-4/13A	,622***	,522***	,495***	,736***	1,000	,688***	,465***	,486***	0,098	,660***	,679***	,557***
IL-8	,667***	,649***	,587***	,551***	,688**	1,000	,332***	,347***	,306***	,687***	,806***	,782***
IL-10	0,131	0,019	0,085	,292***	,465**	,332***	1,000	,979***	-0,016	,458***	,791***	,475***
IL-22	,176*	0,071	0,106	,330***	,486**	,347***	,979***	1,000	-0,011	,487***	,767***	,492***
MMP9	0,095	,331***	,467***	,192***	0,098	,306***	-0,016	-0,011	1,000	,175*	0,165	,238***
IFN-γ	,449***	,531***	,685***	,635***	,660**	,687***	,458***	,487***	,175*	1,000	,622***	,532***
TNF-α	,488***	,461***	,464***	,571***	,679**	,806***	,791***	,767***	0,165	,622***	1,000	,744***
IL-1β	,562***	,489***	,411***	,428***	,557**	,782***	,475***	,492***	,238**	,532***	,744***	1,000

c.

Spearman's rho Correlation coefficient (n = 213)

Spleen	IgM	IgT	CD4	CD8α	IL-4/13A	IL-8	IL-10	IL-22	MMP9	IFN-γ	TNF-α	IL-1β
IgM	1,000	,821***	,464***	,354***	,492***	,797***	,437***	,429***	,547***	,473***	,784***	,772***
IgT	,821***	1,000	,277***	,487***	,569***	,726***	,454***	,487***	,328***	,476***	,677***	,688***
CD4	,464***	,277***	1,000	,347***	,400***	,529***	,325***	,330***	,574***	,551***	,530***	,457***
CD8α	,354***	,487***	,347***	1,000	,787***	,361***	,650***	,694***	,146*	,614***	,331***	,302***
IL-4/13A	,492***	,569***	,400***	,787***	1,000	,497***	,724***	,753***	,269***	,670***	,474***	,428***
IL-8	,797***	,726***	,529***	,361***	,497***	1,000	,460***	,459***	,648***	,565***	,939***	,933***
IL-10	,437***	,454***	,325***	,650***	,724***	,460***	1,000	,963***	,386***	,560***	,433***	,417***
IL-22	,429***	,487***	,330***	,694***	,753***	,459***	,963***	1,000	,346***	,570***	,446***	,418***
MMP9	,547***	,328***	,574***	,146*	,269***	,648***	,386***	,346***	1,000	,393***	,620***	,597***
IFN-γ	,473***	,476***	,551***	,614***	,670***	,565***	,560***	,570***	,393***	1,000	,567***	,475***
TNF-α	,784***	,677***	,530***	,331***	,474***	,939***	,433***	,446***	,620***	,567***	1,000	,910***
IL-1β	,772***	,688***	,457***	,302***	,428***	,933***	,417***	,418***	,597***	,475***	,910***	1,000

Statistical analysis using Spearman's rho correlation coefficient showed an overall strong correlation between all the genes expression studied, post vaccination in skin and spleen and also with some exceptions (IL-10, IL-22 and MMP9) in head kidney as shown in Table 1. This demonstrates a facilitated cross-talk between immune genes in vaccinated group for improved vaccine efficacy of the candidate vaccine.

Overall, the gene expression results showed the modulation of both innate and adaptive as well as systemic and mucosal immune gene responses, with strong correlation between them, thus reflecting their important role in host-parasite interaction and its mode of protection.

Conclusion

In conclusion, the evidences from the antibody levels, transcriptional levels and sampling results post challenge in the present project showed that TT-P0 antigen used in this study is a potentially good vaccine candidate against salmon lice (*L. salmonis*) with minimal side effects thus fulfilling the main purpose and goal of the project at the 1st small-scale laboratory validation. The cumulative sampling result showed an overall vaccine efficacy of 86 % in the vaccine injected group. In addition, the results also revealed the simultaneous involvement of both systemic and mucosal immunity for protection in the 1st generation of infection. Consequently, the vaccine showed visible impact on the 1st generation of sea lice infection, which resulted in delayed hatching pattern and reduced copepodite count in the 2nd generation in vaccinated group. This indicates a larger impact on 2nd parasite generation by reduced re-infection ability and re-infection load, delayed production of parasite transmission stages via less females, delayed reproduction, and more decreased fecundity, thus eventually leading to exponential decrease in lice re-infection load for sustainable protection. However, long-term challenge trials post vaccination is necessary to fully understand and explore the ultimate limit of protection of TT-P0 candidate vaccine and the underlying molecular mechanism of protection at the gene level.

The results obtained from this pilot validation project, can be exploited for the future use in the follow-up project to have a full overview of the TT-P0 vaccine potential, if given opportunity. Therefore, project initiative will be made to FHF focusing on, least individual variations and long-term protection.

The future project initiatives based on the results of this validation project will therefore further explore the ultimate potential of the vaccine for field application and better welfare, thus being actively exploited for the benefit and sustainable growth of the salmon industry and vaccine companies.

These data documented here should shed further light on our understanding of the function of IgT and IgM and their cross-talks in mucosal and systemic immunity and thus will provide new insights into the vaccine design and vaccination methods in fish aquaculture.

Moreover, the results obtained in this vaccine validation project is in preliminary stage of potential effectiveness but it shows the future potential of long term effectiveness in controlling salmon lice infestation in a eco-friendly and sustainable manner and thus can be utilized by the salmon industry and vaccine companies nationally and internationally with good social acceptance, which is in line with FHF's vision of sustainable and profitable growth of seafood industry.

6 Main findings/Hovedfunn

English version

The main findings from the project are listed below:

- Vaccine affected mostly female numbers and female fecundity in 1st generation followed by delayed hatching pattern and reduced copepodite count in the 2nd generation, indicating larger impact of the vaccine on 2nd parasite generation as revealed by the sampling data. This was also supported by antibody response and gene expression data.

- Minimal side effects of the vaccine were observed in the vaccinated group.

- High upregulation of IgM gene with corresponding higher specific IgM antibody titer in serum against P0 antigen were found in vaccinated groups as compared to control.

- High upregulation of IgM gene in skin with corresponding higher P0 antigen specific IgM antibody titer found in the mucus of the vaccinated groups compared to the control.

- Transcript levels of IgM and IgT and other immune genes studied were strongly correlated in the skin and lymphoid tissues of the vaccinated groups. This reveals the important cross-talk between mucosal and systemic immunity as well as innate and adaptive immunity showing mixed Th1/Th2 type response, thus contributing to reduced adult female numbers and their fecundity in 1st generation.

Norsk versjon

Hovedfunnene fra prosjektet er oppsummert nedenfor:

- Vaksinen påvirket hovedsakelig antall hunnlus og deres reproduksjonsevne i 1. generasjon, etterfulgt av forsinket klekkemønster og redusert antall 2. generasjons kopepoditter, noe som indikerer større effekt av vaksinen på 2. parasittgenerasjon som avdekkes av prøvetakingsdataene. Dette ble også støttet av antistoffrespons og genuttrykksdata.

- Lite bivirkninger av vaksinen ble observert i den vaksinerte gruppen.

- Høy oppregulering av IgM-genet med tilsvarende høyere spesifikke IgM antistoff titere i serum mot P0 antigen ble påvist i vaksinerte grupper sammenlignet med kontrollgruppen.

- Høy oppregulering av IgM-genet i huden med tilsvarende høyere P0 antigen-spesifikt IgM antistoff titer i slim ble påvist i vaksinerte grupper sammenlignet med kontrollgruppen.

- Transkripsjonsnivåer av IgM og IgT og andre immungener som ble undersøkt, var sterkt korrelert i hud og lymfoidvev i de vaksinerte gruppene. Dette avdekker den viktige sammenhengen mellom mukosal og systemisk immunitet, samt medfødt og adaptiv immunitet som viser blandet Th1 / Th2 type respons, og derved bidrar til redusert antall hunnlus og deres reproduksjonsevne i 1. generasjon.

7 Deliveries

Detailed overview of the deliveries in the project are:

	Deliveries	Delivery date
1.	Reference group and partners meeting	First meeting on 30th Jan 2018, second and last meeting on 18th June 2018
2.	Summary from the meeting	Revised minutes from the respective meeting submitted on 12th February and 11th July 2018.
4.	Manuscript submission	Total one manuscript. Targeted to submit in relevant open access peer-reviewed journal. It is in preparation phase and targeted to submit in December 2018.
5.	Presentation at the national and international conference	International Conference: Bioaqua2018: from basic research to applied science, Havana, Cuba, 28thOctober -1st November 2018. Cuban collaborator in this project -Yamila Carpio has given the oral presentation on 31st October. The result will be also presented at the coming National Conference: Lusekonferansen 2019, Trondheim, 21st - 23rd Jan. 2019.
6.	Status reports / Summary of results	The overall summary of the results except qPCR and mucus IgM were submitted in the summary from the last meeting on 11th July 2018
7.	Final scientific report (according to the FHF guidelines)	Submitting on 13th November 2018
8.	Administrative final report (according to the FHF guidelines)	Submitted on 31st October 2018

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ISBN 978-82-8296-570-5 (pdf)
ISSN 1890-579X