Infectious salmon anemia (ISA) is a systemic disease caused by an orthomyxovirus, which has a significant economic impact on the production of Atlantic salmon (Salmo salar). Currently, there are several commercial ISA vaccines available; however, those products are applied through injection, causing stress in the fish and leaving them susceptible to infectious diseases due to the injection process and associated handling. In this study, we evaluated an oral vaccine against ISA containing a recombinant viral hemagglutinin-esterase and a fusion protein as antigens. Our findings indicated that oral vaccination is able to protect Atlantic salmon against challenge with a high-virulence Chilean isolate. The oral vaccination was also correlated with the induction of IgM-specific antibodies. On the other hand, the vaccine was unable to modulate expression of the antiviral related gene Mx, showing the importance of the humoral response to the disease survival. This study provides new insights into fish protection and immune response induced by an oral vaccine against ISA, but also promises future development of preventive solutions or validation of the current existing therapies.

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necrosis virus (IPNV) both in vitro and in vivo, the mechanism is not fully elucidated [11,12]. In addition, the protective role of Mx, which has been demonstrated against IPNV, has not been described in ISAV [13–16].

The aim of this work is to evaluate in-vivo different formulations containing various components of a recombinant oral vaccine against ISA and its effect in experimental challenges against a Chilean highly virulent isolate.

2. Material and methods

2.1. Fish maintenance

Disease free Atlantic salmon average weight 40 g were maintained at Centrovet animal facilities in a fresh water recirculation system (Santiago, Chile). Before trials, fish were acclimated to controlled environment during 2 weeks in 1 m³ tanks at a density of 15 kg/m³, and a water exchange rate of 1 m³/hour. Water condition for acclimation and trials were: 12.1 ± 0.6°C, pH 7.4 ± 0.3 and oxygen saturation 80–120%. Two weeks after acclimation fish were placed on 100 L tanks in groups of 55 at a density of 50 kg/m³ where the experiments were conducted. Fish were fed ad libitum (Golden Activa 2.0 mm, Biomar).

2.2. Vaccine and virus

The vaccine was developed using recombinant DNA technology in Saccharomyces cerevisiae, the conserved regions of surface proteins of the virus HE and protein F from different ISAV isolates were cloned as synthetic nucleotide-optimized genes for yeast on an expression vector (DNA 2.0, USA). Yeast expressing the antigens were subjected to disruption using a cell disintegrator (DYNO- MILLS, Glenn Mills), and ISAV protein-containing fractions were encapsulated in a cationic polysaccharide matrix (Micromatrix™, Advanced BioNutrition, Columbia, MD, USA), according to procedure described elsewhere [17,18]. Feeds were formulated with five different components (Table 1) including control groups.

Fish were fed with vaccine-formulated feed prepared in a final concentration equivalent to 6 mg vaccine/fish/day for 10 days, as described previously [17]. For the experimental challenge, virulent ISAV isolate (HPR7b strain) isolated from a field outbreak (X Region, Chile) was used. The virus was expanded in cell line SHK-1 according to Eliassen et al. [18].

2.3. Experimental design and samples

The challenges were conducted in Centrovet animal facility (Santiago, Chile). 55 fish per group were stocked in 100 L tank, at a density of 22 kg/m³. Experimental design comprised 12 groups of 55 fish per tank, 6 groups challenged and 6 groups unchallenged (performed in duplicate and in separate tanks). The transmission model was IP, since it is highly reproducible and reliable method in efficacy evaluation [19]. 450 days (DD) post vaccination the challenged groups were inoculated by IP injection, 0.2 mL of ISAV (3 × 106 TCID50/fish). The non-challenged group was inoculated with 0.2 mL of L-15 medium.

Mortality was recorded daily until day 53 post challenge and necropsy was performed as a diagnosis method of the disease according to characteristic lesions [20,21] and confirmed by PCR analysis according to Mikalsen et al. [22]. Blood and kidney tissue were sampled from 3 fish per group at different sampling times (pre-vaccination, post vaccination and 150, 300, 560 and 740 DD post vaccination) for antibody evaluation and gene expression respectively. Kidney tissue were stored at −20 °C in RNA Later (Ambion, California, USA) and the blood was centrifuged for serum extraction and stored at −20 °C. Sampling and monitoring of ISAV-induced mortality were performed in parallel experimental groups in order to avoid influences of stress in mortality due to handling.

The trials were performed in accordance with the Chilean legislation for animal experimentation under the manual “Bioethical aspects of animal experimentation” issued by the National commission of scientific and technological research.

2.4. Antibody ELISA

Nunc Maxisorp (Nunc, Roskilde, Denmark) plates were activated with 5 µg of an equimolar mixture of vaccine antigens in bicarbonate buffer, pH 8.5. The plates were blocked with PBS containing 1% BSA and test sera diluted 1:50 were added and incubated at 4 °C overnight. The following day the plates were washed with PBS and incubated with monoclonal mouse anti-salmon (dilution 1:500) IgM isotype IgG1 (BiosChile, IGSA, Chile) for 1 h at 30 °C. The plates were then washed again and incubated at 30 °C with horseradish peroxidase-conjugated goat anti-mouse (dilution 1:1000) IgG (KPL, Maryland, USA). Serum antibody titers were determined using 3,3’,5,5’-tetramethylbenzidine as a chromogenic substrate and H2SO4 to stop the reaction. Values were obtained by measuring the absorbance at 450 nm. Sera from experimentally ISAV-infected and from healthy non-immunized fish were used as positive and negative controls, respectively and all sera were tested in triplicates.

2.5. RNA extraction and cDNA synthesis

RNA was extracted from kidney tissues using the kit AxyPrep Multisource Total RNA (Axygen, Massachusetts, USA) according to the manufacturer’s instructions. For reverse transcription 1 µg of template RNA was used (ImProm-II, Reverse Transcription, 

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Immunization treatments, vaccines and controls used in this study.</th>
</tr>
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<tbody>
<tr>
<td><strong>Treatment</strong></td>
<td><strong>Details</strong></td>
</tr>
<tr>
<td>Encapsulated recombinant antigen (Vaccine)</td>
<td>Freeze dry extract of recombinant ISAV antigens expressed in Saccharomyces cerevisiae and encapsulated in polymeric matrix (Micromatrix™)</td>
</tr>
<tr>
<td>Empty microcapsule</td>
<td>Freeze dry extract that contains the polymeric matrix (Micromatrix™) without antigen.</td>
</tr>
<tr>
<td>Recombinant yeast non encapsulated (Yeast RN)</td>
<td>Freeze dry extract of recombinant ISAV antigens expressed in Saccharomyces cerevisiae non-encapsulated.</td>
</tr>
<tr>
<td>Non recombinant yeast non encapsulated (Yeast NE)</td>
<td>Freeze dry extract of wild type Saccharomyces cerevisiae non-encapsulated.</td>
</tr>
<tr>
<td>Control</td>
<td>Oil coated feed.</td>
</tr>
</tbody>
</table>

NR: Non recombinant, RN: Recombinant, NE: Non Encapsulated, EN: Encapsulated, Micromatrix: Commercial name of the cationic polysaccharide matrix used for encapsulation of antigens.
Promega) following the manufacturer’s instructions.

2.6. Real-time PCR

RT-qPCR reaction was performed using One Step Real Time PCR system (Applied Biosystems, USA). The primers sequence and thermal cycling conditions were performed according to McBeath et al. (14). The tested transcripts were the Mx protein (Mx) and the Elongation Factor-1α (ELF) as a reference gene. Relative mRNA expression was calculated using the method of ΔΔCt adjusted to primers efficiency.

The diagnostic PCR form ISAV segment 8 was performed according to Mikalsen et al. [22].

2.7. Data analysis

Statistical analysis was performed using the statistical software GraphPad Prism 5 (Graphpad Software, Inc). Survival curves were analyzed using Kaplan-Meier and group differences were analyzed by the log-rank test, using the Bonferroni correction for multiple comparisons. To assess the effectiveness of the different formulations the following parameters were evaluated, the relative percent survival (RPS), absolute risk reduction (ARR) these indicators estimate the risk reduction of death by ISAV in the different treatments (in relative and absolute terms, respectively). We also evaluated the number of animals necessary to treat (NNT), which is defined as the number of animals to treat experimentally in order to prevent the development of the disease, in this case death by ISAV. All formulas used are described in [Supplement Table 1]. Differences in antibodies were calculated using analysis of variance (ANOVA) for repeated measures and Bonferroni comparison for each treatment. The analysis of the RT-qPCR results was calculated relative to ELF transcript, and presented as a relative expression (ΔΔCt).

Differences between groups were analyzed by ANOVA with Newman-Keuls test. Differences between groups were analyzed by ANOVA with Newman-Keuls test. Statistical analysis was performed using the statistical software GraphPad Prism 5 (Graphpad Software, Inc). Survival curves were analyzed using Kaplan-Meier and group differences were analyzed by the log-rank test, using the Bonferroni correction for multiple comparisons. To assess the effectiveness of the different formulations the following parameters were evaluated, the relative percent survival (RPS), absolute risk reduction (ARR) these indicators estimate the risk reduction of death by ISAV in the different treatments (in relative and absolute terms, respectively). We also evaluated the number of animals necessary to treat (NNT), which is defined as the number of animals to treat experimentally in order to prevent the development of the disease, in this case death by ISAV. All formulas used are described in [Supplement Table 1]. Differences in antibodies were calculated using analysis of variance (ANOVA) for repeated measures and Bonferroni comparison for each treatment. The analysis of the RT-qPCR results was calculated relative to ELF transcript, and presented as a relative expression (ΔΔCt).

Differences between groups were analyzed by ANOVA with Newman-Keuls test. P ≤ 0.05 was considered significant.

3. Results

3.1. Post challenge survival of oral ISA vaccinated fish

Mortality curve of the control group was almost identical to the immunized groups with non-recombinant yeast (non-encapsulated) and with the empty microcapsule at 42 DPC, resulting in similar cumulative mortalities (Table 2). The group immunized with recombinant non-encapsulated antigens presented a cumulative mortality of 70% at 21 DPC and the group immunized with the vaccine showed a cumulative mortality of 33.3% at 20 DPC. Mortality percentages remained unchanged until the end of the trial (Fig. 1). The unchallenged groups showed no mortalities during the course of the trial.

Differences between the survival curves were evaluated using Kaplan-Meier (Fig. 1). Pairwise comparisons relative to control indicated that fish treated with the vaccine have a significantly higher survival rate (log-rank P < 0.0001). The survival rate of all other treatments was not significantly different than the control (Supplement Table 2).

3.2. Efficacy evaluation of the oral vaccination

To evaluate the efficacy of the different formulations, RPS and ARR were calculated, these indicators estimate the risk reduction of death by ISAV in the different treatments (in relative and absolute terms, respectively). We also evaluated the NNT, which is defined as the number of animals to treat experimentally in order to prevent the development of the disease, in this case death by ISAV (Table 2).

The vaccination effectiveness showed a variable reduction in mortality. The orally immunized group with the vaccine presented an RPS of 64.3%, ARR of 60% and NNT = 2, the unencapsulated antigen group presented a 25% RPS, ARR 23.3% and NNT = 5. The other experimental treatments had an RPS less than 7.1% and ARR lower than 6.7% and confidence intervals with negative lower limits, indicating that all those treatments did not provide any additional protection in relation to the control group (Table 2).

3.3. Humoral response to vaccination and challenge

No specific antibodies were detected before vaccination in any of the experimental groups. Only the orally immunized group with the vaccine showed a significant increase (P < 0.001) in antibodies. The antibodies were detectable in fish serum, even at 330 DD post challenge (Fig. 2). The increase of antibodies in the vaccinated group was significant compared to the control (P < 0.001).

Table 2

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Mortality</td>
<td>Vaccine Empty microcapsule Recombinant yeast non encapsulated Non recombinant yeast non encapsulated Non recombinant yeast encapsulated Control</td>
</tr>
<tr>
<td>% RPS</td>
<td>33.3</td>
</tr>
<tr>
<td>% ARR</td>
<td>89.7</td>
</tr>
<tr>
<td>IC 95% ARR</td>
<td>70.0</td>
</tr>
<tr>
<td>NNT</td>
<td>93.3</td>
</tr>
<tr>
<td>IC 95% NNT</td>
<td>2.4–21.4</td>
</tr>
</tbody>
</table>

Indicators calculated in relation to control group.

RPS: Relative percentage survival, ARR: Absolute risk reduction, NNT: Number necessary to treat.
3.4. Post challenge Mx transcript expression

In challenged and non-challenged groups, the pre and post vaccination level of Mx transcript remained relatively low (Fig. 3). However, at 450 DD the challenged groups, shows an increase in transcript expression reaching 30 fold at 630 DD (Fig. 3B). This dramatic increase in Mx transcript expression was not observed in the unchallenged group (Fig. 3A), the relative expression of the transcript remained low and stable over the course of the trial. No significant difference was observed in Mx transcript induction between the different treatments in the challenged group.

4. Discussion

The results showed that the RPS obtained after oral immunization is related to the presence of the antigens and encapsulation. The encapsulated antigen group (vaccine) exhibits an RPS of 64.3%, showing that the formulation is capable of inducing a significant protection against ISA. Mortality curves of non-encapsulated formulations including the non-vaccinated control group were similar without significant differences between treatments, demonstrating that oral immunization with encapsulated antigens is an effective delivery system.

Among the challenged fish, first fish died at 13 DPC, mortality rate then increased. This results obtained are in agreement with the previously reported incubation period for ISAV infection of 10–20 days [23–26]. The cumulative mortality of the unvaccinated control fish was 93.3%, indicating the high virulence of the virus [27].

The viral isolate used in the current study corresponds to HPR7b Chilean strain and comparison of results with other studies reported in the literature is difficult, because most of them used Norwegians isolates, which differ from the Chilean in their genetic background virulence [23,28]. However there is a recent report, where an oral vaccine based on inactivated virus is tested against a Chilean strain [26].

Few studies evaluated the efficacy of vaccine formulations against ISAV and just one the oral delivery [26]. Mikalsen et al. [6] evaluated a DNA vaccine, obtaining an RPS of 60.5% with a cumulative mortality of 41.3% in the control group post IP challenge. Jones et al. [29] evaluated an injectable inactivated vaccine,
showing an RPS of 92–96% after challenged by cohabitation, but the RPS was 0 after an IP challenge. Lauscher et al. [25] evaluated different injectable inactivated vaccines, obtaining an 86% RPS after cohabitation challenge with a cumulative mortality of 71.7% in the control group [25]. Rivas-Aravena et al. [26] evaluated an oral inactivated vaccine and used an alphanivirus replicon as adjuvant, obtaining a RPS of 77% evaluated by IP challenge. The ISAV isolate used was from Chilean origin belonging to HPR7b strain and challenged with $1 \times 10^5 \text{TCID}_{50}$/fish. In contrast, our work used a viral isolate with a similar genetic background and origin but in a 30-fold higher challenge dose, equal to $3 \times 10^5 \text{TCID}_{50}$ obtaining a cumulative mortality for the unvaccinated control group of 93.3% in contrast to 48% obtained in Rivas-Aravena’s work, which is expectable due to the lower challenge dose used. Although our RPS values obtained are quite acceptable, it is likely that these are underestimated due to the lethality of the challenge. Besides of these clear differences in terms of the aggressiveness of challenge conditions Rivas-Aravena et al. [26] and our work demonstrate that oral vaccination represents an effective approximation to prevent ISA outbreaks.

The efficacy of vaccine formulations, either in experimental and field studies, has been traditionally assessed by the RPS method, which allowed to objectively evaluate the efficacy [19]. However, it has disadvantages such as showing benefits of treatments in relative terms [30]. The results depend upon factors that are unique to the different testing models and that interact dynamically with each other during the experiment [31]. Making difficult to compare RPS results of different vaccines and different environments [32]. Because of these reasons, the study also evaluated other parameters, such as ARR and NNT, which allowed an objective evaluation of the preventive treatment against the control. In the present study, the ARR and NNT evaluation gave good results for vaccine group with an ARR of 60% and NNT of 2. The non-capsulated antigen group provided an ARR of 23.3% and a NNT of 28%, both with relatively high CI values (Table 2). Other Immunization treatments with empty microcapsule and non-recombinant yeast encapsulated presented very low values and confidence intervals with negative values, which restricted us to determine whether the treatment is effective or even generated adverse effect. The vaccination effect of the non-recombinant non-encapsulated yeast treatment was not different from the control (Table 2).

Oral immunization with encapsulated antigen formulations was able to induce specific antibodies in both challenged and unchallenged groups. In teleost it has been described the importance of mucosal specialized immunoglobulin, IgT [32–35] which plays an important role in disease resistance [36]. However, due to the limited availability of analytical methods to evaluate the mucosal immune response in salmonids, assessment was not carried out and only systemic IgM was evaluated. On the other hand, by administering an oral vaccine a stimulation of mucosal associated lymphoid tissue and mucosal antibody production is quite expected, and hence, their important role in determining fish survival [17], in future works we expect to evaluate its induction.

The present study considered only a primary vaccination, which partially explains the low levels of antibodies obtained in challenged and unchallenged groups. Although the challenged group showed a large induction of antibodies in the vaccinated group after 290 DD post-challenge indicating a secondary response to the antigen (Fig. 2). It is known that repeated exposure to the antigen increases the magnitude and specificity of the immune response. In this work we demonstrated that oral immunization is capable of inducing the production of specific ISAV antibodies prior to challenge, in agreement with Lauscher results using an inactivated injectable vaccine [24]. Additionally, in this study an induction of antibodies was observed 290 DD post-challenge, suggesting that some degree of immunological memory is generated against the primary vaccination. The important role of disease specific antibodies determining the survival of infected fish has been described elsewhere by either passive immunization by transferring convalescent serum fish [37] or active immunization using an inactivated vaccine [24]. The ability of salmon to elicit neutralizing antibodies against ISA has been described in other studies, assuming that would be directly against HE [24,37,38] but antibody titers are generally low [6,39]. Interestingly, the vaccine tested by Rivas-Aravena et al. [25] also used an oral delivery platforms and was not able to induce antibodies in the blood, cutaneous, Gill, intestinal mucus, nor bile [25], which suggest that exist different mechanisms of action between delivery platforms: This work, focused on stimulating specific immunity, at humoral and probably cellular level, due IFN-I induction, and, probably by generating an antiviral state modulated by the alphanavirus replicon, rather than a specific effect conferred by the vaccine antigen [25].

As in most host pathogen interactions, host resistance is highly dependent on both, virulence of the infectious agent as well as the ability of the host to mount an effective immune response. Several studies have evaluated the interaction between ISAV and Atlantic salmon, either through in vitro model using various cell lines [15,40,41] or in vivo model with different isolates of the virus [40,42]. All the results showed an increase in the expression of various genes associated with innate immunity during the incubation period of the disease. However, in spite of the presence of a potent antiviral response, most of the fish succumbed to high virulence isolates [42].

The head kidney of salmonids is the major site of B cells generation and consists mainly of hematopoietic tissue. For this reason it was selected as the target organ for gene expression. The Mx protein role in antiviral defense has been extensively documented [43]. The protein is induced by IFN-I, and can be used as a marker of the innate immune response against viral infections. A similar Mx protein response has been shown in infection with IPNV, where it determines the susceptibility/resistance states of infection [11]. This protein has also been shown to have an inhibitory effect on viral replication [14]. Nevertheless, it has shown to have little or no effect on ISAV replication both in vitro and in vivo studies [44,45]. In the present study we observed a strong induction of Mx transcript in challenged groups but not in the unchallenged. However, the expression was not significantly different between treatments, indicating that none of the formulations was able to modulate the antiviral pathway and the high levels of Mx transcript expression are merely due to ISAV infection.

Activation of adaptive immune system is assumed to play a central role in viral clearance and the survival of the host. Future work should be focused on the evaluation of the response to booster effect and subsequent challenge with different routes of infection as cohabitation.

5. Conclusions

Results presented in the current study demonstrate that immunization with an oral vaccine induces significant protection against a highly virulent ISAV isolate. The protection was dependent on is the delivery form and the vaccine components. Attributes were given to the combination of recombinant antigens and the oral delivery technology. Encapsulated antigen in MicroMatrix™ gave the best results, whereas none of the other treatments presented comparable levels of protection. Survival of orally vaccinated fish was similar to, or even higher than, those vaccinated with an injectable formulation reported in the literature [6,24,28].
Conflict of interest statement

The authors have not potential conflicts of interest to disclose.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jfi.2016.03.009.

References