

RESEARCH ARTICLE

Increased survival and reduced bacterial shedding following single but not repeated oral immunizations against *Piscirickettsia salmonis* in Atlantic salmon *Salmo salar*

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Outbreaks of *Piscirickettsia salmonis* in cultured salmon populations result in high mortality and high economic loss. An efficacious vaccine against this pathogen is highly desired; however there has been limited success in this regard. In the current study, Atlantic salmon (*Salmo salar*) smolts were vaccinated by single, double, or quadruple immunization with a commercial oral vaccine developed using a Chilean isolate of *P. salmonis*. Fish were then challenged by immersion with a Canadian isolate of *P. salmonis*. Significantly greater survival was observed 300 degree-days (DD) following single immunization (30.9 relative percent survival - RPS) and 370 DD following quadruple immunization (21.1 RPS). Increased survival in the former group was associated with reduced bacterial shedding, and in the latter group, with increased mean days-to-death. Bacterium-specific serum antibodies were not influenced by vaccination in any treatment. No protection was elicited following double immunization. The relatively low protection observed following oral immunizations may be related to antigenic heterogeneity between the isolates used in vaccine manufacture and laboratory challenge. The results of this study highlight the difficulties in production of an efficacious vaccine against *P. salmonis*.

Introduction

Piscirickettsia salmonis (Gammaproteobacteria, Piscirickettsiaceae) is the etiological agent of salmonid rickettsial septicemia (SRS), and the most frequently diagnosed pathogen in seawater-farmed salmonids in Chile (Henriquez et al. 2016). The organism is a Gram-negative, non-motile, facultative intracellular bacterium first isolated from SRS-affected coho salmon (*Oncorhynchus kisutch*) (Fryer et al. 1992). SRS outbreaks have also been reported in farmed Atlantic salmon (*Salmo salar*) from eastern and western Canada, Norway, Scotland and Ireland (Brocklebank et al. 1993; Rodger and Drinan 1993; Grant et al. 1996; Olsen et al. 1997; Cusack, Groman, and Jones 2002; S. R. M. Jones et al. 2020).

Management of SRS requires antibiotic treatment, and 82-90% of all antibiotic use in Chilean salmon aquaculture is for the treatment of this infection (Rozas and Enriquez 2014; Price et al. 2018). In Chile, the dependency on chemotherapeutic intervention is explained in part by poor

efficacy of the available commercial SRS vaccines under salmon production conditions (Evensen 2016; Rozas-Serri 2022; C. Figueroa et al. 2022). Although vaccination theoretically increases host resistance to *P. salmonis*, the efficacy achieved by experimental *P. salmonis* vaccines is inconsistent under laboratory conditions (C. Figueroa et al. 2022). Efficacious oral vaccines have been developed against multiple salmonid pathogens (Mutoloki, Munangandu, and Evensen 2015; Bøgwald and Dalmo 2021) and elicit protective immunity following primary or secondary applications under controlled conditions (Ghosh et al. 2016; Jaafar et al. 2019). Oral vaccines are highly desirable because, in comparison to injectable vaccines, they are easily administered to fish of varying ages with minimal stress.

Recent evidence of genetic heterogeneity among isolates of *P. salmonis* (J. Figueroa, Castro, Lagos, et al. 2019; Schober et al. 2023) raises questions about the ability of commercial SRS vaccines to provide cross-protection against heterologous strains. The present study explored the relative efficacies of single and multiple applications of a commercial oral vaccine from Chile against a Canadian strain of *P. salmonis* in laboratory challenged Atlantic salmon.

Materials and Methods

Fish

All research was conducted in the biosecure research aquarium at the Pacific Biological Station (PBS) in accordance with the Canadian Council on Animal Care guidelines and approved by Fisheries and Oceans Canada's Pacific Regional Animal Care Committee (AUP 20-002). A total of 560 fish were used in this experiment. Atlantic salmon (mean weight = 41.9 g) from a commercial hatchery on Vancouver Island were held in 2000 L tanks of dechlorinated, municipal freshwater (12 °C) at 25 L/min under a natural photoperiod and fed a commercial pelleted diet (EWOS Canada) at 1% total biomass/d. Fish were transitioned to sand-filtered, UV-treated flow-through seawater 16 d prior to the onset of the first immunization. Nine days post-seawater transition, water temperature was increased to 15 °C.

Vaccine administration

Three treatment groups were initially established in separate tanks: control (Control, n = 160), single vaccinate (1xV, n = 80), and double vaccinate (2xV, n = 240). At 865 degree-days (DD), 80 salmon from the 2xV group were moved to a separate tank to become a quadruple vaccinate (4xV) group. See [Table 1](#) for the timings of vaccine administration, blood sampling (n = 10), and immersion challenges with *P. salmonis*.

Table 1. Timing of oral vaccinations, blood sampling and bacterial challenges for Atlantic salmon *Salmo salar* immunized with a commercial oral vaccine against *Piscirickettsia salmonis*

Treatment*	Degree-Days**									
	0	450	700	851	1000	1400	1850	2100	2251	2370
Control (n=160)		S		S	C1		S		S	C2
Single vaccinate (1xV; n=80)		S	V	S	C1					
Double vaccinate (2xV; n=240)	V	S	V	S	C1		S		S	C2
Quadruple vaccinate (4xV; n=80)						V	S	V	S	C2

*80 fish moved from double vaccinate group into a separate tank at 865 degree days (DD) to form the quadruple vaccinate group. **Product of days and temperature (15 °C) since onset of study. V, vaccine administered for 10 days (150 DD). S, blood sampled (n=10/treatment). C1 and C2, immersion challenges (1.52×10^4 colony-forming units/ml (cfu/ml) and 1.28×10^5 cfu/ml, respectively).

Preparation of vaccine-treated feed

Commercial feed pellets were top-coated with Blueguard SRS Oral® (Virbac Chile), anchovy oil (Taplow Ventures; 3% w/w), and freeze-dried krill (San Francisco Bay Brand, Inc.; 1.5% w/w). New batches of vaccine-treated feed were prepared for each 10 d regimen at a daily dose of 6 mg vaccine/fish per manufacturer's recommendations ([Table 1](#)). Controls were fed the pelleted diet top-coated with anchovy oil and krill during the vaccine administration period. Fish were fed the commercial diet at 1% total biomass/d during non-vaccine administration periods.

Immersion challenge

At 865 DD, fish (Control, 1xV, 2xV) were moved to the biocontainment laboratory and challenged with *P. salmonis* 10 d later. At 2265 DD, fish (Control, 2xV, 4xV) were moved to the biocontainment laboratory and challenged with *P. salmonis* 8 d later.

Upon transfer to the biocontainment laboratory, fish were stocked into triplicate 400 L tanks (20 fish/tank) per vaccine treatment. All tanks received sand-filtered and UV-treated flow-through seawater (7-8 L/min) at 15 °C with a mean salinity (range) of 29.2 (24.7 – 30.8) ppt. The photoperiod was set at 12 hr:12 hr. Two immersion challenges with *P. salmonis* (BC isolate SR-1) were carried out as described in Long, Goodall, and Jones (2021). The exposure dose was calculated by standard plate counts of serially-diluted inoculum. The exposure doses for Challenges 1 and 2 were 1.52×10^4 colony-forming units/ml (cfu/ml) and 1.28×10^5 cfu/ml, respectively.

Fish were monitored daily, moribund fish were euthanized by immersion in 400 mg/L tricaine methanesulphonate (TMS; Syndel, Canada) and all fish removed daily contributed to the cumulative percent mortality and morbidity (CMM) in each tank. All dead fish were examined for external lesions and a subsample of liver was preserved in 95% ethanol and screened for *P. salmonis* by qPCR (S. R. M. Jones et al. 2020). The challenges were terminated 60

(1900 DD) and 59 (3270 DD) d post-immersion (dpi), respectively. Surviving fish were euthanized in TMS and liver samples from a sub-sample of fish were screened for *P. salmonis* by qPCR. DNA was extracted from liver using the Qiagen DNeasy® Blood and Tissue kit following the manufacturer's instructions for purification of total DNA from animal tissues using approximately 25 mg of tissue.

Water sampling

Water samples were collected every 2 d from all tanks during both challenges to measure bacterial burdens. To do so, the water flow to each tank was stopped for 30 min after which a 40 ml water sample was collected. Water samples were kept at -80°C pending DNA extraction (Long, Goodall, and Jones 2021).

Blood collection

Blood samples were collected from vaccinated fish at four timepoints ($n = 10$ fish/treatment; [Table 1](#)), as well as from challenge survivors following trial termination. Blood was collected into heparinized vacutainers and plasma recovered the same day. To do so, samples were centrifuged at $1500 \times g$ for 15 min. Plasma was recovered and stored at -80°C until needed.

Serological response

ELISA plates (Corning) were coated with $100 \mu\text{l/well}$ of freeze-thaw inactivated cultured *P. salmonis* cells ($10 \mu\text{g/ml}$) in coating solution (LGC SeraCare) and incubated at 4°C overnight. Plates were then blocked by adding $300 \mu\text{l}$ of a blocking solution (2% bovine serum albumin (BSA; LGC SeraCare) in phosphate-buffered saline with tween-20 (PBST; Thermo Scientific) to each well and incubated for 2 hr at room temperature (RT). Plasma samples (diluted 1:100 in 0.7% BSA + PBST) were added to duplicate wells at $100 \mu\text{l/well}$ and plates incubated for 2 hr at RT. An HRP-conjugated mouse anti-salmonid IgG (ImmunoPrecise Antibodies; 1:2000 in 0.7% BSA + PBST) was added to each well at $100 \mu\text{l/well}$ and plates incubated for 1 hr at 37°C . Plates were washed 5 times with PBST ($300 \mu\text{l/well}$) between each step. To develop plates, $100 \mu\text{l}$ of 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate solution (LGC SeraCare) was added to each well. Plates were incubated in the dark for 15 min at RT after which the reaction was stopped by adding $100 \mu\text{l}$ of TMB stop solution (LGC SeraCare) each well. The absorbance was read immediately at 450 nm.

Each plate included four blank and four positive control wells in addition to duplicate substrate and chromogen control wells. For each plate, the average blank absorbance value was subtracted from each sample well to provide the blank-subtracted optical density value (OD), which was used for all statistical

analyses. To determine the positive-negative threshold of the assay for that day, blank values were averaged between plates and the average blank OD was multiplied by 2.

Data analysis

Relative percent survival (RPS) was calculated using the formula: $RPS = [1 - (\% \text{ vaccinate morbidity-mortality} \div \% \text{ control morbidity-mortality})] \times 100$. All analyses were done in R version 4.2.2 and results were considered statistically significant if $P \leq 0.05$. Graphs were prepared in R using the ggplot2 package (Wickham 2016). Kaplan–Meier survival curves and log-rank analysis of differences in mortality were generated using the survminer package in R (Kassambara, Kosinski, and Biecek 2021) which generated Bonferroni-adjusted P values of the pairwise comparisons. A one-way ANOVA was used to determine if differences in mean days-to-death (MDD) or CMM among treatments were significant. A Kruskal-Wallis test was used to determine the statistical significance of differences in antibody titers or $\log_{10}$ transformed bacterial burden data. Differences in infection prevalence were evaluated using Fisher's exact test. If differences were significant, post-hoc analysis was carried out using a Tukey's HSD test (ANOVA) or Dunn's test (Kruskal-Wallis).

Tank bacterial burden was normalized to sample volume and the number of fish (live and dead) in the tank at the time of sampling using the following equation: $((\text{copies/reaction} \times (1/\text{genome copies}) \times (\text{extraction elution volume} / \text{qPCR template volume})) / \text{water sample volume}) / \text{number of fish}$. Resulting values were $\log_{10}$ transformed and reported as genome equivalents/ml seawater/fish (GEq/ml SW/fish). For each challenge, a general linear model was used to assess variation in bacterial load with treatment and dpi as the explanatory fixed factors. Consistent detection of *P. salmonis* in tank water occurred after 14 (Trial 1) and 16 (Trial 2) dpi. As such, water samples taken prior to these timepoints were not included in the analysis. Post hoc analysis was carried out using the package emmeans (Lenth 2023) in which pairwise contrasts of dpi and treatment were generated and the adjusted P values (Tukey) were then used for the analysis.

Results

There was no mortality in either trial prior to challenge. In Challenge 1, the CMM in the control treatment was 62.7%. Log-rank analysis of the Kaplan-Meier survival curves determined that survival in the 1xV treatment was significantly higher than both the control ($P = 0.03$) and the 2xV ($P = 0.0014$) treatments ([Figure 1](#)). The difference in survival between the control and the 2xV treatments was not significant. Conversely, CMM was not significantly different between the control and the 1xV treatment or the 2xV treatment ([Table 2](#)). However, CMM was significantly lower in the 1xV as compared to the 2xV treatment ($P = 0.009$; [Table 2](#)), and

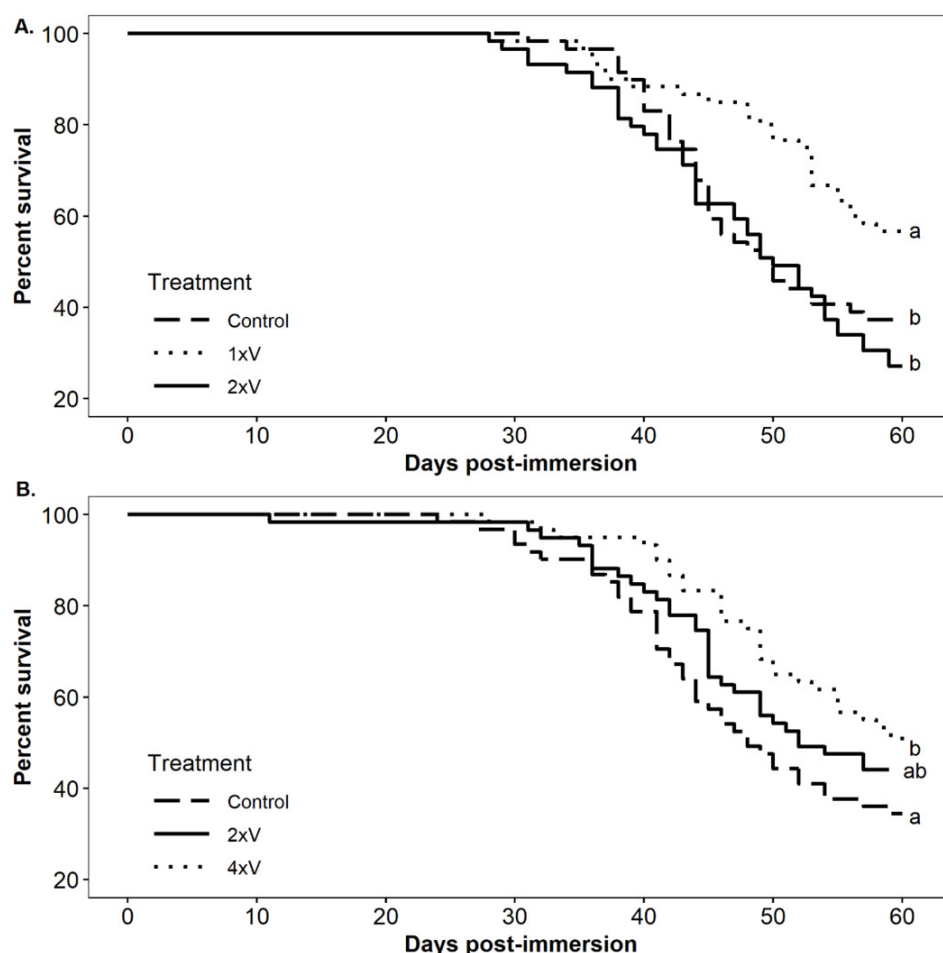


Figure 1. Survival curves of vaccinated Atlantic salmon from (A) Challenge 1 and (B) Challenge 2. Letters after each survival curve reflect statistical significance ($P \leq 0.05$).

differences in MDD between the treatments were not statistically significant. The bacterium was detected in 100% of mortalities from all treatments and in 35% of examined survivors. Differences in bacterial tissue loads (median genome equivalents) among vaccine treatments were not significant for either survivors or mortalities.

In Challenge 2, the control CMM was 65.5%. Log-rank analysis of the Kaplan-Meier survival curves determined that survival was significantly higher in the 4xV treatment as compared to control ($P = 0.05$) (Figure 1); however, differences in CMM among treatments were not significant (Table 2). MDD in the 4xV treatment was significantly higher as compared to controls ($F_{2, 103} = 3.64$, $P = 0.031$; Table 2). Similarly, the bacterial load in mortalities from the 4xV treatment was significantly lower than that of 2xV vaccinates ($\chi^2 = 6.46$, $df = 2$, $P = 0.024$) although there was no difference in load between the control and either vaccine. The bacterium was detected in 45% of survivors and 100% of tested mortalities. As in Challenge 1, there was not a significant difference in infection prevalence among treatments (Table 2).

Tank bacterial burdens provided a measure of bacterial shedding from infected fish. Burdens in both trials varied significantly between treatments depending on dpi (Challenge 1: $F_{22,138} = 30.45$, $P < 0.001$; Challenge 2: $F_{21,131} = 33.5$, $P < 0.001$) and treatment (Challenge 1: $F_{2,138} = 66.7$, $P < 0.001$; Challenge 2: $F_{2,131} = 10.2$, $P < 0.001$), and the interactive effect of the two (Challenge 1: $F_{44,138} = 1.5$, $P = 0.032$; Challenge 2: $F_{42,131} = 2.1$, $P < 0.001$). Post-hoc analysis revealed that in Challenge 1, the burden in Control and 2xV tanks was significantly greater than in 1xV tanks at multiple time points (Figure 2A, Table S1). In Challenge 2, there were significant differences among treatments at varying timepoints with no consistent vaccine-induced effect on bacterial burden (Figure 2B, Table S2). The timing of peak shedding varied between treatments (Figures S1 and S2). In Challenge 1, peak shedding in the control treatment occurred 12-14 d earlier than that of the 1xV and 2xV treatments. Conversely, in Challenge 2, peak shedding in the control treatment occurred at 48 dpi while peak shedding in the 2xV and 4xV treatments occurred at 40 and 50 dpi, respectively.

In neither challenge was there a significant difference in median plasma antibody titers among treatments whether survivors or post-vaccinated fish were examined (Figures S3, S4).

Discussion

Vaccine effectiveness can be evaluated by several metrics including survival in vaccinated fish following exposure, shedding rates of infected fish, and ability of the vaccine to provide cross-protection, i.e. protect the host against multiple different pathogen strains. The genetic heterogeneity among *P. salmonis* isolates is well-documented (J. Figueroa, Castro, Lagos, et al. 2019; Schober et al. 2023), however little is known about the impact of this genetic heterogeneity on vaccine effectiveness. The present study tested whether Atlantic salmon that were vaccinated with a commercial oral vaccine developed using a Chilean *P. salmonis* strain were protected against laboratory exposure to a Canadian *P. salmonis* strain. Single and quadruple oral immunization (1xV, 4xV) of Atlantic salmon smolts elicited a protective response against *P. salmonis* as evidenced by increased survival of the 1xV and 4xV salmon and by reduced bacterial shedding in 1xV salmon. In these groups, fish were challenged at 300 and 270 DD following final immunization, and in neither case was the duration of this early onset protective immunity assessed. An adequate test of vaccine efficacy was achieved in both trials, as assessed by control CMM of approximately 65%, however the elicited protection was low (maximum RPS of 30.9 in the 1xV treatment). Further research is recommended to assess persistence of the protection. In contrast, protective immunity was not observed in double immunized (2xV) salmon challenged at 300 or 1670 DD after challenge.

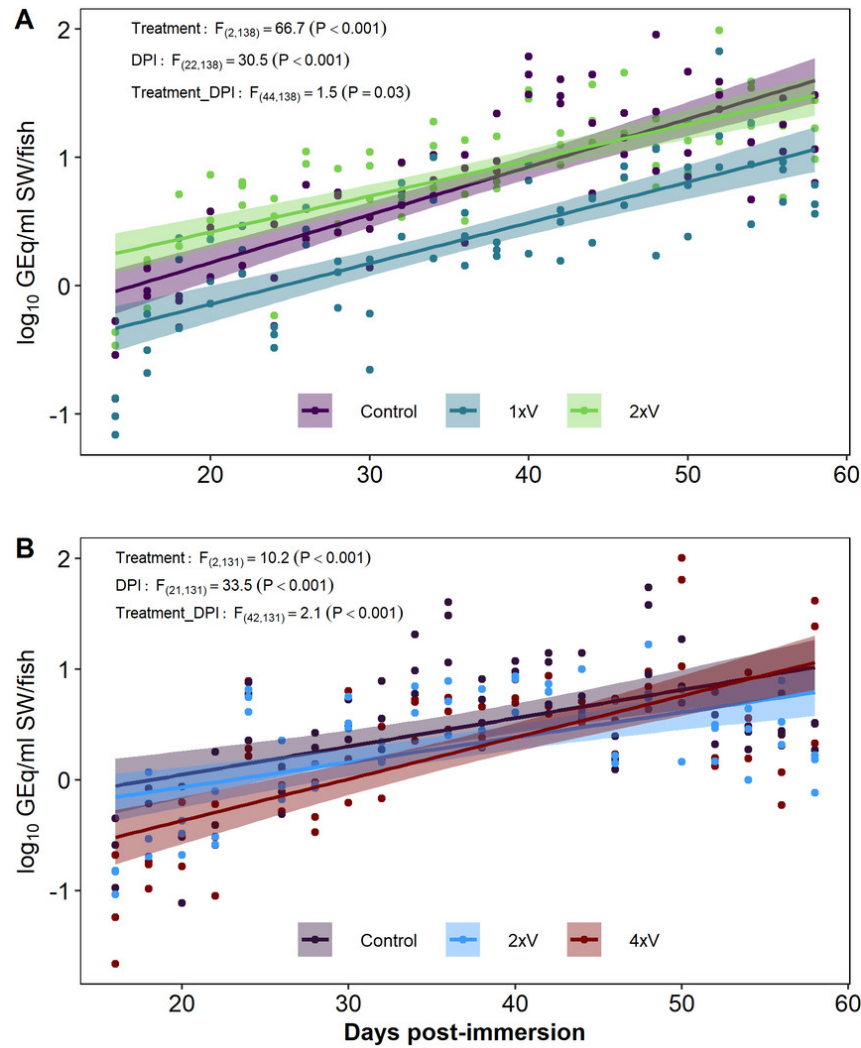


Figure 2. Tank water bacterial burdens (log₁₀ genome equivalents/ml seawater/fish) for **(A)** Challenge 1 and **(B)** Challenge 2. Each dot represents an individual tank. The shaded area represents the 95% confidence level interval. The general linear model indicated significant direct and interactive effects of treatment and days post-immersion (DPI) on tank bacterial load.

Table 2. Cumulative percent morbidity and mortality (CMM), mean day-to-death (MDD), relative percent survival (RPS), prevalence, and median bacterial burden in liver from survivors and mortalities from Challenges 1 and 2.

Challenge*	Treatment	CMM**	MDD**	RPS	Survivors		Mortalities	
					Infected/ examined	Bacterial burden***	Infected/ examined	Bacterial burden***
1	Control	62.7 ^{a,b}	44.5		9 / 22	4.0 (5.1)	7 / 7	5.7 (5.9)
	1xV	43.3 ^a	47.5	30.9	9 / 34	2.1 (3.5)	5 / 5	5.3 (4.6)
	2xV	72.9 ^b	45	-16.3	7 / 16	4.4 (4.8)	9 / 9	5.1 (5.5)
2	Control	65.5	42.5 ^c		13 / 20	2.4 (3.4)	8 / 8	6.1 (6.0) ^{e,f}
	2xV	56	43.3 ^{c,d}	14.5	12 / 26	2.6 (3.9)	7 / 7	6.3 (6.3) ^e
	4xV	51.7	47.7 ^d	21.1	9 / 29	3.3 (5.6)	6 / 6	5.3 (5.7) ^f

*See Table 1 for challenge doses; **Letters denote significant difference in the variable of interest between treatments within a challenge ($p < 0.05$); ***Median Log₁₀ genome equivalents/ μ g DNA (interquartile range).

Consistent with our findings, single oral immunization with the same vaccine elicited an RPS of approximately 81.3% and outperformed an injectable vaccine in Atlantic salmon when challenged by intraperitoneal injection at 300 DD (J. Tobar et al. 2011). In the same study, vaccination by both routes performed equally well following challenge at 600 DD. Unlike J. Tobar et al. (2011) or Sotomayor-Gerding et al. (2020), who used alginate-encapsulated *P. salmonis* antigens, we found no statistical evidence of a systemic antibody response in any orally-immunized treatment group despite several individual samples which responded with OD > 2.0. Thus, the present study suggests that the early protection against *P. salmonis* acquired by oral immunization in Atlantic salmon is not dependent on serum antibodies, similar to protection in European sea bass *Dicentrarchus labrax* following oral vaccination against *Vibrio anguillarum* (Galindo-Villegas et al. 2013).

The low protection and lack of systemic antibody response reported in the current study may be due in part to antigenic heterogeneity between the Canadian challenge isolate and the Chilean vaccine isolate. Numerous studies have documented genetic variability among *P. salmonis* isolates such that the Norwegian and Canadian isolates form a separate genogroup from the LF and EM genogroups (Mauel, Giovannoni, and Fryer 1999; Reid, Griffen, and Birbeck 2004; Otterlei et al. 2008; Schober et al. 2023). Analysis of 73 *P. salmonis* isolates from Chile, Canada, and Norway, including the SR-1 isolate used in the present study, found genogroup-specific differences in genes associated with host-pathogen surface interactions including O-antigen biosynthesis (Schober et al. 2023) raising the possibility of structural differences in surface antigens among the genogroups. Similarly, the failure of vaccines against winter ulcer disease in salmon has been explained by antigenic heterogeneity between the vaccine strains of *Moritella viscosa* and newly emerging isolates (Furevik et al. 2023).

Prolonged administration of the vaccine and/or higher dosing may also explain differences in reported antibody titer between the current study and previous work. In the current study, fish were fed the vaccine-coated feed every day for 10 d at 1% total biomass/day. Conversely, in the original study characterizing this vaccine, fish were fed vaccine-coated feed every three days at 2% of the total biomass for 30 d (J. Tobar et al. 2011). In other vaccine studies, fish have been fed over a similar timescale but at a higher feed rate (Sotomayer-Gerding et al. 2020).

In the present study, oral immunization with *P. salmonis* antigen was not an efficacious means of immunological priming given the decreased survival and low antibody titres. Conversely, in an earlier study evaluating the efficacy of a different oral vaccine against *P. salmonis*, priming by intraperitoneal injection enhanced the response to oral immunization (I. Tobar et al. 2015). In the *Yersinia ruckeri* model, antigen priming by the oral route successfully enhanced protective immunity in Atlantic salmon but failed to do so in

rainbow trout (Ghosh et al. 2016; Jaafar et al. 2019), suggesting that the efficacy of immunological priming by the oral route depends on the bacterial antigen, host species and possibly the presence of immunostimulants or adjuvants (Bøgwald and Dalmo 2021). In the current study, survival was not significantly enhanced in Atlantic salmon given booster oral immunizations. A similar trend has been reported in rainbow trout administered repeated oral immunizations with *Y. ruckeri* bacterin (Jaafar et al. 2019). Jaafar et al. (2019) hypothesized that repeated oral administrations result in host tolerance to the pathogen. The mechanisms of immunological tolerance as a result of repeated administrations of a low concentration of antigen is not well understood in salmonids (E. M. Jones and Cain 2023), and further research is warranted.

The observation of significant reduction in bacterial shedding in some groups was the first evidence that vaccination may reduce transmission of *P. salmonis*. We have recently shown that shedding of *P. salmonis* from infected salmon reflects disease severity and maximum shedding coincides with acute mortality in affected populations (Long and Jones 2021; Long, Goodall, and Jones 2021). Rozas-Serri (2022) suggested that the role of vaccination in controlling the transmission of SRS should be studied under field conditions. Similarly, vaccination and genetic selection of Atlantic salmon reduced but did not eliminate the transmission of infectious salmon anemia virus under laboratory conditions (Chase-Topping et al. 2021). The reduced shedding of *P. salmonis* from vaccinated fish shown here was evidence of a survival-independent measure of vaccine efficacy relevant to the population benefits derived from vaccine-mediated reduction of pathogen propagation.

Conclusions

Efficacy of an oral *P. salmonis* vaccine in Atlantic salmon, as defined by reduced bacterial shedding and increased survival following laboratory challenge, was demonstrated in fish administered either a single dose or quadruple doses. Efficacy was not achieved by double immunizations when challenged at 300 or 1670 DD. Antigenic heterogeneity between Canadian and Chilean *P. salmonis* isolates is suggested to explain the relatively inconsistent efficacy as well as the absence of an antibody response after repeated immunizations.

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SUPPLEMENTARY MATERIALS

Supplementary figures

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Supplemental Table 1

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Supplemental Table 2

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Supplementary Figure Captions

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