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Current vaccination strategy against *Piscirickettsia salmonis* in Chile based only on the EM-90 genogroup shows incomplete cross-protection for the LF-89 genogroup

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ABSTRACT

Piscirickettsia salmonis, the primary bacterial disease in Chilean salmon farming, necessitates a constant refinement of control strategies. This study hypothesized that the current vaccination strategy for SRS control in the Chilean Atlantic salmon aquaculture industry, which has been in place since 2017 (ALPHA JECT® 5.1 plus LiVac®), solely relies on vaccines formulated with the EM-90 genogroup of *P. salmonis* (PS-EM-90), triggering a partial cross-immunity response in fish infected with the LF-89 genogroup (PS-LF-89). Relative Percent Survival (RPS) and cell-mediated immune (CMI) response were evaluated in Atlantic salmon post-smolts vaccinated with the standard vaccination strategy but challenged with both PS-EM-90 and PS-LF-89, in addition to other vaccination strategies considering primo vaccination and booster with other commercial vaccines and the possible enhancing effects of the combination with a natural immunomodulator (PAQ-Xtract®) administered orally. The intraperitoneal (I.P.) challenge was performed after 2395°-days (DD) after the start of the immunostimulant delivery, 1905 DD after the primo vaccination, and 1455 DD after the booster vaccination. Unvaccinated fish showed 73.6 and 41.7 % mortality when challenged with PS-EM-90 and PS-LF-89, respectively. Fish infected with PS-LF-89 died significantly faster (21 days post-infection, dpi) than fish challenged with PS-EM-90 (28 dpi) ($p = 0.0043$) and had a higher probability of death (0.4626) than fish challenged with PS-EM-90. RPS had a significant positive correlation with the PS-EM-90 load of the *P. salmonis* genogroup ($r = 0.540$, $p < 0.01$) but not with the PS-LF-89 load ($r = 0.155$, $p > 0.05$). This demonstrated that the immunization strategies were more effective in lowering PS-EM-90 loads, resulting in higher survival rates in fish challenged with PS-EM-90. The current industry vaccination strategy recorded a 100 % RPS when fish were challenged with PS-EM-90, but the RPS dropped significantly to 77 % when fish were challenged with PS-LF-89, meaning that the strategy did not show complete cross-protection. But after adding PAQ-Xtract®, the RPS improved from 77 % to 92 % in fish that were vaccinated with the standard method but then challenged with PS-LF-89. The most effective vaccination strategy was based on LiVac® as primo vaccination and ALPHA JECT® 5.1 plus LiVac® as booster vaccination, with or without PAQ-Xtract®, in both PS-EM-90 (100 %) and PS-LF-89 (96 %) challenged fish. The serum concentration of anti-*P. salmonis* IgM did not show a correlation with the protection of immunization strategies expressed in survival. Low serum IL-12 and high serum IFNγ concentrations showed a correlation with higher bacterial loads and lower survival. Aggregate analysis showed a significant correlation between higher numbers of $CD8^+$ cells in the head-kidney, higher fish survival, and a lower bacterial load. The immunization strategies were safe for fish and induced only mild microscopic lesions in the gut. Taken together, our results help to better understand the biological interaction between *P. salmonis* and post-smolt vaccinated Atlantic salmon to deepen the knowledge on vaccine-induced protection, CMI immune response, and cross-immunity applied to improve the current immunization strategy for SRS control in the Chilean salmon industry.

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1. Introduction

The Gram-negative facultative intracellular bacterium *Piscirickettsia salmonis*, a member of the subdivision of *Gammaproteobacteria*, family *Piscirickettsiaceae*, order *Piscirickettsiales*, is the causative agent of Piscirickettsiosis (SRS) [1]. *P. salmonis* has been described in farmed salmonid species in Norway, Canada, Scotland, Ireland and Chile [2]. However, SRS particularly affects Chilean salmon farming, accounting for 14 % of total Atlantic salmon mortality in the first half of 2023 [3]. The number of infected farms and the extent of the outbreak in a specific farming area, followed by salinity and water temperature, are the main determinants of SRS prevalence [4]. The prevalence of SRS in a group of salmonid concessions reaches 100 % at an average of 46 weeks after stocking in seawater farms [5].

The pathogenesis of SRS has been described systematically using different experimental infection challenges and *P. salmonis* isolates [6–9]. There is now a greater and better understanding of how *P. salmonis* enters and replicates in fish cells and tissues and how these modulate an immune response [10]. The main issue remained that this immune response often remains ineffective, even in vaccinated fish, leading to disease outbreaks, high mortality rates, and the prescription of antibiotic therapies.

Upon dissecting the official data from the National Fisheries and Aquaculture Service of Chile, Sernapesca, and focusing solely on the mortality data of Atlantic salmon, two distinct phases have emerged since 2012. Circumstantial evidence separated these stages in 2016, when a severe harmful algal bloom (HAB) recorded high losses of farmed salmon biomass, resulting in an SRS-related mortality rate of only 5.7 %. The average SRS-related mortality rate in the first stage between 2012 and 2015 (pre-HAB) was 18.1 %, while the mean SRS-related mortality rate in the second stage between 2017 and 2022 was 12.3 % (post-HAB). Hence, two important situations coexist in this brief history: 1) The industry reduced SRS-attributable mortality by 7 %, and 2) the SRS-related mortality rate did not fall below 11 % per year in the last 7 years. This is crucial, as SRS control accounted for 92.9 % of the total volume of antimicrobials used between 2017 and 2022 [11].

By comparing the sequences of ribosomal genes [12] and whole genomes [13], two *P. salmonis* genogroups have been described: LF-89 and EM-90. Schober et al. [14] recently confirmed the LF-89 and EM-90 genogroups from 73 complete *P. salmonis* genomes, but also described a separate genogroup with the Norwegian and Canadian (NC) isolates, and grouped the EM-90 isolates into four subgroups, EM1 to EM4. According to SRS epidemiology, *P. salmonis* LF-89 and EM-90 have different patterns of geographic distribution and susceptibility to salmon species in Chile [15,16]. In addition, Rozas-Serri et al. [15] demonstrated that SRS may be the result of a complex co-infection between *P. salmonis* genogroup LF-89 and EM-90 isolates in Atlantic salmon at farm, fish, and tissue levels, and evidenced that the detection rate of *P. salmonis* LF-89-like increased significantly between 2017 and 2021, becoming the most prevalent genogroup in Chilean salmon aquaculture. Currently, results from our laboratory show that the prevalence of the *P. salmonis* LF-89-like genogroup will increase to 94 % in 2023.

There have been attempts to use replicating or non-replicating vaccines in the field, but they have only temporarily boosted the humoral immune response and, very poorly, the cell-mediated immune (CMI) response. These responses are not strong or long-lasting enough to effectively control SRS [2,10,17–25]. In 2016, the Chilean market saw the launch of the first live attenuated vaccine, which solely relied on an EM-90-like isolate [15]. Since 2017, the industry has adopted a vaccination strategy based on this vaccine in conjunction with a pentavalent vaccine that also features an EM-90-like but inactivated *P. salmonis* component. Therefore, since *P. salmonis* LF-89 and EM-90 belong to the same species but have distinct virulence genes and distinguishable surface antigenic structures, a biological process could have initiated in 2017 and increased the infection pressure of LF-89-like isolates [13,14]. Thus, studies have described differences in the immune response against *P. salmonis* genogroups and vaccines formulated with either genogroup [8,18–20,22,24], potentially leading to partial cross-immunity [15]. Based on this line of thinking, the rise in EM-90 genogroup isolates seen in the Chilean salmon industry in the early 2010s—which some people thought was a new species of Piscirickettsia—could be the same biological process seen with LF-89 isolates. This is because all vaccines used in the 2000s (since 1999) were made with isolates from the LF-89 genogroup of *P. salmonis*.

Hence, this study hypothesized that the current vaccination strategy for SRS control in the Chilean Atlantic salmon aquaculture industry, which solely relies on vaccines formulated with *P. salmonis* EM-90 genogroup (PS-EM-90), induces partial cross-protective immunity in fish infected with *P. salmonis* LF-89 genogroup (PS-LF-89). To confirm this hypothesis, the following objectives were proposed (1) To evaluate the efficacy in terms of relative percent survival (RPS) and CMI immune response of the current industry vaccination strategy based on commercial vaccines formulated only with the PS-EM-90 genogroup (LAV plus KCV in pentavalent format), in post-smolt Atlantic salmon challenged intraperitoneally (I.P.) with PS-EM-90 and PS-LF-89; (2) To evaluate the efficacy of other vaccination strategies considering primo vaccination and booster with commercially available vaccines using an I.P. challenge model with PS-EM-90 and PS-LF-89 genogroups after 1455°-days (DD) post–booster; (3) To evaluate the efficacy of the same vaccination strategies but combined with the oral administration of a commercial immunostimulant product based on purified extracts of *Quillaja saponaria*.

2. Materials and methods

2.1. Experimental fish

The study took place at the Cargill Innovation Center (CIC) hatchery at VESO Aqualab, located at Ruta 5 Sur Km 1070, Colaco Km 5, Calbuco, Los Lagos, Chile. The VESO Global Ethics Committee reviewed and approved the clinical trial design under Study Number VCC-0158. Every effort was made to provide the best farm conditions and minimize suffering. A total of 1137 specimens of Atlantic salmon parr belonging to the ST2202 Benchmark Genetics Chile batch were transferred to the hatchery (Fig. 1). All fish were non-vaccinated and never treated with antibiotics. A qPCR-based screening to confirm the free-pathogen status of fish was performed on a sample of 30 fish from original populations. Thus, all fish were free for *P. salmonis* [26], *Renibacterium salmoninarum* [27], *Flavobacterium psychrophilum* [28], Piscine orthoreovirus (PRV) [29], Infectious Salmon Anemia Virus (ISAV) [30], and Infectious Pancreatic Necrosis virus (IPNV) [31]. Sexually matured, injured, or deformed fish were excluded from the study upon stocking or during primo vaccination.

2.2. Feed preparation and fish group setting

The feeds were prepared using an uncoated dry commercial feed (Cargill, Coronel, Región Bío Bío, Chile), with the optimum size for the fish according to the stage of the study. Experimental feed was based on a commercial product based on purified extracts of Quillaja saponaria, PAQ-Xtract®, batch number H121120RA0 (Desert King Chile, Quilpué, Región Valparaíso, Chile), which was suspended in an oil-mix (blend of fish oils) by using UltraTurrax® T-50 (IKA-Works, Inc., Wilmington, NC, USA) (with a dispersion tool). Then, feed pellets were coated with the blended mix oil by a Forberg vacuum coater (Forberg International AS, Oslo, Norway). The temperature in the oil mix, non-coated dry feed pellets, and during the dispersion and coating process was kept below 40 °C. Experimental feed was prepared to reach an inclusion level of 0.2 % in feed. The control feed was coated with the same oil mix and at the same oil inclusion level as the experimental feed. Experimental and control feeds were coated with 18 % weight-based oil mix and delivered to the fish after the acclimation stage for 2395 DD (Fig. 1). Thereafter,

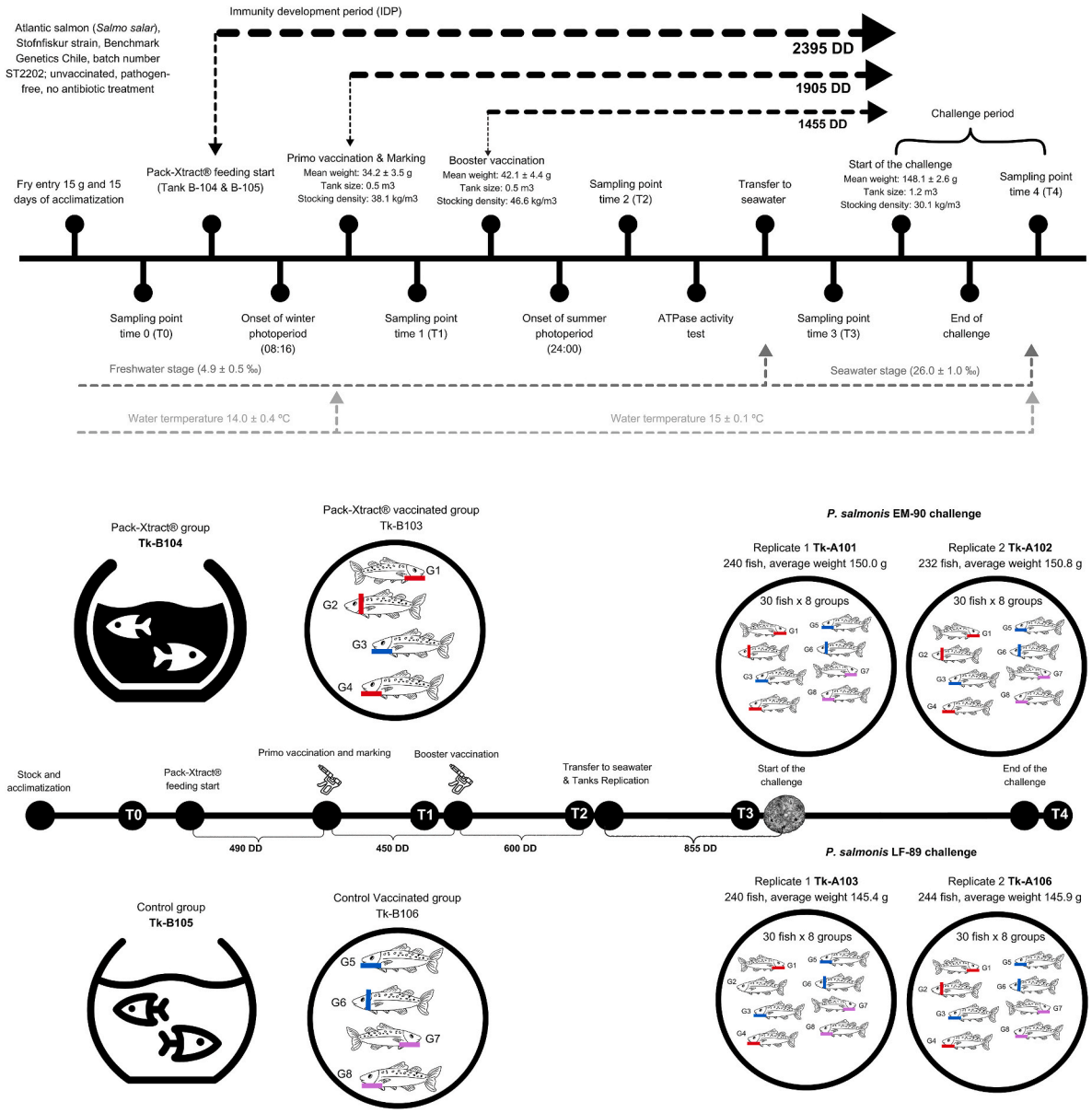

Fig. 1. A general experimental design was used to challenge different immunization strategies in Atlantic salmon postmolts with the PS-EM-90 and PS-LF-89 genogroups described in Chile. The different activities and stages of the study are described: animal model specifications, acclimatization period, immunity development period (IDP), fish marking, experimental group assignment, primo-vaccination and booster, transfer from freshwater to seawater, main environmental and husbandry variables, time points of fish sampling for immunological study, and challenge period with PS-EM-90 and PS-LF-89.

569 and 568 fish were arranged in two 0.5 m^3 tanks identified as B-104 (experimental group) and B-105 (control group), respectively (Fig. 1). After that, regular Cargill commercial feed was used until the end of the study. Fish were always fed to satiety during the experimental feeding period, except during starvation days prior to vaccination and sampling procedures. The feeding activity of fish within each tank was visually assessed each week.

2.3. Husbandry management during the study

Fish weighing an average of ~15 g were acclimatized for 15 days (Fig. 1), monitored daily, and fed through automatic feeders (1–2% of the biomass each day). At each sampling time defined in the study, the weight of all fish was recorded. Fish started primo vaccination, booster vaccination, and challenge stages with 34.2 ± 3.5 g, 42.1 ± 4.4 g, and 148.1 ± 2.6 g in weight, respectively (Fig. 1). Fish were kept in two 0.5 m^3 tanks during the acclimatation, primo vaccination, smoltification, and booster vaccination stages. After that, fish were replicated and challenged in four 1.2 m^3 tanks (Fig. 1). The stocking density was 17.1 kg/m^3 at the acclimatation stage, 38.1 kg/m^3 at the primo vaccination stage, 46.6 kg/m^3at the booster vaccination stage, and 30.1 kg/m^3 at the challenge stage. Salinity was 4.9 ± 0.5 ‰ during the acclimatation, primo vaccination, smoltification, and booster vaccination stages (freshwater phase), and 26.0 ± 1.0 ‰ during the challenge stage (seawater phase) (Fig. 1). During acclimation until primo vaccination stages, the water temperature registered a mean of 14.0 ± 0.4 °C, while during primo vaccination until challenge stages, it was noted at a mean of 15.0 ± 0.4 °C (Fig. 1). The dissolved oxygen was 79.6 ± 13.2 % during the whole study, while the water turnover flow was ~1.0 tanks per hour. Finally, the photoperiod regime during acclimation and the first four weeks of experimental feeding was 24:0, one week before primo vaccination and for six weeks afterwards was 8:16, and 24:0 again after the winter signal to finish the smoltification process and throughout the rest of the study (Fig. 1). Smolts were transferred to

seawater when they presented an average of 15.7 U/mg of Na^+K^+ ATPase enzyme activity in the gills [32]. All dead fish were examined by necropsy and recorded at the respective stages of the study. At the end of the challenge, all surviving fish were euthanized, necropsied, and recorded. Environmental parameters were recorded automatically, specifically water temperature and oxygen saturation inside each tank, salinity, and pH in the header tank.

2.4. Fish sampling time points, vaccination, and immunization strategies

The experimental design included five sampling time points (T), as shown in Fig. 1. On the last day of the acclimation period and one day before the beginning of experimental feeding (T0), (30 naïve fish); on booster vaccination day (5 fish/group) (T1); ~600 DD after booster vaccination (5 fish/group) (T2); ~1455 DD after booster vaccination; and *P. salmonis* challenge day (5 fish/group) (T3); surviving fish at the end of the challenge (5 fish/group/challenge) (T4). Primo vaccination and group marking took place approximately 490 DD after the start of experimental/control feeding.

For analytical purposes, "vaccination strategies" designated G1 to G8 were defined depending on the commercial vaccines considered in the study (Table 1), and "immunization strategies" designated S1 to S3 were defined to evaluate each vaccination strategy with and without PAQ-Xtract® supplementation (Table 1). Strategy 4 (S4) was solely assessed with additive supplementation. Each manufacturing laboratory supplied the commercial vaccines in a ready-to-use format and the vaccines were kept refrigerated until administration. All vaccines were removed from the refrigerator, allowed to acclimate to room temperature and shaken vigorously before administration to the fish. Sterile saline solution (NaCl 0.9 %, batch number 75PA0101) (Fresenius Kabi, Santiago, Chile) was used as a placebo in the control group of fish.

2.5. Primo vaccination

After a 48-h starvation period, fish were anesthetized with 20 % benzocaine (15–20 mL per 100 L of water) until stage III (~2 min). Then, fish were identified with intradermal visible implant elastomer (VIE) tags (Northwest Marine Technology, Inc., Anacortes, WA, USA), weighed, and vaccinated to form the experimental groups detailed in Table 1. Briefly, fish were I.P. vaccinated according to the manufacturer's laboratory instructions using Socorex® semi-automatic syringes (Socorex Isba SA, Écublens, Switzerland) with 0.5 × 3 mm needles. Fish in the group previously fed experimental feed formed groups G1 to G4 (Tank B-103), whereas fish fed control feed formed groups G5 to G8 (Tank B-106) (Fig. 1). All fish were tagged during the primo vaccination (P.V.) procedure, regardless of whether they received any vaccination (Table 1). Subsequently, the fish were kept in their respective tanks until the immunity development period was completed, prior to booster administration (Fig. 1).

2.6. Booster vaccination

After ~450 DD post-P.V., fish were subjected to a booster vaccination (B.V.). Fish from all groups were anesthetized, weighed as previously described, and vaccinated using Socorex® semi-automatic syringes (Socorex Isba SA, Écublens, Switzerland) with 0.6 × 4 mm needles, forming the groups detailed in Table 1. Subsequently, fish from groups G1 to G4 were placed into the tanks until the immunity development period (IDP) was completed (Fig. 1). On the day that all study fish completed ~600 DD after the B.V., the experimental/control feeding period ended, and fish from the two tanks were distributed into four identical replicates/tanks (A-101, A-102, A-103, and A-106) with fish from all eight groups (Fig. 1). Briefly, all fish were removed from the tanks, anesthetized as previously described, identified according to their tags, and finally arranged in the 1.2 m^3 tanks awaiting challenge with the *P. salmonis* genogroups.

2.7. Experimental challenge

2.7.1. Preparation of inocula of P. salmonis genogroup EM-90 and LF-89

P. salmonis EM-90 (PS-EM-90) and *P. salmonis* LF-89 (PS-LF-89) isolates were grown on Austral-TSH agar at 18 °C for 6 days [33]. Colonies of bacterial growth were picked from the agar and suspended in 1 mL of Leibovitz L-15 medium supplemented with 10 % fetal bovine serum (FBS). The biomass produced was quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific Inc., Wilmington, DE, USA) [9]. This OD625 corresponded to approximately 1.37 × 10^6 CFU/ml for *P. salmonis* EM-90, and 1.9 × 10^7 CFU/ml

Table 1
Identification of fish and classification of experimental groups according to vaccination strategy in the primary vaccination and booster stage.

Tank ID	Vaccination strategy	Immunization strategy	Primo vaccination (P. V)	VIE-tag color/ position	Fish number	Weight (g) Mean ± SD (CV%)	Booster vaccination (B.V.)	Fish number	Weight (g) Mean ± SD (CV%)
B103 (PackTract®)	G1	S1	Non-vaccinated	Red/RM	139	33.5 ± 3.8 (11.4)	ALPHA JECT® 5.1 + LiVac®	134	44.8 ± 5.5 (12.2)
	G2	S2	LiVac®	Red/LM	139	34.1 ± 3.1 (8.9)	ALPHA JECT® 5.1 + LiVac®	134	43.0 ± 3.9 (9.1)
	G3	S3	AGROVAC®	Red/LE	138	33.8 ± 3.4 (10.0)	ALPHA JECT® 5.1 + AGROVAC®	131	41.9 ± 4.6 (11.0)
	G4	S4	X-KCV	Blue/RM	138	34.3 ± 3.7 (10.8)	ALPHA JECT® 5.1 + X-KCV	129	43.6 ± 4.7 (10.9)
B106 (Control)	G5	S1	Non-vaccinated	Blue/LM	139	33.4 ± 3.3 (9.6)	ALPHA JECT® 5.1 + LiVac®	134	43.6 ± 4.4 (10.2)
	G6	S2	LiVac®	Blue/LE	138	34.5 ± 3.8 (11.1)	ALPHA JECT® 5.1 + LiVac®	131	41.0 ± 4.4 (10.6)
	G7	S3	AGROVAC®	Pink/RM	138	34.6 ± 3.2 (9.2)	ALPHA JECT® 5.1 + AGROVAC®	132	40.4 ± 4.1 (10.3)
	G8	Control	Non-vaccinated	Pink/LM	138	34.4 ± 3.5 (10.2)	NaCl 0.9 %	131	43.5 ± 4.6 (10.6)

ALPHA JECT® 5.1: Killed-cell vaccine (KCV) against Infectious Pancreatic Necrosis (IPN), Piscirickettsiosis (SRS, *P. salmonis* EM-90 genogroup), Atypical Furunculosis, Vibriosis, Infectious Salmon Anemia (ISA); Pharmaq AS, Overhalla, Norway.
ALPHA JECT LiVaC® SRS: Live-attenuated vaccine (LAV) *Piscirickettsia salmonis* EM-90 genogroup; Pharmaq AS, Overhalla, Norway.
AGROVAC® IPN-SRS: Killed-cell vaccine (KCV) against IPN and SRS; Agrovet SpA, Santiago, Chile. Bivalent killed-cell vaccine (KCV) (PS-EM-90 plus PS-LF-89) against *P. salmonis.*
X-KCV: Monovalent Killed-cell vaccine (KCV) against SRS (*P. salmonis* EM-90 genogroup); Laboratory X.
Abbreviation of the tagging position: RM: right maxillae; LM: left maxillae; LE: (beside) left eye.

for *P. salmonis* LF-89.

2.7.2. Piscirickettsia salmonis intraperitoneal challenge

A total of 956 post-smolt were challenged by I.P. inoculation with *P. salmonis* after a total of 1905 days since the first vaccination, also known as the immunity development period (IDP). Of these, 480 were injected with genogroup PS-EM-90 and the other 476 were injected with genogroup PS-LF-89 (Fig. 1). Thirty post-smolts from each of the eight groups of fish previously tagged, as indicated in Table 1, were placed in each of the four tanks or replicates (Fig. 1). Hence, tank A-101 was made up of 240 fish of 150.1 g average weight, tank A-102 with 232 fish of 150 g average weight, tank A-103 with 240 fish of 145.4 g average weight, and, finally, tank A-106 with 244 fish of 145.9 g average weight (Fig. 1). Briefly, after 24 h of starvation, subgroups of 15 fish from each vaccination strategy were collected from each tank (one tank at a time) and placed in an external container with the same water as the source tank and emergency oxygenation system. These fish were anesthetized as previously described, weighed, and individually injected with 0.1 ml of PS-EM-90 inoculum to complete the 480 fish and then with PS-LF-89 inoculum to complete the remaining 476 fish before returning them to each of the original tanks (A-101, A-102, A-103, and A-106). Mortality was observed daily for a period of 44 and 36 days after the inoculation of fish with PS-EM-90 and PS-LF-89, respectively.

2.7.3. Fish tissue samples and laboratory analyses

2.7.3.1. qPCR Piscirickettsia salmonis. Liver and head-kidney samples (5 mm3) from each fish were collected in 2 mL microcentrifuge tubes containing 70 % v/v ethanol and stored at −80 °C until further analysis. *P. salmonis* was detected by Taqman®-based qPCR as previously described by Karatas et al. [26]. This PCR assay has been used in our previous studies in both field [15,34,35] and experimental conditions [9,18–20]. Each run included a positive control (*P. salmonis* DNA), negative control without DNA, and negative extraction control. Cycle Threshold (CT) values up to 40 were recorded, and a CT $<$ 33.01 was considered positive, otherwise it was considered negative [15]. A low CT value indicates a high abundance of *P. salmonis* 16 rRNA gene transcripts, which determines a high bacterial load in the head-kidney. On the contrary, a high CT value indicates a low abundance of genetic material, which is associated with a lower bacterial load.

2.7.3.2. Enzyme-linked Immunosorbent assay (ELISA). Whole blood samples were collected from the caudal vein of each fish and placed in 1.5 ml Eppendorf tubes. Serum levels of *P. salmonis*-specific secreted IgM, interleukin-12 (IL-12), and interferon-gamma (IFNγ) were quantified by solid-phase sandwich ELISA (Suppl. Fig. 1). The total protein concentration in each serum sample was measured by the QuantiPro™ BCA Assay Kit (Sigma-Aldrich, Merck Group, Burlington, MA, USA) following the manufacturer's instructions. Samples were diluted in carbonate/bicarbonate buffer (50 mM NaHCO3, pH 9.6), seeded at 50 ng/mL (100 μL) in duplicate on Nunc® MaxiSorp™ plates (Sigma-Aldrich, Merck Group, Burlington, MA, USA), and incubated at 4 °C overnight. Plates were blocked with 200 μL of 5 % non-fat milk solution (NFM) per well for 1 h at 37 °C. Self-made primary antibodies for IL-12 and IFNγ were then added and incubated at 37 °C for 90 min. HRP-conjugated secondary antibody diluted 1:5000 was added to the IgG of the first antibody and incubated at 37 °C for 60 min. The plates were incubated at room temperature for 30 min with 100 μl of chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich, Merck Group, St. Louis, MO, USA) per well. Reaction was stopped with 50 μl 1N sulfuric acid and read at 450 nm on an Infinite® M200 PRO microplate reader (Tecan US Inc., Morrisville, NC, USA). Between steps, plates were washed five times with PBS containing 0.05 % Tween-20 (Sigma-Aldrich, Merck Group, Burlington, MA, USA). Nunc® MaxiSorp™ plates were incubated overnight at 4 °C with 50 ng/mL of *P. salmonis* total protein extract (diluted in 100 μL carbonate-bicarbonate buffer) per well to detect *P. salmonis* specific IgM. A dilution of 1:1000 was used up to serum concentrations of 8000 μg/mL and above was 1:3000. After blocking with 200 μL of NFM per well, 50 μL of diluted serum sample was incubated for 90 min at 37 °C. Subsequently, they were incubated with the HPRO-conjugated Mouse Salmonid Ig (H) Monoclonal Antibody (MBS520453, Mybiosource, San Diego, CA, USA) for 90 min at 37 °C. Finally, the ELISA protocol described previously was followed to develop the reaction.

2.7.3.3. Histopathological processing and semi-quantification of intestinal histoscore. Hindgut segment samples (0.5–1 cm^3) were collected from 5 fish per experimental group (n = 8) from each tank (n = 4) and sampling time (n = 5) (Fig. 1) and disposed in 10 % formalin buffer for 24 h. Samples were then dehydrated in alcohol graded series and processed by standard histological examination. Samples were dehydrated in a graded alcohol series and processed by standard histological examination. Sections 4μm thick from each tissue were stained with hematoxylin and eosin (H&E). The slides were scanned with a MoticEasyScan Pro 6 scanner (Motic, Fujian, China) and the images were read with DSServer software (Motic, Fujian, China). To provide a more unbiased analysis, a semiquantitative indicator of tissue damage was developed. The histoscore for posterior intestine (hsINT) considered the histologic changes described in Table 2. The individual histopathological scores were used to calculate the mean hsINT ± standard deviation (SD) at each sampling point time for each experimental group in each tank (n = 5). All assessments and count measurements were performed by a single, trained, and calibrated examiner.

2.7.3.4. Immunohistochemistry (IHC) and CD8 T-cells semi-quantification. Head-kidney samples from each fish (0.5–1 cm^3) were collected, placed in 10 % formalin buffer for 24 h, and processed into a paraffin-embedded tissue block as described above. Paraffin-embedded sections (4 mm) were placed on positively charged frosted glass slides (Hardy Diagnostics, Santa María, CA, USA) and dried at 70 °C for 2 h. After a standard dewaxing procedure, antigenic recovery was performed using a multi-cooker Oster® Bioceramic® 6801 (Oster®, China) in citrate buffer (10 mM citric acid monohydrate, pH 6.0, Vector) at 121 °C for 15 min. The sections were allowed to cool for 3 min in a water bath, and peroxidase blocking was performed using 3 % hydrogen peroxide (Merck Millipore, Burlington, MA, USA).

Immunostaining based on a monoclonal antibody against CD8 (anti-CD8 Mab, clone 10-2G, rat IgG2a isotype) previously described by Takizawa et al. [36] was used to characterize and semi-quantify $CD8^+$ cells in the head-kidney of post-smolt Atlantic salmon vaccinated and subsequently challenged with PS-EM-90 and PS-LF-89. Heat-induced epitope retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) at 95 °C for 20 min to expose target proteins. To block nonspecific binding and ensure binding of the specific antibody with subsequent signal amplification, Blocker™ BSA based on purified 10 % bovine serum albumin solution (Invitrogen, Frederick, MD, USA) was used according to the manufacturer's instructions. The primary anti-CD8 antibody was incubated for 2 h at 25 °C in a 1 × 100 dilution of the primary antibody diluent (phosphate, green, ScyTek-Prolab). Detection was with HRP-conjugated goat anti-rat IgG-HRP secondary antibody (Invitrogen, Frederick, MD, USA) according to manufacturer's instructions. In situ chromogenic detection was performed using the ImmPACT NovaRED® substrate kit (Vector Laboratories, Newark, CA, USA) and Mayer's hematoxylin. The slides were then mounted in Entellan® mounting medium (Merck Millipore, Burlington, MA, USA).

Slides were then scanned with the MoticEasyScan Pro 6 (Motic, Fujian, China) scanner, and images were read with DSServer software (Motic, Fujian, China). A semiquantitative evaluation of CD8-positive T-cells was performed using Mercator software (Explora Nova, La Rochelle, France). Slide scanning and semiquantitative staining

Table 2
Histopathological criteria and semi-quantitative weighting were used to define hsINT. Abbreviations: NHC: no histological changes; NIC: no inflammation changes. Interpretation: hsINT ≤0.9 means a mild cardiac damage; hsINT >0.9 but ≤1.8, means a moderate damage; and hsINT >1.8 means a severe damage.

Histoscore	Mucosal layer				Submucosal layer	Serosal layer
	Decreased enterocyte cytoplasmic vacuoles	Villi atrophy	Chorion thickening	Inflamatory cells infiltrate	Inflamatory cells infiltrate	Chronic fibrohistiocytic inflammation
0	NHC	NHC	NHC	NIC	NIC	NIC
1	<10 % enterocyte cytoplasm	<10 % villi length	<10 % of chorion surface	<10 % of mucosal surface area	<10 % of submucosal surface	<10 % of serosal surface
2	10–50 % enterocyte cytoplasm	10–50 % villi length	10–50 % of chorion surface	10–50 % of mucosal surface area	10–50 % of submucosal surface	10–50 % of serosal surface
3	>50 % enterocyte cytoplasm	>50 % villi length	>50 % of chorion surface	>50 % of mucosal surface area	>50 % of submucosal surface	>50 % of serosal surface
Relative weighting	0.26	0.26	0.26	0.09	0.05	0.10

evaluation were performed on the Pathovet Labs histology platform. A scoring algorithm was developed and related to the number of CD8-stained T-cells in the head-kidney tissue sample previously scanned. Five non-overlapping fields of vision avoiding areas of cell clumping were evaluated in each sample with a 40X objective using optical microscopy. Then, the mean number of CD8-positive T-cells per field of vision was used to assign CD8-scores on a scale of 0–5: 0 (none), 1 (0.1–1.0), 2 (>1.0 to 5.0), 3 (>5.0 to 20.0), 4 (>20.0 to 50.0), and 5 (>50.0). The CD8-scores of each fish were used to calculate the mean CD8-score ± SD at each sampling time for each experimental group in each tank. All assessments and count measurements were performed by a single, trained, and calibrated examiner.

2.8. Statistical analysis

Survival of fish subjected to different immunization strategies based on different commercial vaccines with and without immunostimulant and challenged with *P. salmonis* genogroups PS-EM-90 and PS-LF-89 was evaluated by the Kaplan-Meier survival model and hazard ratio (HR) using Cox proportional hazards regression models and Log-Rank multiple comparisons test. For the analyses, the G8 control group was considered a point of comparison, and for the *P. salmonis genogroups*, the PS-EM-90 genogroup was used as a control. The efficacy of immunization strategies was measured as the survival rate expressed as relative percentage of survival (RPS) calculated as $RPS_{end\text{-}point}$ = (1-(cumulative mortality experimental group/cumulative mortality control group) x 100) and as the effectiveness of immunization strategies (EIS) in reducing the risk of dying or reducing SRS mortality events calculated as EIS = (1-HR) x 100). Both RPS and EIS indicators can measure the performance of vaccination strategies, but the latter is more robust due to its use of Cox models. The "Survival" [37] and "Survminer" [38] libraries implemented in R-Studio were used for these analyses.

Each biomarker was evaluated using the interaction between experimental groups (gl = 7) and *P. salmonis* genogroups (gl = 1), and a log (x+1) transformation was performed. The use of the immunostimulant was considered an independent factor. Tukey's post hoc test (α = 0.05) was performed for significant ANOVA factors for each independent time using the "Car" library in R-Studio [39]. To explore the degree of association of the immune response between vaccination strategies and immunostimulant use, Spearman's multiple correlations were performed between time, IL-12, IFNγ, CD8 Score, IgM PS-EM-90, IgM PS-PS-LF-89, bacterial load (CT) and RPS per group, additive use, and vaccination strategy as categorical variables (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). The "Corrplot" [40] and "GGally" [41] libraries implemented in R-Studio were used for this analysis. All statistical analyses were performed using R Core Team [42].

3. Results

3.1. The current industrial vaccination strategy against SRS in Chile did not show complete cross-protection when challenged with PS-LF-89

Supplementary Tables 1–4 detail mortality at each stage of the study and in each experimental tank, while Supplementary Tables 5 and 6 detail fish weights and macroscopic findings at necropsy examination. Supplementary Table 7 presents the consolidated results of all biomarkers evaluated in the study. Table 3 displays the survival rates in RPS of fish infected with the PS-EM-90 and PS-LF-89 genogroups, subjected to various immunization strategies using commercial injectable vaccines and an oral immunostimulant additive. The unvaccinated group of fish (G8) showed 73.6 and 41.7 % mortality when challenged with PS-EM-90 and PS-LF-89, respectively. Fig. 2 shows the survival function models that show how genogroups and PAQ-Xtract® work in each immunization strategy. It was important to note that the immunization strategies, the immunostimulant, and the *P. salmonis* genogroup all interacted with each other ($\chi2 = 279$; gl = 15; $p < 0.0001$) (Table 3). When fish in the unvaccinated control group were challenged with PS-EM-90, they died 21 days after infection (pdi) much faster than when they were challenged with PS-LF-89 (28 dpi) (Fig. 2A) (LogRank $\chi2 = 3.3$; df = 1; p = 0.0043). However, fish challenged with PS-LF-89 had a higher chance of dying (0.4626) than fish challenged with PS-EM-90 (Table 3).

Regardless of the immunization strategy (S1 to S4), fish challenged with PS-LF-89 had a lower chance of survival and a higher risk of death compared to fish challenged with PS-EM-90. Adding immunostimulants did not change this outcome (Fig. 2). The average survival rate for Strategy 1 (G1 and G5) was 36 days after infection. This rate was different for each P. salmonis genogroup and did not change when immunostimulants were added (LogRank $\chi2 = 9.2$; gl = 3; p = 0.026) (Fig. 2B). The fish in group G1, supplemented with PAQ-Xtract® and vaccinated with ALPHA JECT® 5.1 + LiVac®, demonstrated an RPS of 100 % when challenged with PS-EM-90. However, the RPS decreased by 8 % when challenged with PS-LF-89 (92 %) (Fig. 2B) (Table 3). Even though the fish in group G5 had received vaccinations with ALPHA JECT® 5.1 + LiVac®, the current standard in Chile, their RPS dropped by 23 % when challenged with PS-LF-89 (77 %).

There were no significant differences in the survival rates of strategy 2 (G2 and G6) after 36 days, and there were also no significant differences in the survival rates of different *P. salmonis* genogroups or immunostimulant supplements (LogRank $\chi2 = 9.2$; gl = 3; p = 0.63; Fig. 2C). The fish in group G2, supplemented with PAQ-Xtract® and vaccinated with LiVac® as P.V. and ALPHA JECT® 5.1 + LiVac® as B.V., showed an RPS of 100 % when challenged with PS-EM-90. However, the RPS dropped to 96.0 % when challenged with PS-LF-89 (−4%) (Fig. 2C) (Table 3). Fish in group G6 without immunostimulant and vaccinated with the same plan presented an RPS of 100 % when fish were challenged with PS-EM-90, but the RPS dropped to 95 % when fish were challenged with PS-LF-89 (−5%) (Fig. 2C) (Table 3).

Table 3
Summary of immunization strategy effectiveness (EIS) in reducing SRS mortality events and relative percent survival (RPS) of different immunization strategies challenged with PS-EM-90 and PS-LF-89. EIS and RPS results are shown pooled as a single group from the two replicates per treatment.

Vaccination Strategy	*P. salmonis* Genogroup	Predictor	coef	exp (coef)	se (coef)	Confidence Interval (25 %–95 %)		z	Pr(>|z|)	LogRank	EIS = 1-HR	RPS
	LF-89	*P. salmonis* Genogroup	−0,7708	0,4626	0,2534	0,2816	0,7602	−3,0420	**0,0023**		54 %	
Strategy 1	EM-90	Control feed (G5)	−20,240	0,0000	3986,0	0,0000	Inf	−0,0050	0,9959	*a*	100 %	100 %
	EM-90	PaqXtraxt® (G1)	−20,240	0,0000	3986,0	0,0000	Inf	−0,0050	0,9959	*a*	100 %	100 %
	LF-89	Control feed (G5)	−1,3180	0,2675	0,4547	0,1097	0,6522	−2,9000	***0,0037***	*b*	73 %	77 %
	LF-89	PaqXtraxt® (G1)	−2,4030	0,0904	0,7349	0,0214	0,3818	−3,2700	***0,0011***	*b*	91 %	92 %
Strategy 2	EM-90	Control feed (G6)	−20,240	0,0000	3986,0	0,0000	Inf	−0,0050	0,9959	*a*	100 %	100 %
	EM-90	PaqXtraxt® (G2)	−20,240	0,0000	3986,0	0,0000	Inf	−0,0050	0,9959	*a*	100 %	100 %
	LF-89	Control feed (G6)	−3,0980	0,0451	1,0200	0,0061	0,3332	−3,0380	***0,0024***	*a*	95 %	95 %
	LF-89	PaqXtraxt® (G2)	−3,0980	0,0452	1,0200	0,0061	0,3332	−3,0380	***0,0024***	*a*	95 %	96 %
Strategy 3	EM-90	Control feed (G7)	−2,0400	0,1301	0,4733	0,0514	0,3289	−4,3100	***0,0000***	*a*	87 %	89 %
	EM-90	PaqXtraxt® (G3)	−3,6530	0,0259	1,0120	0,0036	0,1883	−3,6100	***0,0003***	*a*	97 %	98 %
	LF-89	Control feed (G7)	−1,9960	0,1359	0,6111	0,0410	0,4502	−3,2660	***0,0011***	*a*	86 %	88 %
	LF-89	PaqXtraxt® (G3)	−1,9960	0,1359	0,6111	0,0410	0,4503	−3,2660	***0,0011***	*a*	86 %	88 %
Strategy 4	EM-90	PaqXtraxt® (G4)	−2,2880	0,1015	0,5235	0,0364	0,2831	−4,3710	**0,0000**	*a*	90 %	91 %
	LF-89	PaqXtraxt® (G4)	−0,5541	0,5746	0,3420	0,2939	1,1233	−1,6200	0,1052	*a*	43 %	48 %

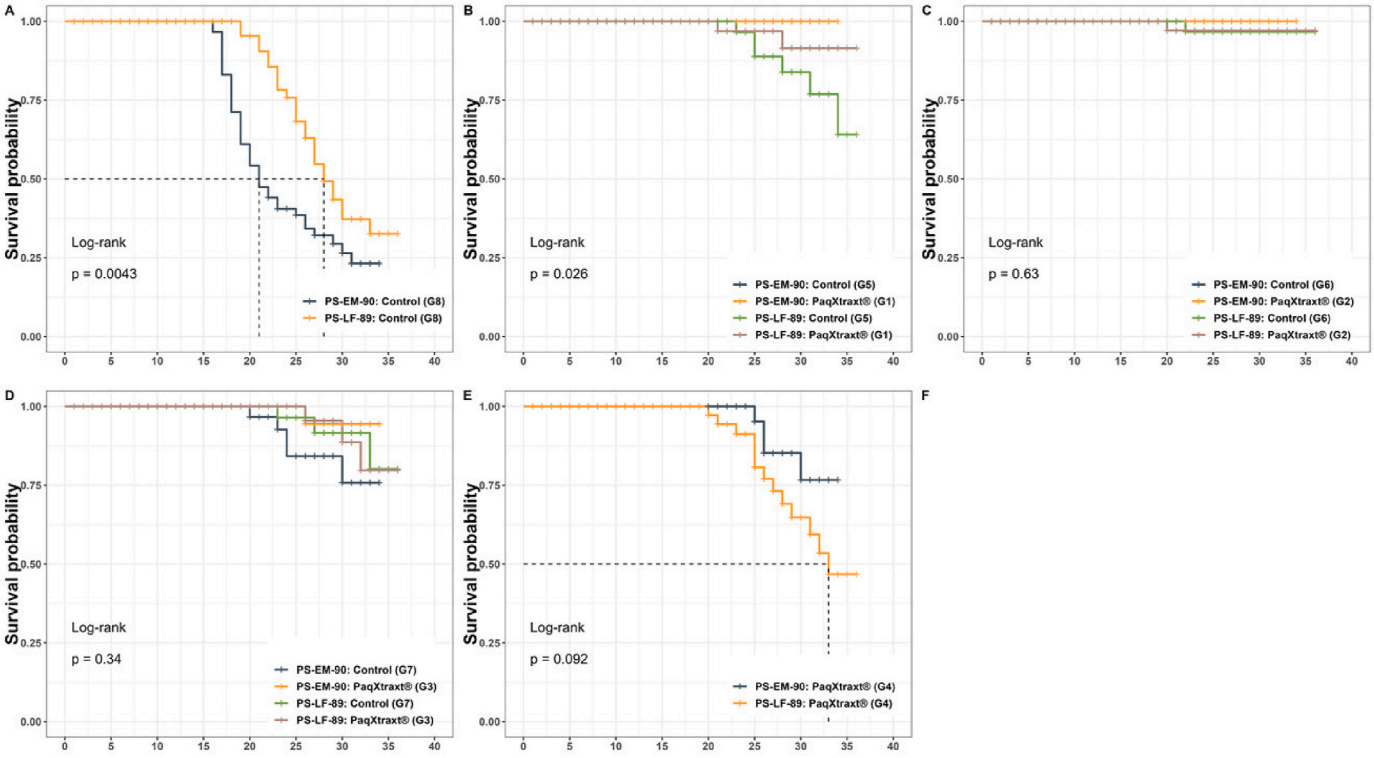

Fig. 2. Survival study and mortality probability model in Atlantic salmon post-smolts subjected to different immunization strategies and challenged with PS-EM-90 and PS-LF-89, and with and without immunostimulant. (A) Kaplan-Meier estimator of the cumulative survival function for control group non-vaccinated and non-supplemented fish (G8). (B) Kaplan-Meier estimator of the cumulative survival function of fish subjected to strategy 1, vaccinated with ALPHA JECT® 5.1 + LiVac® without immunostimulant (G1 = current industry strategy) and with immunostimulant (G5). (C) Kaplan-Meier estimator of the cumulative survival function of fish subjected to strategy 2, vaccinated with LiVac® as primo vaccination and ALPHA JECT® 5.1 + LiVac® as booster vaccination, without immunostimulant (G2) and with immunostimulant (G6). (D) Kaplan-Meier estimator of the cumulative survival function of fish subjected to strategy 3, vaccinated with Agrovac® as primo vaccination and ALPHA JECT® 5.1 + Agrovac® as booster vaccination, without immunostimulant (G3) and with immunostimulant (G7). (E) Kaplan-Meier estimator of the cumulative survival function of fish subjected to strategy 4, vaccinated with X-KCV as primo vaccination and ALPHA JECT® 5.1 + X-KCV as booster vaccination, with immunostimulant (G4).

Mean survival of strategies 3 (G3 and G7) showed no significant differences between the P. *salmonis* genogroup and immunostimulant supplementation (33 dpi) (LogRank $\chi^2 = 3.3$; df = 1; p = 0.340) (Fig. 2D). The fish in group G3, supplemented with PAQ-Xtract® and vaccinated with Agrovac® as P.V. and ALPHA JECT® 5.1 + Agrovac® as B.V., showed an RPS of 98 % when challenged with PS-EM-90, but their survival dropped by 10 % when challenged with PS-LF-89 (88 %) (Fig. 2D) (Table 3). Fish in group G7 without immunostimulant and vaccinated with the same strategy presented a similar RPS of 89 and 88 % when challenged with PS-EM-90 and PS-LF-89, respectively (Fig. 2D) (Table 3). Fish subjected to immunization strategy 4 (G4) showed a mean survival of 33 dpi with no significant differences between genogroups (LogRank $\chi2 = 2.8$; df = 1; p = 0.092) (Fig. 2E) (Table 3). Fish immunized with X-KCV as P.V. and ALPHAJECT 5.1® plus X-KCV as B.V. showed significant differences in RPS in the group challenged with PS-EM-90 (91 %) and PS-LF-89 (48 %), whereby fish challenged with PS-LF-89 would have a higher probability of death (0.5746; 95 % CI = 0.2939–1.1233) (Fig. 2E) (Table 3). This immunization strategy was not evaluated without the immunostimulant.

Lastly, a significant positive correlation was observed at the overall data analysis level between the head-kidney *P. salmonis* load expressed as CT value and survival expressed in RPS (r = 0.258, p < 0.001), especially in the non-vaccinated control group (i.e., G8) (r = 0.562, p < 0.01) (Fig. 3) and non-treated PAQ-Xtract® group (r = 0.317, p < 0.01) (Suppl. Fig. 1). Then, the unvaccinated group presented the lowest survival of the study and was correlated with the lowest CT values, i.e., a higher amount of bacterial genetic material in the fish with a higher mortality rate. Interestingly, none of the immunization strategies showed this correlation, suggesting that vaccination maintains low bacterial loads regardless of the strategy (Fig. 3). Complementarily, RPS showed a significant positive correlation specifically with *P. salmonis* genogroup PS-EM-90 load (r = 0.540, p < 0.01), but not in PS-LF-89 (r = 0.155; p > 0.05) (Suppl. Fig. 2), which would demonstrate that the immunization strategies were more effective in reducing PS-EM-90 loads in the anterior kidney and, consequently, resulted in higher survival in fish challenged with the PS-EM-90 genogroup.

3.2. The most effective vaccination strategy was based on LiVac® as a primo vaccination and ALPHA JECT® 5.1 plus LiVac® as a booster vaccination

The immunization strategy that used two shots of LiVac® (S2: G2 and G6), without adding any immunostimulants, helped the most fish survive both PS-EM-90 and PS-LF-89 infections (Fig. 2C), though the latter had a higher chance of dying (0.451) (Table 3). Although under experimental conditions, the results in S2 showed no difference in survival of fish without LiVac® primo vaccination (S1: G1 and G5) when challenged with PS-EM-90 (RPS was 100 % in both strategies) (Fig. 2C), therefore, from a bioeconomic point of view, primo vaccination with LiVac® and even the use of the immunostimulant might not add additional protection if the challenge is PS-EM-90. However, when fish underwent PS-LF-89 challenge, either with or without immunostimulant, the S2 group exhibited superior survival compared to the S1 group without primo vaccination (Fig. 2B) and the S3 group vaccinated with the bivalent killed-cell vaccine-based strategy for *P. salmonis* (S3: G3 and G7) (Fig. 2D). At the same time, strategy 2 fish showed a significant negative correlation between serum IL-12 and IFNγ concentration (r = −0.396, p < 0.01) and between IL-12 concentration and $CD8^+$ cell number (r = −0.315, p < 0.05). IFNγ concentration showed a significant negative correlation with CT (r = −0.318, p < 0.05) and, consequently, a positive correlation with bacterial load (Fig. 3).

3.3. Supplementation with PAQ-Xtract® improved the survival of fish vaccinated with the current industry strategy when challenged with the PS-LF-89 genogroup

Regardless of the vaccination strategy, the groups of fish supplemented with PAQ-Xtract® consistently recorded the best survival results

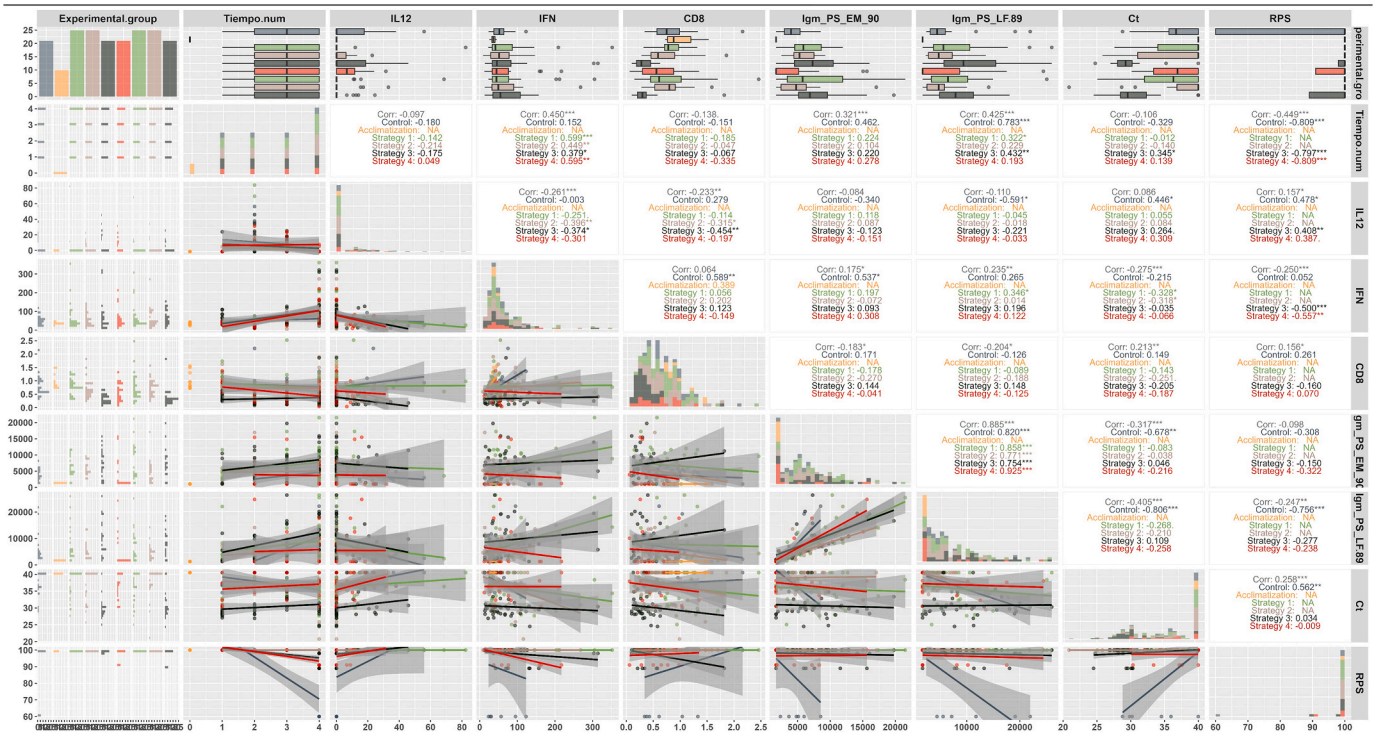

Fig. 3. Pearson's correlation coefficient (r) and p-value (p) between head-kidney *P. salmonis* head-kidney load expressed in CT and serum concentrations of IL-12, IFNγ, anti-PS-EM-90 IgM and anti-PS-LF-89 IgM, the number of $CD8^+$ cells in the head-kidney, and the RPS achieved in each immunization strategy (S1 to S4) evaluated in Atlantic salmon post-smolts infected with the PS-EM-90 and PS-LF-89 genogroups (*p < 0.05, **p < 0.01, ***p < 0.001).

(Table 3). However, fish supplemented with PAQ-Xtract® and challenged with PS-EM-90 showed higher RPS (100 % in G1, G2, 98 % in G3, and 91 % in G4) than those infected with PS-LF-89 (RPS of 92 % in G1, 96 % in G2, and 88 % in G3) (Table 3) (Fig. 2). The survival rate of non-supplemented fish challenged with PS-LF-89 remained at 96 % in the G6 but significantly decreased to 77 % in the G5 (Table 3). There was a 23 % difference in survival rates between groups of fish vaccinated with the current industry standard strategy and challenged with PS-EM-90 and PS-LF-89. There was also a 15 % difference in survival rates between groups vaccinated with the same strategy and challenged with PS-LF-89, with 92 % of the fish getting the immunostimulant and 77 % not getting it. Simultaneously, PAQ-Xtract® boosted the survival rate of fish vaccinated with Agrovac®, ALPHA JECT® 5.1 + Agrovac® in response to PS-EM-90 challenge (RPS increased from 89 % to 98 %), but not in response to PS-LF-89 challenge (RPS remained at 88 %) (Table 3). Thus, the bivalent KCV (PS-EM-90 + PS-LF-89) had the same RPS when challenged with PS-EM-90 (89 %) and PS-LF-89 (88 %), but the immunostimulant only increased the chance of survival in fish when challenged with PS-EM-90 (98 %).

Serum IL-12 concentration showed a significant negative correlation with IFNγ concentration ($r = -0.411$, $p < 0.001$) and $CD8^+$ cell count ($r = -0.333$, $p < 0.01$) in PAQ-Xtract® fish (Suppl. Fig. 2). In the PAQ-Xtract® group of fish, RPS was negatively related to serum IFNγ concentration ($r = -0.354$, $p < 0.001$) and positively related to $CD8^+$ cell number ($r = 0.230$, $p < 0.05$). In the control group, RPS was positively related to bacterial load ($r = 0.317$, $p < 0.01$) (Suppl. Fig. 2). A significant positive correlation between $CD8^+$ and *P. salmonis* load was observed in the PAQ-Xtract® group ($r = 0.233$, $p < 0.05$) and in the "feed" factor in genogroups ($r = 0.350$, $p < 0.001$) (Suppl. Fig. 3), indicating that there was a significant increase in $CD8^+$ cells in the pre-challenge time, but while the correlation increased after challenge with PS-EM-90 ($r = 0.400$, $p < 0.05$), the association was lost in the PS-LF-89 group ($r = -0.287$, $p > 0.05$) (Suppl. Fig. 3). Collectively, the results suggest that fish supplemented with PAQ-Xtract® exhibited increased survival, which was associated with a decrease in serum IFNγ concentration, an increase in $CD8^+$ cell count, and a decrease in head-kidney bacterial load.

3.4. Serum anti-P. salmonis IgM concentration does not correlate with the survival rates of immunization strategies

The mean serum concentration of anti-PS-EM-90 IgM showed significant differences with time ($F = 3.299$, $p = 0.0444$) and immunization strategy ($F = 4.126$, $p = 0.0009$) but showed no differences with the use of immunostimulant ($F = 1.249$, $p = 0.2660$) and *P. salmonis* genogroup PS-EM-90 ($F = 2.226$, $p = 0.1385$) or their interaction ($F = 1.161$, $p = 0.3146$) (Table 4). Likewise, the mean serum concentration of anti-PS-LF-89 IgM did not show significant differences over time ($F = 3.038$, $p = 0.0518$) or with the use of immunostimulant ($F = 0.185$, $p = 0.6679$) but showed a significant difference between immunization strategy ($F = 3.798$, $p = 0.0017$) and *P. salmonis* PS-LF-89 genogroup ($F = 4.330$, $p = 0.0397$) and their interaction ($F = 1.958$, $p = 0.0271$) (Table 4).

Neither the concentration of IgM specific for PS-EM-90 nor that of PS-LF-89 showed any correlation with the rest of the biomarkers evaluated, with bacterial load, or with survival in any of the immunization strategies (Fig. 3). Only genogroup-specific IgM concentrations showed a significant positive correlation with each other in all immunization strategies ($r = 0.885$, $p < 0.001$) (Fig. 3). Fish in the control group showed a significant negative correlation between bacterial load and the concentration of anti-PS-LF-89 IgM ($r = -0.806$, $p < 0.001$) and anti-PS-EM-90 IgM ($r = -0.678$, $p < 0.01$) (Fig. 3). The control fish, on the other hand, had a strong negative relationship between RPS and anti-PS-LF-89 IgM concentration ($r = -0.756$, $p < 0.001$), but not with anti-PS-EM-90 IgM ($r = -0.308$, $p > 0.05$) (Fig. 3).

The concentration of anti-PS-LF-89 and anti-PS-EM-90 antibodies showed a significant negative correlation with bacterial load in both PAQ-Xtract®-treated ($r = -0.342$, $p < 0.01$ for PS-LF-89 and $r = -0.273$, $p < 0.01$ for PS-EM-90) and untreated groups ($r = -0.471$, $p < 0.001$ for PS-LF-89 and $r = -0.394$, $p < 0.001$ for PS-EM-90) (Suppl. Fig. 3). Complementarily, no significant differences were observed between the concentrations of specific antibodies against PS-EM-90 (Suppl. Fig. 4) and PS-LF-89 (Suppl. Fig. 5) among the PAQ-Xtract®-treated fish group and the untreated group. At the genogroup level, the concentration of anti-*P. salmonis* PS-LF-89 IgM showed a significant negative correlation with bacterial load ($r = -0.405$, $p < 0.001$) and RPS ($r = -0.247$, $p < 0.01$), whereas the concentration of anti-*P. salmonis* PS-EM-90 IgM showed a significant negative correlation only with bacterial load ($r = -0.317$, $p < 0.001$). Taken together, the results would confirm that the immunization strategies activate the adaptive humoral response, but the antibody concentration did not correlate with the survival rate of the immunization strategies evaluated.

Table 4
Summary of repeated-measures ANOVA results for biomarkers of humoral and cellular adaptive immune response between immunization strategies (IS) (vaccines and use of immunostimulant), *P. salmonis* genogroup (PS) and interaction of immunization strategies and genogroup (IS*PS).

Biomarker	Predictor	Df	SS	MS	F value	Pr(>F)
IL-12	Time (T)	3	4360	1453	1425	0,2380
	Feed (F)	1	1960	1958	1919	0,1680
	Immunization strategies (IS)	6	7540	1257	1233	0,2930
	P. salmonis Genogroup (PS)	1	0,290	0,292	0,286	0,5940
	IS*PS	14	8330	0,595	0,583	0,8750
	Residuals	154	157,100	1020		
IFNγ	Time (T)	3	10,690	3563	17,302	***0,0000***
	Feed (F)	1	0,070	0,075	0,363	0,5476
	Immunization strategies (IS)	6	0,840	0,140	0,678	0,6676
	P. salmonis Genogroup (PS)	1	11,070	11,067	53,739	***0,0000***
	IS*PS	14	5380	0,384	1865	***0,0342***
	Residuals	154	31,720	0,206		
CD8 Score	Time (T)	3	0,522	0,174	5923	***0,0008***
	Feed (F)	1	1951	1951	66,424	***0,0000***
	Immunization strategies (IS)	6	1705	0,284	9678	***0,0000***
	P. salmonis Genogroup (PS)	1	0,392	0,392	13,357	***0,0004***
	IS*PS	14	0,685	0,049	1666	0,0681
	Residuals	154	4523	0,029		
IgM PS-EM-90	Time (T)	2	2500	1253	3200	***0,0444***
	Feed (F)	1	0,490	0,489	1249	0,2660
	Immunization strategies (IS)	6	9690	1615	4126	***0,0009***
	P. salmonis Genogroup (PS)	1	0,870	0,871	2226	0,1385
	IS*PS	14	6360	0,454	1161	0,3146
	Residuals	116	45,410	0,391		
IgM PS-LF-89	Time (T)	2	2680	1339	3038	0,0518
	Feed (F)	1	0,080	0,082	0,185	0,6679
	Immunization strategies (IS)	6	10,050	1675	3798	***0,0017***
	P. salmonis Genogroup (PS)	1	1910	1909	4330	***0,0397***
	IS*PS	14	12,080	0,863	1958	***0,0271***
	Residuals	116	51,140	0,441		

3.5. Low serum IL-12 and high serum IFNγ concentrations would correlate with increased bacterial load and decreased survival

The mean serum IL-12 concentration did not show significant differences over time ($F = 1.43$, $p = 0.236$), immunostimulant ($F = 1.93$, $p = 0.167$), *P. salmonis* genogroups ($F = 0.92$, $p = 0.455$), or immunization

strategy (F = 0.29, p = 0.593) (Table 4). Overall, fish belonging to all immunization strategies showed an increase in mean serum IL-12 concentration at the beginning of the IDP but a rapid decrease at the end of the IDP, especially at the end of the challenge with both *P. salmonis* genogroups (Suppl. Fig. 6). On the other hand, the mean serum IFNγ concentration showed significant differences over time (F = 17.302; p = 0.000), but not between immunization strategies (F = 0,678, p = 0,6676) (Fig. 4; Table 4). Serum IFNγ concentration did not show significant differences between PAQ-Xtract®-treated and untreated groups (F = 0.363, p = 0.5476), but fish challenged with PS-LF-89 showed a significantly higher concentration than those challenged with PS-EM-90 (F = 53.739, p = 0.000) (Fig. 4; Table 4). In the case of the strategies that considered primo vaccination, there were increases in the mean serum IL-12 and INFγ concentration after primo vaccination and after booster, but without significant differences between the groups with and without immunostimulant (Table 4).

Serum IL-12 and IFNγ concentration showed a significant negative correlation at the global level (r = 0.261, p < 0.001), but at the immunization strategy level, it was observed in S2 (r = 0.396, p < 0.01) and S3 (r = 0.374, p < 0.05) (Fig. 3). Similarly, serum IL-12 concentration showed a significant negative correlation at the global level with the number of CD8$^+$ cells (r = −0.233, p < 0.001), as well as at the levels of S2 (r = −0.315, p < 0.05) and S3 (r = 0.454, p < 0.01) (Fig. 3). Interestingly, control fish in the unvaccinated group (i.e., G8) showed a significant positive correlation of serum IL-12 concentration with bacterial load in the anterior kidney (r = 0.446, p < 0.05) and RPS (r = 0.478, p < 0.05) (Fig. 3). Serum IFNγ concentration increased significantly with time (F = 17.302; p = 0.000), and regardless of immunostimulant use, fish that survived challenge with PS-LF-89 showed significantly higher serum IFNγ levels than fish that survived challenge with PS-EM-90 (Fig. 4; Table 4). At the same time, serum IFNγ concentration globally showed a significant negative correlation with *P. salmonis* load (r = −0.275, p < 0.001), and then IFNγ significantly increased with the bacteria load, especially in fish immunized with LAV in S1 (r = −0.328, p < 0.05) and S2 (r = −0.318, p < 0.05) (Fig. 3). Globally, IFNγ concentration showed a significant negative correlation with RPS (r = −0.250, p < 0.001), especially with killed-cell vaccine-based strategies in S3 (r = −0.500, p < 0.001) and S4 (r = −0.557, p < 0.01) (Fig. 3). Similarly, higher serum concentrations of IFNγ were correlated with lower survival rates, especially in groups of fish vaccinated with killed cell vaccines. Taken together, these results would indicate that low serum IL-12 and high serum IFNγ concentrations would correlate with higher bacterial loads and lower survival.

3.6. *Aggregate analysis shows a significant correlation between higher numbers of CD8$^+$ T cells and higher fish survival and lower bacterial load*

The number of CD8$^+$ cells showed significant differences over time (F = 5.923, p = 0.0008), between *P. salmonis* genogroups (F = 13.357, p = 0.0004), and immunization strategies (F = 9.678, p = 0.0000) (Table 4). Surviving fish challenged with PS-EM-90 showed significantly higher numbers of CD8$^+$ cells in the head-kidney in the PAQ-Xtract® treated group than in the untreated group (F = 66.424, p = 0.0000) (Table 4), but surviving fish challenged with PS-LF-89 showed no difference between the treated and untreated groups (Fig. 5). The number of CD8$^+$ cells in the head-kidney (Suppl. Fig. 7) showed a slightly significant positive correlation at the global level with the bacterial load in the same tissue (r = 0.213, p < 0.01) and with the RPS (r = 0.156, p < 0.05) (Fig. 3).

In addition, CD8$^+$ cells showed a significantly positive correlation with both bacterial load (r = 0.233, p < 0.05) and RPS (r = 0.230, p < 0.05) in the PAQ-Xtract® fish group (Suppl. Fig. 1). At the same time, the number of CD8$^+$ cells showed a significantly positive correlation with bacterial load (r = 0.400, p < 0.05) and RPS (r = 0.473, p < 0.01) in PS-EM-90-challenged fish but not in PS-LF-89-challenged fish (Suppl. Fig. 2). Similarly, serum IFNγ concentration showed an overall significant positive correlation with the number of CD8$^+$ cells in the unvaccinated fish group (G8) (r = 0.589, p < 0.01) (Fig. 3). A slightly significant negative correlation was observed between the number of CD8$^+$ cells in the head and the amount of serum antibodies specific for

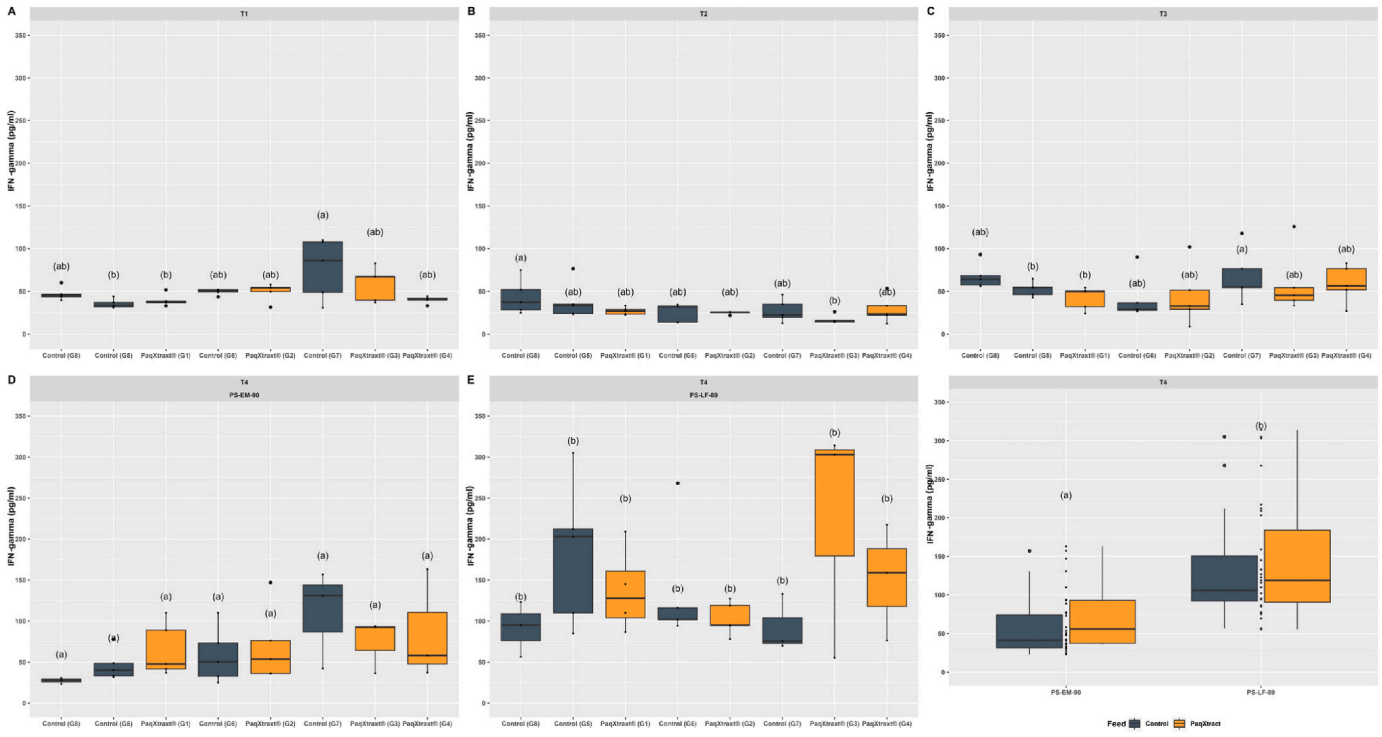

Fig. 4. Serum IFNγ concentration (pg/ml) in Atlantic salmon post-smolt challenged with PS-EM-90 and PS-LF-89 and quantified by ELISA assay. The effect of the immunization strategy (S1 to S4) and the use of the immunostimulant (PAQ-Xtract® or control) on serum IFNγ concentration was evaluated by repeated measures ANOVA considering four time points. Different letters mean significant differences.

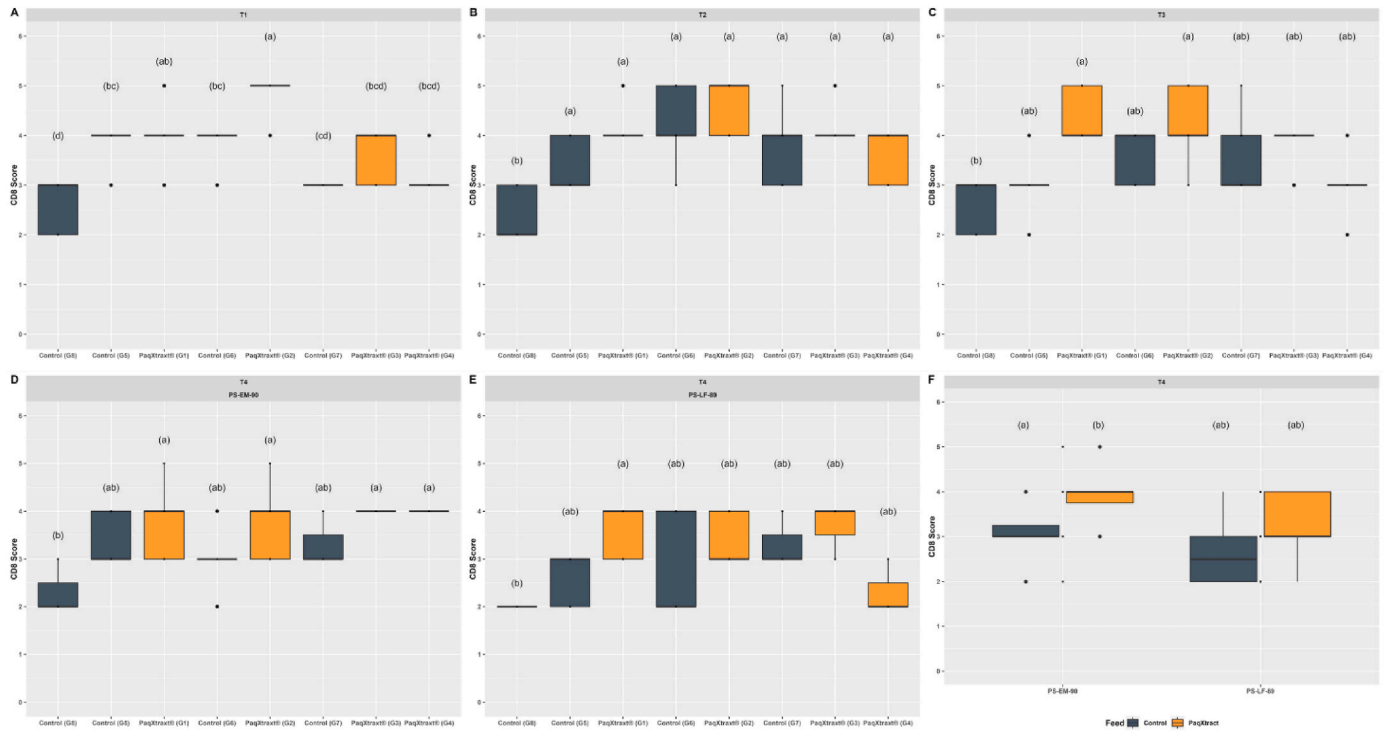

Fig. 5. Number of CD8$^+$ cells (CD8-score) in the head-kidney of Atlantic salmon post-smolt challenged with PS-EM-90 and PS-LF-89 and semi-quantified by IHC assay. The effect of the immunization strategy (S1 to S4) and the use of the immunostimulant (PAQ-Xtract® or control) on CD8$^+$ cells count was evaluated by repeated measures ANOVA considering four time points. Different letters mean significant differences.

both PS-EM-90 ($r = -0.183$, $p < 0.05$) and PS-LF-89 ($r = -0.204$, $p < 0.05$). Taken together, these results would indicate that a higher survival rate can be achieved when a strategy of vaccination and immunostimulants is integrated to increase the number of CD8$^+$ cells to maintain a low tissue bacterial load, especially for the PS-EM-90 genogroup. These results were not observed in fish challenged with PS-LF-89, which would also support partial cross-protection of the current industry heterologous vaccination.

3.7. Immunization strategies are safe for fish and induce only mild microscopic lesions in the gut

Fish subjected to each of the immunization strategies presented mild intestinal lesions (hsINT <0.9), basically characterized by inflammatory lesions of low to moderate extent in the intestinal serosa (Suppl. Fig. 8). However, the mucosal and submucosal layers showed no histopathological changes but rather evidenced adequate cytoplasmic vacuolization in enterocytes and normal integrity of intestinal villi (Suppl. Fig. 7). Before the start of the test (T0), all fish showed an hsINT of 0, with no histopathological changes. Histopathological changes in the intestinal serosa were slightly higher in fish immunized with whole cell vaccines for *P. salmonis* (Agrovac® and X-KCV) than those immunized with single or double doses of LAV, even considering that fish in all experimental groups were immunized with a polyvalent vaccine based on killed viruses and bacteria (ALPHA JECT® 5.1). As such, fish in the control group (G8) had an average hsINT of 0 up to the time before challenge with *P. salmonis* (T3), but in fish supplemented with the immunostimulant, it was 0.12 in G1, 0.38 in G2, 0.62 in G3, and 0.50 in G4, while in fish without the immunostimulant, it was 0.13 in G5, 0.17 in G6, and 0.61 in G7 (Suppl. Table 7). Although fish surviving challenge with both genogroups (T4) showed slightly higher hsINT in the immunostimulant groups, fish surviving challenge with PS-LF-89 showed higher hsINT than PS-EM-90 challenged groups, particularly in G2 and G3.

The hsINT showed a significant positive correlation with serum IL-12 concentration in the S3 group ($r = 0.394$, $p > 0.01$) and with IFNγ concentration in S1 ($r = 0.286$, $p > 0.05$), but a negative correlation with the number of CD8$^+$ T cells in both strategies, S1 ($r = -0.288$, $p > 0.05$) and S3 ($r = -0.487$, $p > 0.01$). Overall, hsINT showed a negative correlation with CT ($r = -0.228$, $p > 0.01$). Finally, fish without additives showed a negative correlation of hsINT with the number of CD8$^+$ cells ($r = -0.405$, $p > 0.001$) and bacterial load ($r = 0.303$, $p > 0.01$). Taken together, the results confirm the high safety of commercial vaccines for the control of SRS, especially given the current vaccination strategy in the industry.

4. Discussion

There are several factors that can influence the evolution of pathogens and their transmission dynamics such as transmissibility, virulence and pathogenicity, duration of infection, population size, productive management, environmental variables, among others, but the dynamics of infectious diseases are further complicated by the presence of multiple strains or variants of a pathogen and/or the simultaneous infection of several types of pathogens, i.e. co-infections. Schober et al. [14] recently performed a comprehensive analysis of 73 high-quality closed genomic sequences from different isolates of *P. salmonis* and showed that most of the sequences were members of the LF-89 and EM-90 genogroups, but the Norwegian and Canadian isolates formed a separate genogroup (NC) related to the Chilean LF-89 isolates; and, the EM-90 genogroup sequences were separated into four EM-90 subgroups (EM1 and EM4). Cross-protection can shape the epidemiological dynamics of multi-strain or genogroup pathogens when one strain temporarily suppresses the transmission of another, which is the situation we are observing with PS-EM-90 and PS-LF-89 in Chilean salmon farming [15]. Temporary or permanent cross-protection, multiple routes of transmission, climatic variability and co-infection levels are some of the key mechanisms that result in altering the fitness for successful pathogen transmission and escape from the host immune response.

Rozas-Serri et al. [15] have shown that Atlantic salmon can be infected by both PS-LF-89 and PS-EM-90 in the same farm, net cage and

tissue from the same fish. Indeed, there has been an increase in susceptibility to SRS in recent years, as evidenced by earlier first detection and antibiotic treatment from week 25 to week 12 after stocking in seawater farms. In this study, the current industry vaccination strategy recorded 100 % RPS when fish were challenged with PS-EM-90, but the survival rate dropped significantly to 77 % when fish were challenged with PS-LF-89, so complete cross-immunity was not observed in fish vaccinated with the PS-EM-90 genogroup and challenged with PS-LF-89 of *P. salmonis*. This lower survival in the PS-LF-89 challenged, even compared to the strategy based on the bivalent KCV (S3), could indicate that PS-LF-89 would modulate mechanisms that would allow it to be more efficient in evading the immune response of fish when vaccinated with PS-EM-90, so it could be hypothesized that fish vaccinated with this genogroup would not protect fish against one or more evasion strategies that PS-LF-89 possesses but PS-EM-90 does not.

The first LAV based solely on an isolate like EM-90 was introduced in Chile in 2016. The vaccination strategy based on this vaccine and a pentavalent vaccine, whose *P. salmonis* component is also similar to EM-90 but inactivated, has been the current industry standard [15]. Accordingly, the LF-89 genogroup isolates may have increased their infection pressure because of this control strategy. Many specific genes of this genogroup are associated with host-pathogen surface interactions (O antigen synthesis, OM proteins, peptidases, defense systems, transporters, etc.), suggesting the presence of different surface antigens between LF-89 and EM-90 isolates [14]. Hence, the differences in virulence and modulation of the immune response observed in PS-EM-90 and PS-LF-89 infections under experimental [8,9,18–20,22] and field [15] conditions may be explained in part by the greater number of icm/dot type IVB gene clusters [43,44], and the greater degree of mutational inactivation by frameshifts in the PS-LF-89 genogroup isolates analyzed by Schober et al. [14].

In addition, the only immunization strategy using a vaccine based on both *P. salmonis* genogroups, albeit of a killed-cell vaccine, resulted in an almost identical RPS for both PS-EM-90 (89 %) and PS-LF-89 (88 %). This immunization strategy using a PS-LF-89 specific vaccine also proved to be more effective in controlling PS-LF-89 infection than the current industry standard strategy in Chile (RPS = 77 %), which would suggest that the inclusion of the PS-LF-89 component in the vaccines would add more protection than the current vaccination strategy. The protection results for PS-LF-89 obtained with the KCV based only on PS-EM-90 (RPS G4 = 48 %) would confirm that cross-protective immunity is significantly lower than that obtained with strategies based only on PS-EM-90, either with live-attenuated or killed-cell vaccines. In addition, Rozas-Serri et al. [20] showed that a KCV *P. salmonis* vaccinated-fish exhibited *MHCI, MHCII,* and *CD4* overexpression but a significant downregulation of *CD8b* and *IgM*, suggesting that the KCV bacteria promoted the $CD4^+$ T-cell response but did not induce an immune response mediated by $CD8^+$ T cells or a humoral response. Taken together, these results could be considered that the current vaccination strategy for the control of SRS in Chile, based on vaccines of different nature (KCV plus LAV), but always based only on the PS-EM-90 genogroup, would works as a heterologous vaccine strategy for PS-LF-89. Therefore, the current strategy based on PS-EM-90 confers only partial cross-protection against the *P. salmonis* genogroup for which it was not designed, i.e., PS-LF-89.

Figueroa et al. [22] challenged fish vaccinated with the same vaccination standard as the present study (S1) with PS-LF-89 and PS-EM-90 by cohabitation, but in different, unrelated, and non-comparable trials. The authors recorded no significant differences in the percentage survival in vaccinated (56.7 %) and unvaccinated fish challenged with PS-LF-89 (60.3 %), nor in vaccinated (60.2 %) and unvaccinated fish challenged with PS-EM-90 (64.6 %), although the latter group of fish was co-infected with sea lice. Thus, the RPS values of −9% for PS-LF-89 and -12 % for PS-EM-90 were significantly different from those obtained in the present study. The differences were basically in the challenge model (I.P vs. cohabitation), co-infection with sea lice, the different isolates of each genogroup of *P. salmonis*, among others. Although the formal registration system for biological products in Chile has historically been based on I.P challenge trials [2,10], which is why this route of infection was prioritized in the present study, our research group described for the first time the cohabitation challenge model for both genogroups of *P. salmonis* [9], promoting its more massive use to evaluate the efficacy of vaccines, immunostimulant products and/or genetic resistance for SRS [2], basically because it is a test that better mimics the dynamics of horizontal transmission of diseases, including the battle with all the components of mucosal immunity in fish.

The best result expressed in RPS was recorded in fish that received first vaccination with LiVac® and booster again with LiVac® plus ALPHA JECT® 5.1, with or without PAQ-Xtract®, both in fish challenged with PS-EM-90 (100 %) and in fish challenged with PS-LF -89 (96 %), which would indicate that repeated immunizations (which is currently not the standard), even with only antigenic components of PS-EM-90, could increase protection for PS-LF-89 (from 77 % in S1 to 96 % in S2). These results were especially remarkable in fish vaccinated first with LAV and challenged with PS-LF-89 (96 %), as in the groups of fish vaccinated first with bivalent killed-cell (S3 = PS-LF- 89 and PS-EM-90) and monovalent (S4 = PS-EM-90) vaccines, 88 and 48 %, respectively, which would also demonstrate that, even using killed-cell vaccines, including both genogroups of *P. salmonis* twice, the RPS increased by 40 % when challenged with PS-LF-89.

Revaccination or booster vaccination is a well-known method to enhance the magnitude and specificity of the immune response and has been previously described with serial oral vaccinations against SRS under both experimental and field conditions [45,46]. Nonetheless, these results support the idea that the adaptive immune response is plausible to be boosted (immunological memory), so the vaccination strategy should consider other immunization routes, but with vaccines of proven efficacy in activating the CMIresponse and with the technical and operational feasibility of being administered by immersion and in the feed. No significant differences were observed in serum anti-*P. salmonis*-specific IgM levels between immunization strategies or between genogroups in our study, and a significant negative correlation was observed between antibody concentration and survival expressed on RPS. Thus, fish with higher survival showed similar anti-*P. salmonis* IgM antibody concentrations to fish with lower survival, findings that would demonstrate that the humoral immune response does not mediate the main protective mechanism against *P. salmonis* infection, confirming that the intracellular nature of the bacteria requires rather a strong CMI response. These results are consistent with the kinetics of the transient antibody response observed under field conditions after vaccination in freshwater, as antibody levels begin to decrease approximately 1800 days after vaccination [45,46], but the mortality begins even 1200-DD after vaccination [2,10,17,25,47]. To our knowledge, the results of our study represent the first in the evaluation of cross-protection between *P. salmonis* genogroups. Taken together, the results of this study suggest the strong necessity to carry out antigenic and serological characterization of isolates of each genogroup of *P. salmonis* in Chile, with the aim of eventually revealing different serotypes within each genogroup. In this way, Morrison et al. [48] described three serologically distinct Tasmanian *Rickettsia*-like organism (TRLO) isolates and linked each serotype to a specific geographic location within Tasmania.

Both IL-12 and IFNγ are key cytokines in Th1 polarization to promote differentiation of $CD8^+$ T cells [49], therefore, they are critical to minimize evasion of the CMI response during *P. salmonis* infection [18,19]. Low serum IL-12 concentrations and high IFNγ concentrations correlated with higher bacterial loads and lower survival in this study, a finding that is consistent with previously described results in vaccinated and *P. salmonis*-challenged fish [18–20,50,51]. Similarly, elevated levels of post-smolt IFNγ gene expression have been described in Atlantic salmon infected with the PS-EM-90 and PS-LF-89 genogroups showing down-regulation of the CD8α gene. [18], severe tissue damage and low survival [9]. Interestingly, IFNγ is the key molecule in Th1 polarization

for the activation of immunity against intracellular pathogens, but it is also a potent proinflammatory cytokine important for increasing Toll-like receptor (TLR) expression, increasing MHC-I antigen presentation, and inducing chemokine secretion, macrophage activation, and increased phagocytosis, all of which could lead to tissue damage [52]. At the same time, low levels of IL-12, associated with high expression of IL-10, has also been described as an anti-inflammatory balance that would facilitate the replication and intracellular survival of *P. salmonis* and that could also promote the differentiation of regulatory T cells [18,53].

Notably, the results obtained with the current industrial vaccination strategy were significantly improved to 92 % survival (from 77 %) when fish challenged with PS-LF-89 were previously supplemented with PAQ-Xtract®, a commercial product based on purified extracts of Quillaja saponaria. The strategy based on the bivalent KCV recorded the same RPS of 88 % in the group challenged with PS-LF-89, with or without PAQ-Xtract, but the additive increased the RPS of fish challenged with PS-EM-90 (from 89 to 98 %). Cortés et al. [54] demonstrated that these extracts had no direct antimicrobial effect on *P. salmonis*, but observed a lower invasion and replication rate of *P. salmonis* in macrophage cell lines, accompanied by down-regulation of bacterial genes encoding virulence factors such as dotB of T4-BSS and the chaperone protein chaPs, as well as modulation of genes encoding IL-12 and IL-10 in an equilibrium that favors phagosome-lysosome fusion [18,53]. Furthermore, Cortés et al. [55] demonstrated that supplementation with PAQ-Xtract modulated key markers in innate and CMI responses under in vivo conditions, increasing the survival rate of fish under both experimental (I.P. and cohabitation) and field challenge conditions. Taken together, these results would support the model that fish vaccinated with the current ALPHA JECT® 5.1 + LiVac® based field strategy be previously immunized using immunostimulant to extend protection of fish against the PS-LF-89 genogroup in the field.

Vaccine efficacy for SRS control from experimental challenges is acceptable [2,10,25], but current field vaccination strategies have consistently shown only transient CMI response activation [10,17,47]. The CMI response can be activated in farmed salmon [56], but it is not a long-term response and probably generates an immunological memory about which we know very little so far. Undoubtedly, the strategy based on single intraperitoneal immunization using the current vaccines should be improved, not only thinking of expanding the spectrum of these vaccines through their formulation with both PS-LF-89 and PS-EM-90 isolates or their respective antigenic components in the same formulation, i.e., bivalent for *P. salmonis*, but also in the development of new vaccines with greater potency to activate CMI and with a nature that allows the design of a multiple vaccination strategy using different routes of administration depending on the productive stage of the farmed salmon. It is implausible to think that effective control of SRS can be achieved using only a single vaccination at the freshwater stage of salmon (50–80 g weight) and expect the efficacy and immunological memory of the response to be maintained until harvest (>5 kg weight). Therefore, the immunization strategy should be based on activating CMI systematically and periodically to induce a relatively protective immune response during most of the grow-out period of Atlantic salmon at sea, i.e. 14–17 months depending on the geographical area. Vaccines against pathogens with high antigenic variability, cross-reactivity is essential in the strategy of designing a vaccine with optimal efficacy. With the advent of new vaccine technologies, e.g. synthetic peptide antigens, cross-reactivity could become the solution to create vaccines against pathogens with high genetic and antigenic variability.

It should always be kept in mind that the immune response and survival results of all vaccination strategies against SRS under field conditions are significantly lower than those results obtained under experimental conditions [10]. However, the actual protection of the current vaccination strategy has been able to maintain controlled EM-90 in the field (low loads and low prevalence), but at the expense of significantly increased loads and prevalence of LF-89 in fish and in the environment. Constant surveillance of the spatiotemporal dynamics of *P. salmonis* genogroups in the industry and early warning of eventual new changes in the epidemiological situation of SRS in the field is fundamental for an adjusted design of the vaccination strategy that provides the necessary guarantees for optimal SRS control [15]. This study partially characterized the $CD8^+$ T-cell response without providing further mechanistic information on cytotoxic responses, so more research regarding the cytotoxic cell-mediated response is needed to improve its understanding in fish vaccinated against *P. salmonis*. Taken together, our results help to better understand the biological interaction of *P. salmonis* and the host and to deepen the knowledge on vaccine-induced protection, CMI response and cross-immunity applied to improve the current immunization strategy against SRS in the Chilean salmon industry.

Data availability

Data will be made available on request.

CRediT authorship contribution statement

Marco Rozas-Serri: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Takahiro Kani:** Methodology, Investigation, Writing – review & editing. **Victoria Jaramillo:** Methodology, Investigation, Writing – review & editing. **Rodolfo Correa:** Investigation, Software, Data curation. **Ricardo Ildefonso:** Methodology, Investigation, Supervision, Writing – review & editing. **Carlos Rabascall:** Methodology, Investigation. **Soraya Barrientos:** Methodology, Investigation. **Darling Coñuecar:** Methodology, Investigation. **Andrea Peña:** Methodology, Investigation, Supervision, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Data availability

All results and databases were included as supplementary material in this manuscript.

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Appendix A. Supplementary data

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