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A pentavalent vaccine for rainbow trout in Danish aquaculture

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ABSTRACT

Mariculture in Denmark is based on production of rainbow trout grown two years in fresh water followed by one growth season in sea cages. Although the majority of rainbow trout are vaccinated against the most serious bacterial pathogens – *Aeromonas salmonicida* subsp. *salmonicida*, *Vibrio anguillarum* and *Yersinia ruckeri*, by the use of commercially available vaccines, disease outbreaks requiring treatment with antibiotics still occur.

The present study tested the potential of a new experimental multicomponent vaccine that is based on local bacterial strains, isolated from rainbow trout in Danish waters, and thus custom-designed for Danish rainbow trout mariculture. The vaccination with the multicomponent vaccine resulted in protection against three relevant bacterial diseases (yersiniosis, furunculosis, vibriosis) under experimental conditions. We showed that i.p. injection of the vaccine induced specific antibody responses in trout against the different bacterial antigens and regulated expression of genes encoding SAA, C3, IL-1β, IL-6, IL-8, IgD and MHCII.

1. Introduction

Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) is the most important aquacultured fish species in Denmark and is reared in both freshwater and marine farms [1]. Even though the majority of rainbow trout in Danish aquaculture farms are vaccinated against the main bacterial pathogens, they still experience disease outbreaks requiring treatment with antibiotics. In mariculture systems *Aeromonas salmonicida* subsp. *salmonicida* causing furunculosis and *Vibrio anguillarum* causing vibriosis are the main pathogens, while *Yersinia ruckeri* – the etiological agent of enteric redmouth disease (ERM), is the main bacterial pathogen responsible for outbreaks in freshwater fish farms [2,3].

A common practice against ERM in Danish aquaculture is immersion vaccination of 4–5 g rainbow trout fry. The vaccine gives protection against ERM, but the protection only lasts for up to 6 months [4]. Thus, the fish in Danish freshwater farms experience disease outbreaks at a later stage of the production cycle and an additional dip vaccination is required in order to maintain high levels of protection [5,6]. However, dip vaccination of larger fish is considered unpractical and economically non-sustainable by many farmers. Vaccination against vibriosis and furunculosis is performed only on fish aimed for sea rearing and takes place prior to transfer from freshwater farms to the marine net cages. Fish are injection vaccinated with a trivalent mineral oil-adjuvanted vaccine emulsion containing *A. salmonicida* subsp. *salmonicida* and *V. anguillarum* serotype O1 and O2a bacterins (Alpha-ject®3000, Pharmaq, Norway). However, vaccinated fish still experience outbreaks of vibriosis and particularly furunculosis in seawater during elevated summer temperatures [2]. Whether the suboptimal protection induced by these vaccines could be partly explained by antigen variability related to the fact that they are based on bacterial strains derived from heterologous fish host species and/or from distant locations (UK and Norway) rather than from Denmark, remains to be determined. An optimized vaccines and improved vaccination strategies [7] are highly requested by the farmers in order to reduce the consumption of antibiotics and minimize the economic losses. This study aimed to incorporate *Y. ruckeri* bacterin antigens into a vaccine with *A. salmonicida* and *V. anguillarum* antigens, based on local bacterial strains, and hereby custom-design a new pentavalent vaccine for Danish rainbow trout aquaculture conditions.

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2. Materials and methods

2.1. Fish

Disinfected eyed rainbow trout eggs, originating from a disease free farm were hatched in a pathogen-free rearing facility at the Bornholm Salmon Hatchery (Nexø, Denmark). Details of retaining the pathogen-free status have previously been described [8]. Fish were vaccinated as described below and reared in the system containing recirculated municipal water at 12 °C in 700 L tanks (total volume 1 m^3). Fish were fed 1% biomass per day with dry pellet feed (BioMar A/S, Denmark). Subsequently, batches of fish were transported to the fish keeping facilities at the University of Copenhagen and the Technical University of Denmark for several challenge experiments. All experiments were approved by the Animal Experiments Inspectorate, Ministry of Environment and Food, Denmark under the license no. 2015-15-0201-01350 and 2014-15-0201-00379.

2.2. Vaccination

An experimental pentavalent vaccine was designed by combining formalin-inactivated bacterial cells (bacterins) of strains of *Y. ruckeri* serotype O1 biotype 1 and 2, *V. anguillarum* serotype O1 and O2a and *A. salmonicida* subsp. *salmonicida*. All strains were isolated from disease outbreaks in Danish rainbow trout farms. The viability of bacterial cells after formalin inactivation was determined by culture trials and plating bacteria on agar plates. The bacterin composition was emulsified with mineral oil adjuvant. A commercial vaccine comprising the three latter bacterin antigens (derived from foreign isolates) emulsified with mineral oil adjuvant (Alphaject®3000, Pharmaq, Norway) was used as positive control while saline was used as negative control. Rainbow trout (average body weight of 34 g) were anaesthetized with Finquel® vet (100 mg L^{-1}) with sodium hydrogen carbonate (100 mg L^{-1}) at pH 7.2, 11.4 °C and vaccinated intraperitoneally (i.p.). Ten fish at a time were vaccinated with 0.1 ml of experimental vaccine followed by 10 fish injected with the commercial vaccine and 10 fish injected with saline. Separate automatic repeater syringes were used for each group. Fish were tagged by removing left maxilla (experimental vaccine), adipose fin (commercial vaccine) or right maxilla (saline).

Fish were vaccinated at two separate occasions, thus fish used for *A. salmonicida* and *Y. ruckeri* challenge belonged to a different batch than fish used for *V. anguillarum* challenge.

2.3. Challenge experiments

2.3.1. Aeromonas salmonicida

At 10 weeks post-vaccination (wpv) fish were transported to the fish keeping facility at University of Copenhagen and kept in 6 glass aquaria (80 L). Each tank accommodated 15 fish, i.e. 5 fish of each treatment group. Thirty fish per treatment group were used for a challenge study. Fish were acclimatized over the course of two weeks, by gradually raising the water temperature to 19 °C. At 12 wpv (1058 degree-days), all fish (average body weight of 81 g) were anaesthetized (75 mg L^{-1} MS-222) and exposed to live *A. salmonicida* strain 111129-1/2 by a tail fin infection method. This method has previously been validated and demonstrated as a reproducible method to induce systemic infection in rainbow trout and elicit symptoms comparable with furunculosis in the field [9]. Briefly, 10 perforations were made through the tail fin using a multi-puncture device. Then 100 µl of bacterial culture (2.0×10^8 CFU) was placed on the puncture site for 90 s and fish were transferred to freshwater for recovery. Fish morbidity was monitored for 4 weeks post-challenge (wpc). The moribund state was defined as a loss of equilibrium, strong discoloration and development of skin hemorrhages. Moribund fish were immediately removed for euthanasia (300 mg L^{-1} MS-222) and recorded as mortalities. Head kidney swabs from freshly euthanized fish were plated onto 5% blood agar plates (SSI Diagnostica, Denmark) for bacteriological analysis. The confirmation was done according to Dalsgaard and Madsen [3] and the re-isolated bacteria from dead fish were confirmed as *A. salmonicida*.

2.3.2. Yersinia ruckeri serotype O1 biotype 1 and 2

At 4 wpv fish were transported to the fish keeping facility at the University of Copenhagen and kept in 6 fiberglass tanks (180 L). Each tank accommodated 30 fish, i.e.10 fish from each treatment group. Thirty fish per treatment group were used for a challenge study with each biotype of *Y. ruckeri*.

At 9 wpv (853 degree-days) fish (average body weight of 54 g) were anaesthetized (75 mg L^{-1} MS-222) and 3 tanks were challenged by intraperitoneal (i.p.) injection of 0.1 ml of *Y. ruckeri* serotype O1 biotype 1 strain 910926-2/2 (5.0×10^6 CFU). Additional 3 tanks were i.p. challenged with 0.1 ml of *Y. ruckeri* serotype O1 biotype 2 strain 020716-2/2a (4.3×10^6 CFU). Morbidity of infected fish was monitored during 3 weeks post-challenge (wpc). The assessment of the moribund state, mortality recordings and bacterial confirmation were performed similarly to *A. salmonicida* challenge. The re-isolated bacteria from dead fish were confirmed as either *Y. ruckeri* serotype O1 biotype 1 or *Y. ruckeri* serotype O1 biotype 2, respectively.

2.3.3. V. anguillarum serotype O1 and O2a

At 6 wpv fish were transported to the fish facilities at the Technical University of Denmark and distributed into 4 aquaria (180 L), so that each aquarium contained 33 fish, i.e. 11 fish from each treatment group.

At 7 wpv (637 degree-days) fish (85–120 g) were anaesthetized with 0.01% benzocaine and challenged by i.p. injection of 0.1 ml of *V. anguillarum* serotype O1 strain 090819-1/28A (2.4×10^6 CFU). At 11 wpv (1000 degree-days) fish (85–130 g) were challenged by i.p. injection with 0.1 ml of *V. anguillarum* serotype O2a strain 090903-1/2B (1.2×10^8 CFU). Morbidity of infected fish was monitored during 10 days post-challenge. In both challenges, fish in one additional aquarium were injected with 0.1 ml sterile bacterial growth medium as negative control. Fish with evident clinical signs were euthanized with an overdose of benzocaine and registered on a daily basis. Re-isolation of the bacteria was performed on a representative number of fish of each aquarium by taking swabs from the head kidney and plating them on blood agar plates, followed by serological identification.

2.4. Sampling

Blood samples for Enzyme-linked immunosorbent assay (ELISA) were taken from unchallenged vaccinated and saline control fish at 8 wpv (6 fish per group) and 26 wpv (10 fish per group). Tissue (spleen, head kidney and liver) samples for quantitative PCR (qPCR) were taken from unchallenged vaccinated and saline control fish at 8 wpv. Blood was collected by caudal vein puncture from euthanized fish (300 mg L^{-1} MS222) and allowed to clot at 4 °C overnight. Serum was separated at 4 °C by centrifugation (3000 *G*) for 10 min and stored at −80 °C until further analysis. For qPCR, samples of spleen, head kidney and liver were aseptically removed and transferred to RNAlater stabilization reagent (Sigma-Aldrich, RO901, Denmark), pre-stored at 4 °C for 24 h and then stored at −20 °C until RNA isolation.

2.5. ELISA

ELISA was performed according to a previously established protocol, applying a specific monoclonal antibody, measuring binding of rainbow trout IgM to bacterial antigens [10]. In brief, 96-well microtiter plates (MaxiSorp™, Nunc, Denmark) were separately coated with one of the following sonicated bacterial lysates (protein conc 5 µg ml^{-1}): 1) *A. salmonicida* strain 090710-1/23, 2) *Y. ruckeri* serotype O1 biotype 1 strain 910926-2/2, 3) *Y. ruckeri* serotype O1 biotype 2 strain 100415-1/4, 4) *V. anguillarum* serotype O1 strain 090602-1/12,

5) *V. anguillarum* serotype O2a strain 090903-1/2B. Based on pilot studies, the 1:1000 dilution was chosen for testing the presence of IgM antibodies against sonicated bacteria. To determine the non-specific binding of antibodies to the solid surfaces of the micro-titer plate wells, all samples were tested in non-coated wells. The latter served as a negative control for measuring non-specific background. The optical density (O.D.) values were subtracted from those obtained with antigen-coated wells. Each plate also included a positive control serum from fish immunized with paraffin oil-adjuvanted bacterin of *A. salmonicida.* Positive control was used as an inter-plate calibrator to normalize variation between the plates. All the samples were normalized according to the plate specific regulation factor i.e. the ratio of the mean absorbance from the entire pool of positive control wells to the mean absorbance of the positive control wells on each plate. The O.D. was measured at 450 nm in an Epoch spectrophotometer (BioTek, USA) in duplicate wells.

2.6. Serum protein measurement

Serum protein was measured by the use of a NanoDrop 2000 (Thermo Scientific, Denmark) by the A280 method. The differences between groups were tested with 2-tailed *t*-test ($p < 0.05$).

2.7. RNA extraction, cDNA production and qPCR

The RNA extraction, cDNA synthesis and quantitative PCR assays were performed as previously described [11]. The transcription levels were studied for genes encoding C3, SAA, MHC I, MHC II, TGF-β, TNF-α, IL-1β, IL-6, IL-8, IL-10, IL-4/IL-13A, IL-17A/7F2, IL-17C1, IL-17C2, IgM, IgT, IgDm, IgDs, CD4 and CD8. The details of used primers and probes are shown in Table 1.

2.8. Statistical analysis

All statistical tests were performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, USA, www.graphpad.com) and p values < 0.05 were considered statistically significant. Mortality data were analysed using Log-rank tests and 1way ANOVA followed by Tukey's multiple comparison test. ELISA results were compared using one-way ANOVA followed by Dunn's multiple comparison test.

Gene expression data were analysed according to the $2^{-\Delta\Delta Ct}$ method [19]. The data are presented as fold increase/decrease in vaccinated group compared to saline group at each sample time point. Changes in the threshold cycles (ΔCt) value were calculated as differences between the gene of interest and reference gene, APR, for each sample. ΔΔCt values were calculated as differences between the ΔCt of the samples in the groups of interest and the average ΔCt of the reference group. Fold change was calculated as $2^{-\Delta\Delta Ct}$ and considered as up-regulation if greater than 1 and down-regulation if less than 1. The degree of down-regulation was calculated as negative reciprocal fold. Minimum 2-fold regulations were considered substantial and differences between the groups were tested with 2-tailed *t*-test ($p < 0.05$). Regulation was considered significant if both requirements were fulfilled. Four genes had groups with low or absent expression resulting in a low number of valid Ct-values (< 3). This was the case for liver samples in the saline group. Thus, quantitative assessment was not feasible and only qualitative assessment was performed.

3. Results

3.1. Challenge experiments

The highest cumulative mortality after challenge with *A. salmonicida* was recorded in the saline group (60.6%). Mortalities in the experimental vaccine group (3.2%) and in the commercial vaccine group (3.3%) were significantly lower compared to the saline group (Fig. 1A)

The cumulative mortality after challenge with *Y. ruckeri* was highest in the saline group (76.7% for biotype 1 and 81.3% for biotype 2) followed by the commercial vaccine group (46.9% for biotype 1 and 80% for biotype 2). No mortalities following *Y. ruckeri* challenge were recorded in the experimental vaccine group (Fig. 1B and C).

The cumulative mortality after the challenge with *V.anguillarum* serotype O1 was highest in the saline group (63.6%) and significantly lower in the experimental and commercial vaccine groups (3.03% and 0%, respectively) (Fig. 1D). The mortality after the challenge with *V.anguillarum* serotype O2a was also highest in the saline group (87.9%), while the mortality in the commercial vaccine group and in the experimental vaccine group was 54.5% and 27.3%, respectively (Fig. 1E).

3.2. Antibody reactivity

3.2.1. 8 Weeks post-vaccination

Antibody reactivity in sera of fish sampled 8 wpv is presented in Fig. 2A and indicates that fish in the experimental vaccine group had elevated serum (diluted 1:1000) antibody reactivity when compared to the saline group (significant for both biotypes of *Y. ruckeri* and both serotypes of *V.anguillarum*). The reactivity against sonicated *A. salmonicida* bacteria in the experimental vaccine group was higher compared to the saline group, although not significantly. In the commercial vaccine group, the reactivity against sonicated *A. salmonicida* bacteria was significantly higher when compared to the saline group.

3.2.2. 26 Weeks post-vaccination

Antibody reactivity in sera of fish sampled at 26 wpv is presented in Fig. 2B. The experimental vaccine group had significantly elevated reactivity against all sonicated bacteria when compared to the saline group. In the commercial vaccine group, the antibody reactivity against sonicated bacteria was significantly higher for *A. salmonicida* and *V. anguillarum* serotype O1, when compared to the saline group.

3.3. Gene expression analysis

Significant regulations in gene-expressions are illustrated in Fig. 3.

In general, only minor regulations of immune gene expression were observed in response to vaccination. Thus, hereby we only mention the results of significantly regulated genes. The gene encoding serum amyloid A (SAA) was up-regulated in the spleen of both vaccinated fish groups. A corresponding expression pattern was also found for liver but was only significant for fish in the commercial vaccine group (Fig. 3A). The gene for the complement component C3 was significantly up-regulated in the liver of both experimental and commercial vaccine group fish (Fig. 3B).

Of the pro-inflammatory cytokines the gene encoding IL-1β was significantly up-regulated in the spleen of both groups of vaccinated fish, while regulation of the genes for IL-8 and IL-6 was less consistent (Fig. 3C–E).

Minor regulations were observed for *igds* and *mhcii*, suggesting up-regulation of IgD and MHC II in liver of fish given the experimental vaccine, while igm and igdm tended to be down-regulated in head kidney of fish given the commercial vaccine (Fig. 3F–I).

3.4. Serum protein measurement

Serum protein concentrations measured in fish from the different groups are presented in Table 2. The serum protein concentration was higher in fish vaccinated with the commercial vaccine when compared to other groups. The difference in serum protein levels between fish vaccinated with the experimental and the commercial vaccine was statistically significant at 26 wpv ($p < 0.019$).

Table 1
Primers and probes used for qPCR assays. All nucleotides are read from 5′ end (labeled with FAM) to 3' (labeled with BHQ1) end. All qPCR assays were optimized to use annealing temperature of 60 °C and have efficiencies of 100 ± 5%.

Gene GenBank acc.no.	Length Bp	Primers 5′end to 3′end	Probes 5′end to 3′end	References
arp	106	FWD: GAAAATCATCCAATTGCTGGATG	CTATCCCAAATGTTTCATTGTCGGCGC	[12]
AY505012		REV: CTTCCCACGCAAGGACAGA		
Igm	72	FWD: ACCCTCCTCTTGGTCGTTTC	TGATGACACCAACAGCAACA	[13]
S63348		REV: TGATGACACCAACAGCAACA		
igt	73	FWD: AGCACCAGGGTGAAACCA	AGCAAGACGACCTCCAAAACAGAAC	[13]
AY870265		REV: GCGGTGGGTTCAGAGTCA		
saa	79	FWD: GGGAGATGATTCAGGGTTCCA	TCGAGGACACGAGGACTCAGCA	[14]
AM422446		REV: TTACGTCCCCAGTGGTTAGC		
c3	85	FWD: ATTGGCCTGTCCAAAACACA	TGGAATCTGTGTGTCTGAACCCC	[15]
AF271080		REV: AGCTTCAGATCAAGGAAGAAGTTC		
il-1β	91	FWD: ACATTGCCAACCTCATCATCG	CATGGAGAGGTTAAAGGGTGGC	[13]
AJ223954		REV: TTGAGCAGGTCCTTGTCCTTG		
il-8	69	FWD: AGAATGTCAGCCAGCCTTGT	TTGTGCTCCTGGCCCTCCTGA	[16]
AJ279069		REV: TCTCAGACTCATCCCCTCAGT		
il-10	70	FWD: CGACTTTAAATCTCCCATCGAC	CATCGGAAACATCTTCCACGAGCT	[13]
AB118099		REV: GCATTGGACGATCTCTTTCTTC		
il-4/13a	138	FWD: ATCCTTCTCCTCTCTGTTGC	CGCACCGGCAGCATAGAAGT	[17]
AB574337		REV: GAGTGTGTGTGTATTGTCCTG		
cd4	89	FWD: CATTAGCCTGGGTGGTCAAT	CAGAAGAGAGAGCTGGATGTCTCCG	[16]
AY973028		REV: CCCTTTCTTTGACAGGGAGA		
cd8	74	FWD: CACCAATGACCACAACCATAGAG	ACCAGCTCTACAACTGCCAAGTCGTGC	[16]
AF178054		REV: GGGTCCACCTTTCCCACTTT		
tgf-β	75	FWD: TCTGAATGAGTGGCTGCAAG	CTGGAGAGGAGCAGGGATTCCAAT	[13]
X99303		REV: GGTTTCCCACAATCACAAGG		
tnf-α	75	FWD: GGGGACAAACTGTGGACTGA	GACCAATCGACTGACCGACGTGGA	[16]
AJ277604		REV: GAAGTTCTTGCCCTGCTCTG		
il-6	91	FWD: ACTCCCCTCTGTCACACACC	CCACTGTGCTGATAGGGCTGG	[16]
DQ866150		REV: GGCAGACAGGTCCTCCACTA		
mhci	73	FWD: TCCCTCCCTCAGTGTCT	CAGAAGACCCCCTCCTCTCCAGT	[14]
AY523661		REV: GGGTAGAAACCTGTAGCGTG		
mhcii	67	FWD: TGCCATGCTGATGTGCAG	CGCCTATGACTTCTACCCCAAACAAAT	[13]
AF115533		REV: GTCCCTCAGCCAGGTCACT		
il-17a/f2	158	FWD: TCAAAAGCAACGTGTCGAAG	TATGCTGCTGGGCCTGACCA	[18]
AJ580842		REV: TCCCTCTGATTCCTCTGTGG		
il-17c1	138	FWD: CTGGCGGTACAGCATCGATA	CGTGATGTCCGTGCCCTTTGACGATG	[17]
CAW30792		REV: GAGTTATATCCATAATCTTCGTATTCGGC		
il-17c2	134	FWD: CTGGCGGTACAGCATCGATA	CGTGGTGTCCAGGCCCTTTAATGATG	[17]
CAW30793		REV: CAGAGTTATATGCATGATGTTGGGC		
igds	120	FWD: GGCACGCCAGGATTTGAC	CCACACCACACAGACTCTGGCCCTGAA	[11]
JQ003979		REV: TCAGAATTGAGTGAACGGACAGACA		
igdm	304	FWD: CAGGAGGAAAGTTCGGCATCA	CCACACCACACAGACTCTGGCCCTGAA	[11]
AY870262		REV: CCTCAAGGAGCTCTGGTTTGGA		

4. Discussion

The Danish mariculture production is based on rainbow trout grown for 2 years in freshwater followed by one growth season in the sea. Although the fish in this type of production are vaccinated with licensed vaccines against the most prevalent bacterial pathogens, disease outbreaks involving these pathogens still occur. Currently, these outbreaks are treated with antibiotics, resulting in undesirably high consumption of antibiotics in both freshwater farming and in the marine rainbow trout production, particularly during warm summers [20]. Furthermore, diseased fish exhibit reduced growth negatively affecting the fish farmers' economy. Hence, improved vaccines and vaccination strategies optimized for Danish aquaculture are warranted.

Rainbow trout is immersion-vaccinated against ERM at the size of 5 g but when fish at freshwater farms grow beyond the fingerling stage, the immunity obtained is no longer sufficiently protective and ERM starts causing problems. Re-vaccination by i.p. injection, which is the most efficacious delivery method for larger sized fish [5,21] has been generally considered impractical and too expensive due to high labor costs. Rainbow trout for sea-rearing are vaccinated against vibriosis and furunculosis by i.p. injection with an oil-adjuvanted trivalent vaccine before they are transferred to the marine net pens. This vaccination is often performed one year before the transfer and some farmers simultaneous inject aqueous ERM bacterin vaccine together with oil-adjuvanted trivalent vibriosis and furunculosis vaccine. However, incorporating *Y. ruckeri* antigens into oil-adjuvanted vaccine as described in current study appears to be a more practical approach.

The insufficient ability of the existing vaccines to provide protection against furunculosis and vibriosis in Danish aquaculture might partly be due to the fact that these vaccines are based on foreign bacterial isolates, which could be antigenically different from the ones causing disease in Danish rainbow trout.

This study tested the protective potential of an experimental multicomponent vaccine developed specifically for Danish marine rainbow trout aquaculture. The bacterial strains included in the vaccine were isolated from disease outbreaks in Danish rainbow trout farms [3,22]. It was anticipated that the tailored nature of the vaccine components might provide superior protection compared to vaccines based on foreign isolates. Besides, including *Y. ruckeri* antigens in the vaccine and aiming at vaccinating the fish one year before sea transfer could extend the benefit of the injection vaccination into the fresh water phase of the production cycle.

The efficacy of the experimental vaccine including 5 inactivated bacterial strains was evaluated by conducting infection studies and by measuring antibody reactivity and regulation of gene expression in response to vaccination.

Laboratory scale challenge experiments demonstrated that vaccination of fish with experimental vaccine led to significantly higher

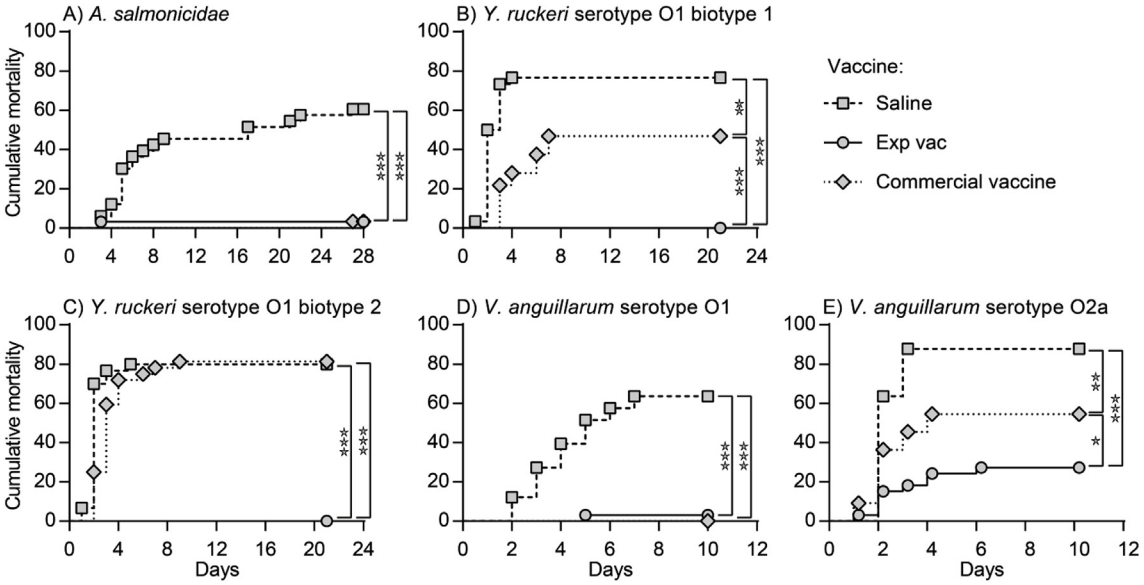

Fig. 1. Cumulative mortality in different groups of rainbow trout following: (A) tail fin puncture infection challenge with *A. salmonicida*, (B) i.p. infection challenge with *Y. ruckeri* serotype O1 biotype 1, (C) *Y. ruckeri* serotype O1 biotype 2, (D) *V. anguillarum* serotype O1 and (E) *V. anguillarum* serotype O2a. Asterisks (*) represent p values between groups (*p < 0.05, **p < 0.01, ***p < 0.001).

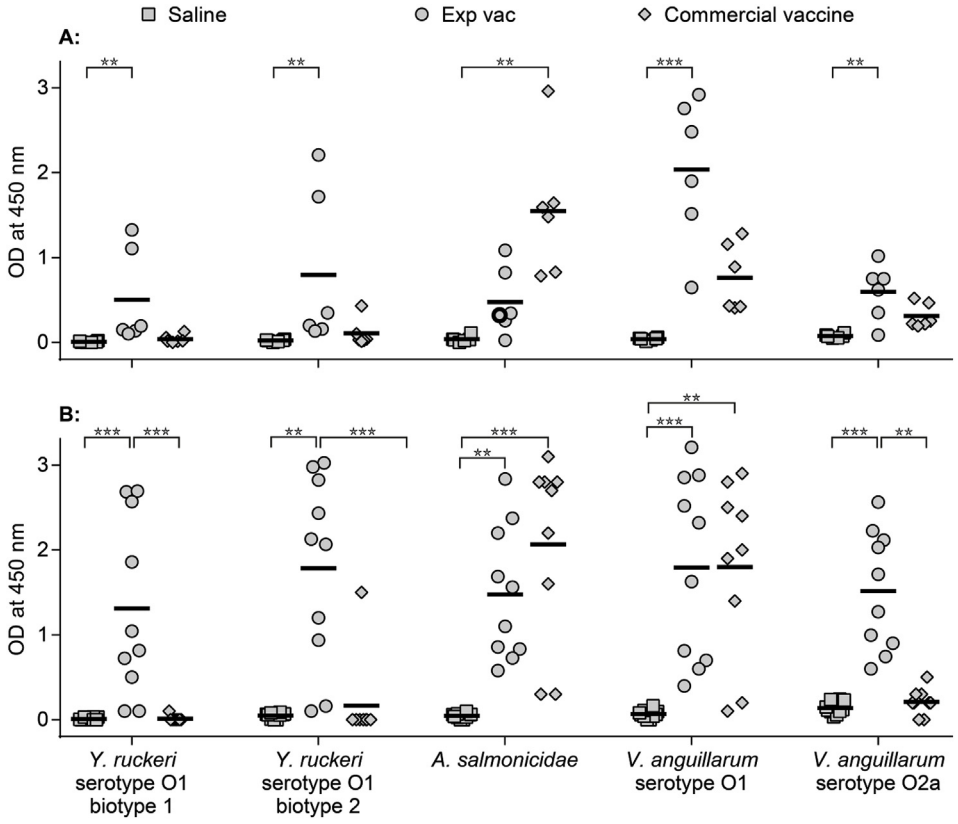

Fig. 2. Antibody reactivity in the sera (1:1000) of rainbow trout against sonicated bacteria measured at (A) 8 weeks post-vaccination and (B) 26 weeks post-vaccination. Asterisks (*) represent p values (*p < 0.05, **p < 0.01, ***p < 0.001) between groups.

survival for all tested pathogens when compared to the saline group. The challenge with *Aeromonas salmonicida* subsp. *salmonicida* showed that the experimental vaccine was as efficient in inducing immunity against furunculosis as the commercial vaccine. The ability of the commercial vaccine to induce high antibody response in vaccinated fish [23], as well as a positive correlation of antibody levels and survival in fish after *A. salmonicida* challenge [24,25] has been reported previously. However, several authors have also demonstrated that antibody levels raised against *A. salmonicida* are not always indicative of protective potential of the vaccine nor correspond to survival [26,27]. The commercial vaccine group had significantly higher UV 280 nm absorbance compared to the experimental vaccine group at 26 wpv. Further analyses are needed to determine whether this was related to a higher antibody concentration in this group, or to elevated levels of

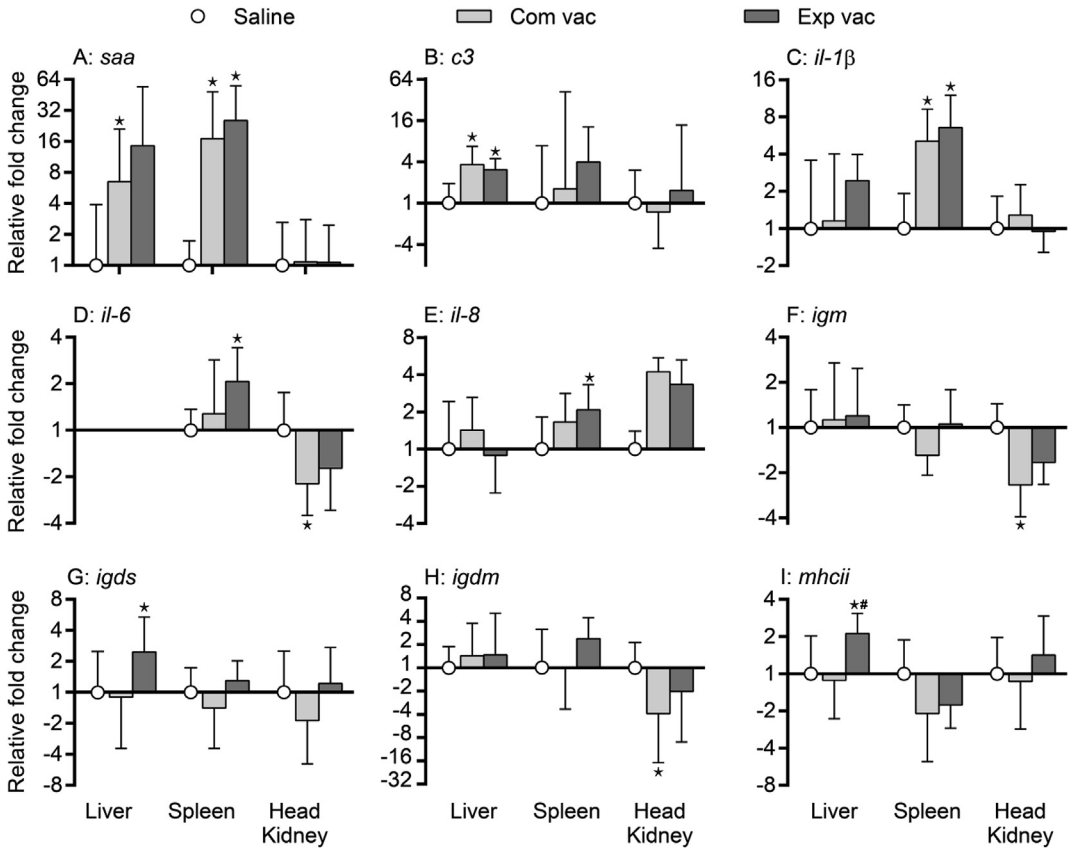

Fig. 3. Expression of significantly regulated genes in rainbow trout at 8 weeks post-vaccination (wpv). A) *saa*, B) *c3*, C) *il-1β*, D) *il-6*, E) *il-8*, F) *igm*, G) *igds*, H) *igdm*, I) *mhcii*. The results are presented as geometric means of folds compared to the saline group. Asterisks (*) and octothorpe (#) indicate significant ($p < 0.05$) differences to saline group and commercial vaccine group, respectively. Error lines represent geometric standard deviations.

Table 2
Average protein concentration in the serum of fish measured at 8 and 26 weeks post-vaccination (wpv). * Significant difference in serum protein levels between the experimental and the commercial vaccine groups.

Sampling time point	Group	Protein conc. mg/ml
8 wpi	Saline	32.8 ± 23.3
	Exp vac	41.8 ± 12.8
	Com vac	48.3 ± 6.6
26 wpi	Saline	40.5 ± 6.6
	Exp vac	37.8 ± 6.8
	Com vac	*46.2 ± 7.2

other serum components.

During the *Y. ruckeri* biotype 1 and 2 challenge trials, all fish vaccinated with the experimental vaccine survived, while saline group fish showed high morbidities in both cases. When fish vaccinated with the commercial vaccine, which does not include *Yersinia ruckeri* antigens in the formulation, were exposed to *Y. ruckeri* biotype 2, the morbidity was similar to that of the saline group, as expected. Surprisingly, when the challenge was performed with *Y. ruckeri* biotype 1, fish vaccinated with the commercial vaccine had significantly lower morbidity compared to the saline group (46.9% and 76.7% respectively), indicating that the commercial vaccine induced some protection against *Y. ruckeri* biotype 1.

Cross-protective effects of polyvalent vaccines have been reported previously. For example, partial but significant protection from *A. salmonicida* bacterin against challenge with *V. anguillarum* and *Y. ruckeri* (Hagerman strain) has been earlier observed by Amend and Johnson, 1984 [28]. Since some mineral oil-based adjuvants are known to elicit non-specific immunity [29], this component in the commercial vaccine, possibly in combination with immunostimulating pathogen associated molecular patterns (PAMPS) like lipopolysaccharides (LPS) from the included bacterins [30], might have triggered the non-specific protective mechanisms. It is especially noteworthy that no protection against *Y. ruckeri* biotype 2 was seen. This pathogen lacks flagellae and thereby flagellin whereas *V. anguillarum* and *Y. ruckeri* biotype 1 possess flagellae. This suggests that cross-reactivity could be due to flagellin on these bacteria.

Both the experimental and the commercial vaccine conferred high protection against vibriosis when the challenge was performed with *V. anguillarum* serotype O1. However, when the challenge was performed with the *V. anguillarum* serotype O2a isolate (homologous to the isolate in the experimental vaccine), superior protection of the experimental vaccine group was observed when compared to the commercial vaccine group. This probably reflects a high antigen variability among the *V. angullarum* O2a isolates derived from rainbow trout, as earlier reported for *V. angullarum* O2a isolates from Atlantic cod [31]. Thus, the protection against *V. anguillarum* O2a from the experimental vaccine was highly dependent on the bacterial isolate used for challenge (data not shown).

We examined the expression of immune-related genes in liver, spleen and head kidney and found the gene expression profile in vaccinated fish to be relatively unaltered which may be explained by the late sampling point post-vaccination in this study. It has been previously shown that the activation of gene expression after i.p. vaccination against ERM [13,32] and furunculosis [33] is rapid and peaks shortly after injection.

The most pronounced gene expression changes occurred for the gene encoding SAA in both spleen and liver of the vaccinated fish. In mammals SAA is produced in response to inflammatory cytokines [34] and has a role in binding and opsonizing Gram-negative bacteria for

macrophages and neutrophils [35]. In salmonids, SAA, expressed by hepatocytes [36] and several extrahepatic immune-relevant tissues, has similar functions [37,38]. Studies have shown that salmonid macrophages, in response to stimulus from bacterial LPS, secrete cytokines that trigger hepatocytes to produce SAA [36]. Thus, the SAA gene expression in salmonids is induced by cytokines such as IL-1β, IL-6 and TNF-α [32,36]. A significant up-regulation of *saa* gene in the spleen of vaccinated rainbow trout has been earlier observed following injection vaccination against ERM [32]. We also found the gene encoding complement component C3 to be up-regulated, which indicated an elevated production of C3 protein in the liver of vaccinated fish. Antibodies are activators of the classical complement pathway and the elevation of C3 expression and antibody levels in this study may reflect their joint involvement in development of immunity in the vaccinated fish.

For other examined immune genes changes in regulation were minor and in some cases observed exclusively in fish given the experimental vaccine e.g. the up-regulation of genes encoding IgDs, IL-1β, MHC II in the liver and IL-6, IL-8, IL-10 in the spleen. Whether these changes were related to the *Y. ruckeri* antigen in the vaccine remains to be examined.

In conclusion, we found that the experimental vaccine induced significantly lower morbidity in vaccinated fish with regard to furunculosis, enteric redmouth disease and vibrosis compared to the saline control group. All vaccinated fish developed antibody reactivity against 5 bacterial isolates incorporated in the vaccine. On top of the efficient protection against ERM, the protection levels against furunculosis and vibriosis induced by the vaccine were equivalent to those seen for fish given the commercial trivalent vaccine, altogether suggesting that the experimental pentavalent vaccine could meet the demand for custom made multivalent vaccine for Danish aquaculture.

Although the protective effect of the experimental vaccine was documented by challenge trials conducted 7–12 weeks post-vaccination, no superior effect against *A. salmonicida* or *V. anguillarum* O1 could be detected when compared to the commercial vaccine. Additional testing of the long-lasting protection as well as testing under field conditions is still needed to fully evaluate the applied potential of the vaccine.

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