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Immersion vaccines against Yersinia ruckeri infection in rainbow trout: Comparative effects of strain differences

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Abstract
The protective effects of autogenous and commercial ERM immersion vaccines (bacterins based on Yersinia ruckeri, serotype O1, biotypes 1 and 2) for rainbow trout (Oncorhynchus mykiss) were compared in order to evaluate whether the use of local pathogen strains for immunization can improve protection. In addition, the effect of the bacterin concentration was established for the commercial product. Following sublethal challenge of vaccinated and non-vaccinated control fish with live bacteria, we followed the bacterial count in the fish (gills, liver and spleen). The expression of genes encoding immune factors (IL-1β, IL-6, IL-8, IL-10, IFN-γ, MHCI, MHCII, CD4, CD8, TCRβ, IgM, IgT, IgD, cathelicidins 1 and 2, SAA and C3) and densities of immune cells in organs were recorded. Both vaccines conferred protection as judged from the reduced bacterial load in exposed fish. Innate immune genes were upregulated in all groups following bacterial challenge but significantly more in non-vaccinated naive fish in which densities of SAA-positive immune cells increased. Immunoglobulin genes were upregulated on day 5 post-challenge, and fish vaccinated with the high commercial bacterin dosage showed increased IgM levels by ELISA on day 14 post-challenge, reflecting that the vaccine dosage was correlated to protection. In conclusion, both vaccine types offered protection to rainbow trout when exposed to live Y. ruckeri and no significant difference between commercial and autogenous vaccines was established.

KEYWORDS
antigen, immunity, rainbow trout, vaccine, Yersinia

1 | INTRODUCTION

Yersiniosis is a globally distributed disease of mainly freshwater fish, causing a high level of mortality and significant economic losses in aquacultures (Ummey et al., 2021). The disease is also termed enteric redmouth disease (ERM) due to the specific clinical signs of subcutaneous haemorrhages in and around the mouth and throat in infected fish (Ross et al., 1966; Warren, 1983; Zorriehzahra et al., 2017). The
aetiological agent of ERM is a Gram-negative rod-shaped facultative intracellular entero bacterium, *Yersinia ruckeri*, which was first isolated in rainbow trout, *Oncorhynchus mykiss*, in the United States in 1956 (Ross et al., 1966). It is highly contagious to rainbow trout of all age classes, and since the first outbreak in central Jutland in 1983, *Y. ruckeri* has spread rapidly in most of the rainbow trout farms in Denmark (Dalsgaard et al., 1984). The bacteria spread with host faeces and survive in water, sediments and biofilms for months, and even surviving fish without signs may serve as carriers, emphasizing that control methods are indispensable in infected systems (Coquet et al., 2002; Valtonen et al., 1992; Willumsen, 1989). Vaccines, immunostimulants, prebiotics and probiotics have been shown to confer some protection against *Y. ruckeri* (Adel et al., 2020; Austin et al., 2003; Bilen et al., 2020; Medina et al., 2020; Ohtani et al., 2019; Rashidian et al., 2020; Yamaguchi et al., 2019; Yilmaz et al., 2020). Among these approaches, vaccination is the most successful strategy providing a high relative percentage of survival (RPS) and the protection may be promoted through optimization of administration methods, water temperature, booster vaccinants and adjuvants (Chettiri et al., 2013; Jaafar et al., 2015; Raida & Buchmann, 2008b; Skov et al., 2018; Soltani et al., 2014; Yamaguchi et al., 2019). So far, six O-serotypes, five outer membrane protein types and two biotypes have been described in *Y. ruckeri*, among which serotype O1 and the two predominant associated biotypes (BT 1 and 2) are recovered from rainbow trout (Austin et al., 2003; Davies, 1990; Davies & Frerichs, 1989; Ormsby et al., 2016). The O antigen is the dominant immunogenic molecule involved in protection against ERM, but distinct phenotypic and antigenic differences exist between *Y. ruckeri* BT 1 and BT 2. This may have implications for the protection of farmed fish according to changes in the prevailing biotypes in aquaculture systems. This was demonstrated by a comparative study on vaccines containing either BT 1 and 2 or merely BT 1 (Tinsley et al., 2011). However, the geographical diversity, environmental changes and the selective pressure generated by continuous immunization may lead to the evolvement of strain differences. This may result in inferior vaccination results as the vaccine strains then differ from the prevailing pathogens in a certain region (Wheeler et al., 2009). With a view to improving the current vaccine used in Denmark, we have compared the protection conferred by a commercial AquaVac® RELERA™ to an autogenous vaccine based on two Danish isolates, both occurring in Danish trout farms. In addition, we elucidated whether the concentration of the vaccine AquaVac® RELERA™ affected protection examining the general notion that vaccine concentration influences the protective status of the fish (Marana et al., 2017; Tatner, 1987; Yamaguchi et al., 2019). The protection levels afforded by the bacterins were assessed following vaccination when we challenged fish with live *Y. ruckeri* and followed the pathogen load in organs (gills, spleen and liver). Further, the host response, reflecting the infection level, was recorded by measuring immune cell densities and immune gene expression in the fish organs together with serum antibody titres. We applied a low bacterin concentration for immersion vaccination in order to detect minor differences in host response mechanisms, if any. In addition, we used a low concentration of bacteria for challenge to reach a sublethal infection whereby all experimental fish could be sampled equally as no fish were lost to the disease. This also secured that fish death could not be used as a humane endpoint.

## 2 | MATERIALS AND METHODS

### 2.1 | Fish

Rainbow trout eyed eggs (certified virus-free) from the Fårup Mølle farm (Jutland) were delivered to the Bornholm Salmon Hatchery (Nexø, Denmark) on 6 September 2019, where eggs were disinfected, incubated (7°C) in trays and subsequently hatched between 13 September and 21 September 2019. The system is disease-free and restarted for each hatching after disinfection following each production cycle. After hatching, the fish were reared 60 days at 13°C (780 degree days) to a body size of 4.8–5.0 g, whereafter the fish were vaccinated as described below. They were then reared 56 days at 13°C (728 degree days) at the hatchery to a body size of 13–14 g, whereafter they were transported to the fish infection facility at the University of Copenhagen. Before challenge, the fish were acclimatized for 7 days at 15°C, and after challenge, the fish were kept at this temperature until termination of the study.

### 2.2 | Vaccines

#### 2.2.1 | Commercial vaccine

The commonly used commercial vaccine is AquaVac® RELERA™ (MSD Animal Health), which contains *Y. ruckeri* serotype O1, biotype 1 (5 x 10^9 cells/ml) and biotype 2 (ES5) (5 x 10^9 cells/ml). The bacterin was diluted 1:10 (high dosage, Com H) and 1:100 (low dosage, Com L) and used for immersion vaccination (high and low dosage applied).

#### 2.2.2 | Autogenous vaccine (AutoVac)

The autovaccine was based on formalin-inactivated Danish isolates of *Y. ruckeri* biotype 1 (isolate 910926-2/2, 5 x 10^9 cells/ml) and biotype 2 (isolate 190620-1/1, 5 x 10^9 cells/ml). Origin of isolates and procedures for formalin inactivation was previously described (Jaafar et al., 2015). The bacterin was diluted 1:10 for immersion vaccination whereby only a low dosage corresponding to the low-dosage commercial vaccine (Com L) was applied for the autogenous vaccine type.

### 2.3 | Bacteria for challenge

*Yersinia ruckeri* (isolate 100415-1/4, serotype O1 and biotype 2) was recovered from an outbreak of ERM in a freshwater trout farm in...
Denmark. The bacteria were inoculated on LB culture medium and incubated in an orbital shaker for 48 h at 20 ± 2°C. Serial 10-fold dilutions with sterile phosphate-buffered saline (PBS) were applied to determine the concentration of Y. ruckeri (1 × 10⁹ CFU/ml).

2.4 | Experimental groups and vaccination

Fish of body weight 4.8–5 g were confirmed free of infection before experimentation by examining five fish with standard bacteriological and parasitological examination (Buchmann, 2007). A total of 1250 fish were divided into five groups (250 fish per group), which were separately subdivided into duplicates (125 fish per subgroup) whereby the fish were kept in a total of ten fish tanks: Group I (unchallenged and non-vaccinated)—fish were neither vaccinated nor challenged; Group II (challenged and non-vaccinated)—fish were not vaccinated but later challenged; Group III (Com H): fish were immersed into a high concentration of the commercial bacterin (1 × 10⁹ CFU/ml) for 30 s (recommended by the manufacturer MSD) and later challenged; Group IV (Com L)—fish were immersed into a low concentration of the commercial bacterin (1 × 10⁸ CFU/ml) for 3 min (to allow uptake of antigen) and later challenged; and Group V (AutoVac)—fish were immersed into the autogenous bacterin (1 × 10⁸ CFU/ml) for 3 min and later challenged. No mortality among fish was observed during or after vaccination.

2.5 | Challenge and sampling

At day 56 post-vaccination, all experimental fish (average body weight 13.7 ± 3.2 g) were transported to the fish keeping facility at the University of Copenhagen, Denmark, and kept in 10 glass tanks (total volume 150 L). The 4 groups of fish (8 tanks), used for challenge with Y. ruckeri, were placed in an infected room. Unchallenged control fish were kept in two similar tanks in an uninfected temperature-controlled room. Following the 7-days acclimatization, fish were exposed to live Y. ruckeri on day 62 post-vaccination (dpv). The water content was lowered to 30 L in each tank, whereafter a 200 ml culture of Y. ruckeri serotype O1 and biotype 2 (isolate 100415-1/4) was added to reach a concentration of approximately 7 × 10⁶ CFU/ml. Exposure time was 6 h, whereafter water was filled into each tank to reach 150 L. Control fish were treated similarly except for adding live bacteria to the tanks.

Fish were monitored for the occurrence of clinical signs every second hour around the clock from day 1 to day 14 post-challenge (dpc) according to the ethical rules of the infection facility (death is not accepted as a humane endpoint). At 2 dpc, two fish from each challenged group were killed to verify Y. ruckeri infection with the specific pathogen (inoculation of headkidney smears on blood agar). The infection levels in all sampled fish were further determined by quantitative real-time PCR (see Sections 2.8 and 3.2).

Blood samples (caudal vein puncture) from five fish per tank (ten fish per group) were taken one day before challenge and then at 8 and 14 dpc. Blood samples were kept at 4°C overnight, whereafter serum was separated by centrifugation (3000 g, 10 min, 4°C) and then stored at −80°C until being processed for ELISA.

Samples for immunohistochemistry (gills, liver, spleen, intestine, skin and muscle) were recovered one day before challenge and then at 1 and 5 dpc (ten fish per group) (Table 1). The samples were fixed in 4% neutral formaldehyde (10% formalin) and after 24 h stored in 70% ethanol at 4°C until processing.

Samples for gene expression (gills, liver and spleen) were taken 1 and 5 dpc from four fish per tank (eight fish per group) (Table 1), fixed in RNAlater (Sigma-Aldrich, Cat. No. R0901), placed at 4°C for 24 h and then stored at −20°C until further processing.

2.6 | Enzyme-linked immunosorbent assay (ELISA)

The level of serum immunoglobulin M (IgM) specific to Y. ruckeri was measured in sera by ELISA (Raida et al., 2011). Briefly, 96-well microplates were coated with 100 µl sonicated Y. ruckeri (isolate 100415-1/4, 5 µg/ml protein concentration) in bicarbonate coating buffer (Sigma-Aldrich, Cat. No. C3041). The coating was followed by blocking, using 2% bovine serum albumin (BSA) for 1 h at room temperature and then rinsed with washing buffer (PBS/Tween). Sera (100 µl, diluted 1:50) were added to duplicate wells and incubated for 12 h at 4°C. Mouse anti-salmonid IgM (dilution 1:500; AbD Serotec, Cat. No. MCA2182, Bio-Rad) and rabbit anti-mouse IgG polyclonal HRP-conjugated antibody (dilution 1:500; AbD Serotec, Cat. No. STAR13B, Bio-Rad) were applied as primary and secondary antibodies, respectively. After washing, 100 µl of TMB PLUS Substrate (AbD Serotec, Cat. No. BUF042B, Bio-Rad) was added. The reaction was stopped after 15 min with 100 µl 1N HCl and absorbance measured at 450 nm using an Epoch Spectrophotometer (Biotek, Holm & Halby). Internal positive and negative control samples were included in all assay plates. The relative OD values were calculated by the formula: Relative OD450 nm = (ODsample − ODnegative) × (average of ODpositive of all plates)/ODnegative.

2.7 | Immunohistochemistry (IHC)

The formalin-fixed tissue samples were dehydrated with graded EtOH series, which subsequently were cleared in Shandon Xylene
Substitute (Cat. No. 10524305, Fisher Scientific), embedded in paraffin blocks and sectioned at 3 μm. Sections were processed following the instructions of UltraVision Quanto Detection System HRP Kit (Cat. No. TL-125-QHL, AH diagnostics as). In brief, after deparaffinization, rehydration, quenching of endogenous peroxidase and antigen retrieval, tissues were blocked in 2% BSA in Tris-buffered saline (Cat. No. 524750-1EA, VWR, Bie & Bernten A/S) (TBS, pH 7.6) for 10 min at room temperature. Slides were incubated overnight with primary monoclonal antibodies (Table 1) at 4°C. Slides were washed in TBS and successively incubated with Primary Antibody Amplifier Quanto and HRP Polymer Quanto. Bound antibodies were visualized using the AEC substrate Chromogen Kit (Cat. No. TA-060-SA, AH diagnostics as) according to the manufacturer’s recommendations. Finally, slides were counter-stained with Mayer’s haematoxylin (Cat. No. 1.085.620.050, VWR) and mounted in Aquatex (Merck). Positive cells were counted in three random fields per sample.

Photographs of all areas were captured with Leica CD300 (Leica Microsystems). The areas of the visual fields at ×200 and ×400 were 0.1032 mm² and 0.4128 mm², respectively.

2.8 | Quantitative reverse transcription–PCR (RT-qPCR)

RNA later fixed tissues were subjected to RNA extraction and cDNA synthesis (Karami et al., 2018). In brief, tissues were homogenized and pretreated (only liver and gills) with Proteinase K, whereafter RNA was purified using GenElute™ Mammalian RNA Kit (Sigma-Aldrich, Cat. No. RTN 350). The RNA was further processed with DNase I (Sigma-Aldrich, Cat. No. AMP-D1) to remove genomic DNA, and the concentration was measured with NanoDrop 2000 spectrophotometer (Thermo Scientific). A total of 1000 ng RNA was used to synthesize cDNA in a 20 μl reaction volume with random hexamers and oligo-d(T)16 using TaqMan™ Reverse Transcription Reagent Kit (Cat. No. N8080234, Thermo Fisher Scientific). Finally, the cDNA was diluted to the working concentration at a ratio of 1:10 in DNase/RNase-free water (Cat. No. EN0521, Thermo Fisher Scientific).

Analysis of gene transcription was conducted using an AriaMx Real-time PCR Machine (AH Diagnostics) using specific primers and probes (Table 2) (Gibello et al., 1999; Marana et al., 2019; Zuo et al., 2020). Briefly, a sample setup (12.5 μl) was composed of 2.75 μl RNase-free water, 2.5 μl cDNA, 1.0 μl primer mixture (10 μM forward primer, 10 μM reverse primer and 5 μM probe) and 6.25 μl Brilliant III Ultra-Fast QPCR Master Mix (Cat. No. 600883, AH diagnostics as). The cycling conditions were as follows: pre-denaturation at 95°C for 3 min, then 40 cycles of denaturation at 95°C for 5 s and one-step annealing and elongation at 60°C for 10 s with endpoint measurement, and the lid was kept at 108°C. Relative gene expression data were analysed using the 2−ΔΔCq method (Livak & Schmittgen, 2001). The average of 3 reference genes (ARP, ELF1α and β-actin) was used as the internal calibrator, which was the best combination according to NormFinder (Andersen et al., 2004), and geometric means were calculated.

2.9 | Ethics

All experiments were performed under the licence 2019-15-0201-01614 obtained from the Experimental Animal Inspectorate, Committee for Experimental Animals, Ministry of Environment and Food, Denmark. Ethical guidelines of the University of Copenhagen were followed. Fish were monitored every second hour around the clock until 14 dpc, securing that any fish showing severe disease signs (moribund fish, with signs including loss of equilibrium, irregular swimming, haemorrhages and discoloration) could be removed from the experiment and killed by an overdose of MS222 (300 mg/L) (Zuo et al., 2020). Fish for sampling of blood and organs were also killed similarly before sampling.

2.10 | Statistical analysis

All statistical tests were performed using GraphPad Prism 8.0.2. Mortality was analysed by the Kaplan–Meier test and the log-rank test. The relative OD450 nm values from the ELISA were used to present the IgM data and were tested using one-way ANOVA with Dunnett’s multiple comparisons test. A 5% probability level was applied whereby p < .05 was considered as a significant difference. The number of IHC-positive cells per mm² reflected the expression of the related proteins, and differences between groups were tested by one-way ANOVA with Dunnett’s multiple comparisons test. Gene transcription levels were measured by qPCR and calculated according to Livak and Schmittgen (2001). Folds were calculated relative to the uninfected non-vaccinated group. Groups were compared by the one-way ANOVA with Dunnett’s multiple comparisons test. The data were visualized in diagrams showing geometric means with geometric standard deviation. Differences between groups were considered significant only when p < .05, and expression showed at least twofold of geometric mean changes (Zuo et al., 2020). Molecular detection of Y. ruckeri 16S by qPCR was based on the presence or absence of Cq values, and differences between groups were compared by the Kruskal–Wallis test and Dunn’s multiple comparisons test. This was supplemented by performing a chi-square test with a 5% probability level.

3 | RESULTS

3.1 | Clinical signs

Yersinia ruckeri was recovered successfully from fish randomly sampled in all challenged groups. Headkidney smears on blood agar
| Gene        | Oligonucleotide(5′−3′)                                                                 | GenBank accession no. | Length (bp) |
|-------------|---------------------------------------------------------------------------------------|-----------------------|-------------|
| ARP         | Fwd GAAAATCATCCAATTGCTGGATG<br>Rev CTTCCCCAGCAGAAGGACAGA<br>Probe CTATCCAAATGGTTTACAGGGGCG<br> | AY505012              | 106         |
| ELF-1α      | Fwd ACCCTTCCTCTGGTCTGTTTCC<br>Rev TGATGACACCAACACAGCA<br>Probe GCTTGGCAGATGAGGTCG<br> | AF498320              | 63          |
| β-actin     | Fwd ACATCGAGAGAAGGCTGCTAC<br>Rev TACGGATGTCAGTGTCACC<br>Probe CTCCTCTAGGAAAGGAGCTG<br> | AB196465              | 241         |
| IL-1β       | Fwd ACATTGGCAAATCATCATCG<br>Rev TGATGACACCAACACAGCA<br>Probe CTCCTCTAGGAAAGGAGCTG<br> | AJ223954              | 91          |
| IL-6α       | Fwd ACTCTCCTGTGACACACC<br>Rev GGGGACAGGATGTCAGGGTCTAC<br>Probe CGCTTGGCATCAGAGGTC<br> | DQ866150              | 91          |
| IL-8 isoform α-e | Fwd AGAATGTCAGCGAGCTTCTTG<br>Rev TCTCAGACTCATCCTCTCT<br>Probe TTGTGCTCTGCGTCCTCTGA<br> | AF16098(2−6)          | 69          |
| IL-10α      | Fwd CGACTTTTAAATCCTCCATCG<br>Rev GCATGACAGATCTCCTTCTT<br>Probe CATCGGAAATCATCCTCCAGCT<br> | AB118099              | 70          |
| MHC I       | Fwd TCTCCCTCTGCTGCTG<br>Rev GGGTAGA AACACACAGG<br>Probe CAGAAGACCCCTCCCTCAGT<br> | AF523661              | 73          |
| MHC II β    | Fwd TGCCATGCTGATGTCAG<br>Rev GTCCCTGACCCAGCAGTCT<br>Probe CTCGGAAAATCATCCTCCAGCT<br> | AF115533              | 68          |
| CD4         | Fwd CATTAGGGTGGTGTCAT<br>Rev CCCTTCCTGGAGAG<br>Probe CAGAAGGAGCATCTGCAGT<br> | AY973028              | 89          |
| CD8a        | Fwd ACACCAATAGCCAAACCAGTAGAG<br>Rev GGTCACACCTTCTACTTCT<br>Probe ACCAGCTTACAAATGCAGAAGCTG<br> | AF178054              | 74          |
| IgDm        | Fwd CAGGAGAGGTCAGGGCAG<br>Rev CACACCAAGGATGTCAG<br>Probe CACACCAAGGATGTCAGG<br> | AY870622              | 304         |
| IgDs        | Fwd TGCCAGGGAAGGTCAGGGCA<br>Rev TCAAGCCTGAAGACCACT<br>Probe CACCCACACAGACTCTGGAGC<br> | JQ003979              | 120         |
| IgMb        | Fwd CTTGGCTTGGTGACAGATGAG<br>Rev TGCTAGTTGTGTAAGTCT<br>Probe TGGAGAAGCAGGACGATCAG<br> | AH014877 Membrane S63348 Secreted | 72          |
| Ig7b        | Fwd AGCACCAGGGTAAACAG<br>Rev GGTCACACCTTCTACTTCT<br>Probe ACCAGCTTACAAATGCAGAAGCTG<br> | AY870265 Membrane Y870263 Secreted | 73          |
| C3          | Fwd ATGGCTCTGCTGCGACAC<br>Rev AGCGAGTCAGCGAGGAC<br>Probe TGGAGAAGCAGGACGATCAG<br> | AF271080 / U61753     | 85          |
| Cathelicidin 1a | Fwd TCTCTCGTCTGGGGTT<br>Rev GTCTAGCTGGTCTGATCTATG<br>Probe TAATGGCTGCTCTGGGGTGG<br> | AY382478              | 189         |

(Continues)
confirmed identity of the pathogen in the fish at this early time point after challenge. No mortality was recorded, but clinical signs were observed for the first time in non-vaccinated fish 2 dpc, when the fish presented light haemorrhages around the anus and at the base of dorsal fins. Thereafter, these fish recovered gradually with merely few clinical signs at 7 dpc and at 14 dpc, and no signs were recorded in any of the groups.

3.2 | Infection recorded as 16S rRNA of *Y. ruckeri*

The occurrence of the pathogen in fish from the different groups was monitored by measuring the presence or non-presence of 16S transcripts in different organs. The Kruskal–Wallis analysis showed that at 1 dpc, only fish vaccinated by the high commercial vaccine dosage harboured a significantly lower infection, and only in the liver (Figure 1).
Five days after challenge, the spleen of vaccinated fish (all groups) showed a lower infection than the non-vaccinated and challenged fish at this time point. At this late time point, the groups vaccinated with the high-dosage commercial vaccine and the autogenous low-dosage vaccine showed a lower infection than the non-vaccinated fish in gills and liver (Figure 1). When analysed by use of a chi-square test, no difference between groups was found at 1 dpc, whereas at 5 dpc, all organs in all vaccinated groups carried a significantly lower bacterial load than those in the non-vaccinated and challenged fish.

3.3 | OD value of IgM specific to Y. ruckeri

No significant IgM-level differences between groups were recorded prechallenge and at 8 dpc, where the antibody level was slightly but non-significantly lower than the time point controls. At 14 dpc, a marked significant increase was recorded in the Com H fish (Figure 2).

3.4 | Immune cells visualized by IHC

The location and number of cells exhibiting different epitopes (six immune-related proteins) were recorded in organs of naive and vaccinated fish before Y. ruckeri challenge and on 1 and 5 dpc. Of all the investigated organs, mainly gills, spleen and liver showed marked reactions, followed by the intestine, whereas the skin and muscle were the least affected. The details are presented below (Table 3 and Figures S1 and S2).

3.4.1 | CD8α+ cells

The CD8α+ cells occurred in higher densities in the spleen than in other organs. Also, sections of the intestine and gills showed clear presence of these cells, whereas only a few were seen in the skin, liver and muscle (Figure S1). When compared to the uninfected and non-vaccinated fish, significant increases in CD8α+ cells were observed in the gills and intestine of the Com H group before infection (Figure S1). No significant differences between groups were found post-infection (Table 3).

3.4.2 | MHCII+ cells

In general, the MHCII+ cells were mainly located in the spleen and gills, some in the intestine, liver and skin, but merely a few in the muscle. Before infection, the MHCII+ cells were more abundant in the vaccinated fish represented by the AutoVac group (spleen and gills), Com L group (gills and skin) and Com H group (spleen) (Figure S1). At 1 dpc, a significant decrease in MHCII+ cells was found in the non-vaccinated group (gills, intestine, liver and spleen) and the AutoVac group (gills). On 5 dpc, the MHCII+ cell occurrence was reduced markedly in the AutoVac group (gills, intestine, liver and spleen) and Com H group (liver) (Figure S1).

3.4.3 | IgM+ cells

The IgM+ cells were primarily located in the spleen of uninfected and non-vaccinated fish. Post-vaccination, the IgM+ cells increased significantly in the spleen, gills, intestine and liver of all vaccinated groups and especially in the skin of the Com H group (Figure S1). The density of IgM+ cells showed no significant differences to that of the unhandled group 1 dpc, and at 5 dpc, only the non-vaccinated group exhibited significant increases in IgM+ cells in the gills and liver (Table 3).

3.4.4 | IgT+ cells

The occurrence of IgT+ cells in the gills and spleen was more clear than in the other organs, but generally, vaccination did not affect their distribution and quantity, except for a decline in the spleen of Com H fish at 5 dpc (Figure S1, Table 3).

3.4.5 | IgD+ cells

The antibody applied in IHC for recognition of IgD recognized both the membrane-bound and secreted forms of the IgD. The positive cells were primarily located in the spleen followed by the gills, then the intestine, liver, skin and muscle. A significant increase in IgD+ cell density was recorded in vaccinated fish including AutoVac (gills, muscle and skin), Com L (gills and skin) and Com H (gills and spleen) (Figure S1, Table 3). At 1 dpc, IgD+ cell density showed no obvious changes in the Com H group, but a marked increase occurred in the unvaccinated group (gills, intestine, liver, skin and spleen), AutoVac
group (gills, intestine, liver and skin) and Com L group (gills and intestine) (Table 3). The IgD+ cell involvement decreased at 5 dpc where only the Com L group showed a significant increase in IgD+ cells in the gills, intestine, liver and skin compared with other groups (Figure S1, Table 3).

### 3.4.6 | SAA+ cells

Before challenge, SAA+ cells remained at a very low level in all organs in all groups. After challenge (1 dpc), a significant elevation of SAA cell density was recorded in the non-vaccinated group (liver and spleen) and Com L group (spleen). At 5dpc, SAA cells were merely noted in non-vaccinates (gills, intestine, liver, muscle and spleen) (Figure S1).

### 3.5 | Immune gene expression

Genes associated with innate responses (proinflammatory cytokines, SAA and cathelicidins) were generally the first to be activated (1 dpc), but at later time points (5 dpc), the expression also increased with regard to the genes encoding adaptive response molecules.

#### 3.5.1 | Cytokines

Non-vaccinated fish showed already at 1 dpc significant upregulation of genes in the gills (IL-1β, IL-6 and IL-10), spleen (IL-1β, IL-8 and IL-10) and liver (IL-8) (Figure 3). The groups receiving the commercial vaccine exhibited elevated cytokine expression (IL-6, IL-8 and IL-10) at this time point, whereas no transcription changes were found in the AutoVac group. At 5 dpc, the non-vaccinated group still presented a significant increase in genes encoding IL-1β, IL-6, IL-10 and IFN-γ in the gills, IL-1β and IL-8 in the liver and IL-1β, IL-6, IL-8 and IL-10 in the spleen. Also, the vaccinated fish reacted at this time point. Significant changes were seen for gills (IL-1β, IL-8, IL-10 and IFN-γ), liver (IL-1β, IL-8 and IL-10) and spleen (IL-1β, IL-6, IL-8 and IL-10).

#### 3.5.2 | C3

The gene encoding complement factor C3 was downregulated slightly in the gills of all infected groups 1 dpc, but on 5 dpc, the transcription was generally upregulated in all samples and with a significant increase in the spleen of Com L fish (Figure 3).

#### 3.5.3 | SAA

Transcription of the serum amyloid protein A (SAA) gene increased significantly 1 dpc in the non-vaccinated group (liver and spleen), and in the two groups vaccinated with the commercial bacterin (spleen). On 5 dpc, all organs in all groups showed elevated expression of this gene (Figure 3).

#### 3.5.4 | Cathelicidins 1 and 2

The genes encoding the antimicrobial peptide genes were generally highest regulated in the non-vaccinated fish (Figure 3). Cathelicidin...
FIGURE 3  A–C. Expression of immune related genes (A: cytokines and MHC, B: innate and adaptive effector molecules, C: adaptive effector molecules) in rainbow trout tissues sampled from experimental groups on 1 and 5 dpc. Bars and error lines represent geometrical means and geometrical standard deviations. ★: significantly different (p < .05), and at least 2-fold of geomean changes, when compared to uninfected non-vaccinated fish.
2 gene expression increased significantly at 1 dpc in the spleen of all groups except for the AutoVac group, but was elevated in all the groups at 5 dpc. Cathelicidin 1 gene expression was also increased at 5 dpc, but the elevation was only significant in the Com L group (gill and spleen) and in the non-vaccinated group (liver).

3.5.5 | TCRβ

Expression of the TCRβ gene, an indirect measure of T-cell activity, increased significantly at 5 dpc in the gills of all groups, except for the AutoVac group, but was significantly upregulated in the liver of AutoVac group and in the spleen of Com H group (Figure 3).

3.5.6 | MHC genes related to antigen presentation

The non-protected non-vaccinated fish presented an upregulation of the MHC1 gene in liver and the MHCII gene in gills at 5 dpc, whereas no significant upregulation was found in the other groups at any time point (Figure 3).

3.5.7 | CD4 and CD8

Upregulation of CD4 genes was recorded in all groups at 5 dpc. At this time point, only the non-vaccinated group and the AutoVac group presented an upregulation of the CD8 gene (liver only) (Figure 3).

3.5.8 | Immunoglobulin genes

Regulation of antibody encoding genes was recorded at 5 dpc when all groups showed a significant increase in IgDm gene expression in the gills. At this time point, the gene encoding IgM was also elevated in the gills of all the groups, but only significantly for non-vaccinated fish. In addition, the fish vaccinated with the commercial vaccine showed upregulation in the spleen of genes encoding IgDs, IgDm, IgM and IgT at this time point (Figure 3).

4 | DISCUSSION

Immersion vaccines based on formalin-killed bacterins (bacterins) confer some immunity to the fish against yersiniosis (ERM) caused by Y. ruckeri (Kumar et al., 2015; Yamaguchi et al., 2019) but disease outbreaks are reported despite prior vaccination (Austin et al., 2003). This suggests that optimization of vaccines and vaccination administration methods is needed. Booster immunization administered by various routes increases protection (Chettri et al., 2015; Schmidt et al., 2016), but it is worthwhile to investigate whether efficacy may be further improved by targeting the antigenic profile and the antigen concentration of the vaccines. Commercial vaccines are based on selected bacterial isolates from specific geographical regions, but their antigens may differ to some extent from strains in other locations (Ormsby et al., 2019). We have previously shown that injection vaccine efficacy is elevated when using Y. ruckeri isolated from Danish rainbow trout farms for antigen preparation (Jaafar et al., 2015; Marana et al., 2019). We therefore hypothesized that an autogenous immersion vaccine, based on local Danish bacterial isolates, could confer better specific immunity against local pathogens than the commercial vaccine currently in use. We tested the hypothesis by comparing the immersion protective potential of an autogenous vaccine and the commercially available vaccine and judged the protection afforded by the bacterial transcripts and immune reactions (qPCR and IHC) in the different vaccine groups. We also tested two antigen concentrations of the commercial vaccine for efficacy reference. This could also frame that the antigen concentration is a critical parameter to be considered (Chettri et al., 2015; Marana et al., 2017, 2020; Tatner, 1987). It is a common practice to evaluate the efficacies of vaccines by calculating RPS by challenging fish with a lethal pathogen dosage. However, to secure that all fish survived the infection and were included in the immunological study we applied a sublethal challenge, and therefore, RPS calculation is not included in the present work. We could here confirm that studies were conducted with other pathogens and formulations (Tatner, 1987; Yamaguchi et al., 2019) and that the antigen concentration is a decisive factor for conferring immunity by immersion vaccination—also for the bacterin containing two biotypes of Y. ruckeri. The bacterial infection, reflected by the number of positive fish with bacterial transcripts in different organs, was significantly reduced in fish immersion-vaccinated with a high bacterin concentration. However, we found no clear difference in efficacy between fish immersion-vaccinated, either by the autogenous or by the commercial immersion vaccines, when applied in a concentration of 1 × 10⁸ formalin-killed cells/ml. This may indicate that the antigenic differences between the geographical of the two biotype isolates are of minor importance when compared to biotype differences (Deshmukh et al., 2013).

It should be noted that the legislation in some countries does not allow the use of autogenous vaccines if licensed corresponding products are available.

Generally, an early infection is considered to induce a series of immune reactions, dominated by innate factors and correlated to the infection level. This was confirmed in the present work as the non-vaccinated fish were highly susceptible to infection and elicited a marked innate response. Densities of SAA- and MHCII-positive cells (putative macrophages) were elevated together with increased expression of inflammatory cytokines (IL-1β), IL-6 and IL-8) and the antimicrobial peptides cathelicidins 1 and 2 in the gill, spleen and liver. These factors are involved in chemotraction of immune cells to infection foci and direct elimination of bacteria and are thereby associated with the acute-phase response to invasion by pathogens (Deshmukh et al., 2013; Kania et al., 2014). The enumeration of immune cells in organs, especially SAA-positive cells, indicated how non-immunized fish recruited innate cells to the sites of pathogen...
exposure. The protective effects were therefore explored primarily by measuring the \( Y. \ ruckeri \) transcripts in the fish groups and secondarily by their responses to the challenge.

Adaptive response patterns were mainly seen in vaccinated fish. The elevated IgM titres measured by the pathogen-specific ELISA were a clear reflection of how the prior vaccination induced an adaptive response. Protective immunity against ERM, induced by immersion vaccination, has previously been found to be associated with an increase in IgM production in fish (Raida et al., 2011; Zuo et al., 2020), but the increased specific IgM titre is low until a marked elevation occurs at 14 dpc (Cossarini-dunier, 1986; Marana et al., 2019; Skov et al., 2012, 2018). This corresponds to the present study in which a significant IgM level was measured after 14 dpc in fish vaccinated with a high antigen concentration. In addition, the present study pointed to other immunoglobulin classes, such as IgT and IgD, as protective elements. IgT is generally considered to be associated with mucosal surfaces, but we showed that internal organs, such as spleen, harbour IgT+lymphocytes, supporting previous work showing elevated IgT-producing cells in the spleen of vaccinated rainbow trout (Schmidt et al., 2016). Correspondingly, IgD+cells increased their presence in vaccinated fish suggesting a protective role of this antibody class.

Previous studies suggested that the vaccinated fish are able to eliminate \( Y. \ ruckeri \) within a few days, while the non-vaccinated fish may harbour bacteria at least for a week (Deshmukh et al., 2013). We confirmed that \( Y. \ ruckeri \) occurrence decreased markedly in the vaccinated groups between 1 and 5 dpc, while the bacteria remained in all organs of the non-vaccinated fish. Shortly after live bacterial exposure, all gills from all fish groups became colonized by \( Y. \ ruckeri \). The gill is one of the important entrance portals of \( Y. \ ruckeri \), through which \( Y. \ ruckeri \) can invade the blood circulation within minutes (Ohtani et al., 2014; Tobback et al., 2009), and we noted invasion of internal organs already at 1 dpc. Also, the spleen (a well-known immune organ for pathogen capture and clearance) was targeted, and we subsequently recorded a clearance until 5 dpc in immunized fish, corroborating previous studies (Castro & Tafalla, 2015; Espenes et al., 1995) and demonstrating that both the commercial and autogenous vaccine induced immunity against \( Y. \ ruckeri \). The same picture was seen in the liver and even in the gills, suggesting that immunity operated both at the systemic and at the mucosal level. The involvement of cellular factors in immunological memory and its role in protection were clearly illustrated by Yamaguchi et al. (2019) performing adaptive transfer of cells from vaccinated to non-vaccinated clonal rainbow trout. In particular, lymphocytes (B and T cells) were able to home to central immune organs and secure protection following \( Y. \ ruckeri \) challenge. Gene expression analysis is another relevant approach for elucidation of the extent to which innate and adaptive factors are involved in protection of immunized fish (Harun et al., 2011; Raida & Buchmann, 2007, 2008b; Wiens & Vallejo, 2010; Zuo et al., 2020). In the present study, both naive fish and vaccinated fish survived the sublethal bacterial challenge, which offers a good opportunity to profile the early immune response in all vaccinated and non-vaccinated fish in a batch after infection. It was clear that innate immune reactions dominate the early infection phase of non-immunized fish as previously noted (Deshmukh et al., 2013; Harun et al., 2011; Raida & Buchmann, 2009). Naïve fish exposed to the live pathogen exhibited significant upregulation of inflammatory cytokines (such as IL-1β, IL-6, IL-8 and IL-10) and innate reactions (SAA and cathelicidins 1 and 2). The cytokine IL-10 is a regulatory factor, known to protect the host from the damage of excessive inflammation, and is therefore coexpressed with inflammatory cytokine genes (Harun et al., 2011; Raida & Buchmann, 2008b). Likewise, immune cells carrying the acute-phase reactant SAA (Jørgensen et al., 2000; Raida & Buchmann, 2009) dominated in organs of non-vaccinated fish after challenge. In contrast, vaccinated fish reacted less markedly with these innate factors presumably because the adaptive factors already offered some protection against the invasion. Thus, the expression of immune genes remained stable in the AutoVac group at 1 dpc. This suggests that these vaccines could induce the host to produce some antibacterial factors, which is able to prevent invasion and thereby subsequent inflammation. One of the protective factors is the production of \( Y. \ ruckeri \)-specific plasma IgM, but additional protective adaptive factors may contribute to protection. Relevant candidates are T cells and IgT+cells (Yamaguchi et al., 2019), indicated by the occurrence of CD8+ cells in the spleen of protected fish at early time points. However, on day 5 post-infection, several of the innate immune genes were still upregulated to some extent emphasizing that clearance of infections in rainbow trout is based on interactions between innate and adaptive factors until adaptive factors (CD8, TCR and Igs) become dominating at later phases.

5 CONCLUSION

Our results show that both types of immersion vaccines offered protection to rainbow trout when exposed to live \( Y. \ ruckeri \). Thus, no significant difference in efficacy between commercial and autogenous vaccines was found. Raising the antigen concentration of formalin-killed bacteria in the commercial bacterin increased the protection markedly, and in this context, the geographical strain differences are clearly of minor importance when performing immersion vaccination. Our approach of using a non-lethal challenge model and subsequently measuring the pathogen level in all fish has been suitable for elucidation of efficacy. When viewed from an ethical perspective, the method is less challenging as it reduces the usage of lethal methods. By applying a combination of immunohistochemical techniques for describing involvement of various types of immune cells in organs combined with detailed gene expression analyses, we were able to show the effect of vaccination on protection and immune response.

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CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest.

ETHICAL APPROVAL
All experiments were performed under the licence 2019–0201–01641 obtained from the Experimental Animal Inspectorate, Committee for Experimental Animals, Ministry of Environment and Food, Denmark. The study followed the ethical guidelines of the University of Copenhagen.

DATA AVAILABILITY STATEMENT
All data used and analysed in this article are available upon reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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