Received: 31 January 2023 | Revised: 1 March 2023 | Accepted: 5 March 2023

DOI: 10.1111/jfd.13782

Journal of Fish Diseases WILEY

RESEARCH ARTICLE

Immune profiling of rainbow trout (*Oncorhynchus mykiss*) exposed to *Lactococcus garvieae*: Evidence in asymptomatic versus symptomatic or vaccinated fish

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Funding information
Partnership for Research and Innovation in the Mediterranean Area

Abstract

Lactococcosis, caused by the Gram-positive bacterium *Lactococcus garvieae*, is a major concern in rainbow trout (*Oncorhynchus mykiss*) farms, which are regularly affected by outbreaks especially during the summer/fall months. In these farms, unvaccinated healthy and symptomatic fish can coexist with vaccinated fish. In the present study, innate (leukogram, serum lysozyme activity, peroxidase activity, antiprotease activity, bactericidal activity, total IgM and total proteins), and specific immune parameters (serum antibodies to *L. garvieae*) were assessed in unvaccinated adult rainbow trout naturally exposed to the pathogen, with or without evidence of clinical signs, or subjected to vaccination. Blood was drawn from all three groups, and blood smears were prepared. Bacteria were found in the blood smears of 70% of the symptomatic fish but not in any of the asymptomatic fish. Symptomatic fish showed lower blood lymphocytes and higher thrombocytes than asymptomatic fish ($p \leq .05$). Serum lysozyme and bactericidal activity did not vary substantially among groups; however, serum antiprotease and peroxidase activity were significantly lower in the unvaccinated symptomatic group than in the unvaccinated and vaccinated asymptomatic groups ($p \leq .05$). Serum total proteins and total immunoglobulin (IgM) levels in vaccinated asymptomatic rainbow trout were significantly higher than in unvaccinated asymptomatic and symptomatic groups ($p \leq .05$). Similarly, vaccinated asymptomatic fish produced more specific IgM against *L. garvieae* than unvaccinated asymptomatic and symptomatic fish ($p \leq .05$). This preliminary study provides basic knowledge on the immunological relationship occurring between the rainbow trout and *L. garvieae*, potentially predicting health outcomes. The approach we proposed could facilitate infield diagnostics, and several non-specific immunological markers could serve as reliable indicators of the trout's innate ability to fight infection.

KEYWORDS
immune profile, innate immunity, lactococcosis, rainbow trout, vaccination

1 | INTRODUCTION

Infection in fish is a complex process involving the interaction among the pathogen, the host and environmental factors. Pathogens have evolved strategies to overcome biochemical barriers, get access to host cells and even use host cells for survival/proliferation. On the contrary, fish defend themselves by adopting immunological strategies such as humoral and cellular pathways, modulation of membrane permeability/fluidity and the synthesis of antimicrobial peptides or enzymes (Ben Hamed et al., 2018; Ellis, 1999). A deeper study of the relationship occurring between the host fish species and a specific bacterium might provide new basic knowledge as well as refine existing prophylactic and therapeutic strategies.

Fish lactococcosis is a hyperacute hemorrhagic septicemia caused by *Lactococcus garvieae* (Austin & Austin, 2012), a pathogen in global fish farming that causes disease in a variety of fish species, including rainbow trout (*Oncorhynchus mykiss*) (Eldar et al., 1996), one of the most important species for aquaculture worldwide (Meyburgh et al., 2017; Vendrell et al., 2006). Signs in affected fish include anorexia, melanosis, exophthalmia and haemorrhages in the periorbital and intraocular areas and at the base of fins, swollen abdomen and anal prolapse (Bekker et al., 2011; Eldar & Ghittino, 1999).

The virulence of *L. garvieae* is linked to its ability to form a capsule, secrete hemolysins and express adhesion factors, as well as the presence of a putative set of virulence factor genes (hly1, hly2, hly3, nox, sod, pavA, psaA) (Gnanagobal & Santander, 2022; Kawanishi et al., 2006; Shahi et al., 2018; Ture & Altinok, 2016). These factors can contribute to host tissue damage by aiding in the infection process through adhesion to host tissues, evasion of the host's innate immunity and systemic invasion (Soltani et al., 2021).

There is limited information on how rainbow trout host reacts to this infection, either by a defined immune response or by inflammation. What we know comes from investigations on spontaneous outbreaks, experimental challenges and post vaccination surveys. *L. garvieae* naturally infects gills and eyes before swiftly spreading to the blood system. Afterwards, it can be detected in all the major internal organs, including intestine, liver, spleen, kidney, brain and heart (Avci et al., 2014). The activation of the immune response of trout against *L. garvieae* includes the modulation of several humoral and cellular immune mechanisms (i.e. lysozyme activity, bactericidal activity, antiprotease activity, phagocytosis, respiratory burst activity, natural and specific antibody synthesis, differential expression of immune-related genes) that show variability due to the lactococcal infection and/or to vaccination with *L. garvieae* antigens (Bulfon et al., 2020; Castro et al., 2019; Halimi et al., 2019, 2020).

So far, vaccination is the safest method to get a proper and effective immune response against the pathogen *L. garvieae*. It is classically performed by intra-celomatic injection of inactivated bacteria (either autovaccines or commercial formulations) when rainbow trout weigh at least 50 g and prior to the water temperature increasing over 15°C (Vendrell et al., 2006). A valuable protection is usually reached 3-week post-vaccination and remains for a period of 3–4 months up to 4–8 months with adjuvanted vaccines (Meyburgh et al., 2017; Pridgwon & Klesius, 2014; Vendrell et al., 2006). Within the general framework of the disease control, selected non-specific immunological parameters could serve as indirect indicators of innate fish ability to fight the infection (Das & Sahoo, 2014; Yáñez et al., 2014), thus facilitating the infield diagnosis of the disease.

Currently, few data are available on immune or haematological profiles detectable by laboratory assessment in symptomatic and asymptomatic rainbow trout naturally exposed to the pathogen *L. garvieae*. To get more insight into this topic, the present study was designed to investigate the profile of non-specific immune parameters (leukogram; serum lysozyme activity, peroxidase activity, antiprotease activity, bactericidal activity; total IgM and total protein), as well as specific immune parameters (serum-specific antibodies to *L. garvieae* antigens) in adult unvaccinated rainbow trout naturally exposed to the pathogen, with or without evidence of clinical signs, or submitted to in-field vaccination. This kind of approach can be useful to evaluate the immune modulation of rainbow trout upon *L. garvieae* infection, as well as for the definition of resistance biomarkers in this fish species.

2 | MATERIALS AND METHODS

2.1 | Fish and infield sampling

During the summer of 2021, a single time point sampling was conducted in an intensive trout farm in northeastern Italy, where lactococcosis outbreaks are repeatedly signalled due to extremely high-water temperatures. Details on the investigated groups of fish, as well as on their number and condition, are reported in Table 1.

Unvaccinated rainbow trout with or without evidence of clinical signs shared the same basin, whereas asymptomatic vaccinated fish were reared in a different basin, under the same environmental conditions: temperature 20–22°C; oxygen 6–8 ppm; pH 7.00.

TABLE 1 Sampled fish groups, number of individuals, size and clinical signs.

Group	Number	Trout size (g)	Main clinical signs
Asymptomatic-unvaccinated	20	500–600	Absent
Symptomatic-unvaccinated	10	500–600	Exophthalmia, ocular haemorrhages, anal prolapses
Asymptomatic—IP vaccinated with Icthiovac® LG Hipra—then sampled at 10-week post vaccination	10	500–600	Absent

Fish were anaesthetized with MS-222 (30 mg/L, Pharmaq), then blood was drawn from the caudal vein using appropriately sized syringes (2.5 or 5 mL). Samples were immediately used to prepare blood smears, then serum was obtained by centrifugation at 1000 *g* for 15 min at RT. Individual sera were aliquoted and stored at −20°C until immunological evaluations. A preliminary bacteriological test performed by IZS Torino according to Pastorino et al. (2019) confirmed the diagnosis of lactococcosis in symptomatic fish.

2.2 | Ethics statement

All of the samples were analysed for diagnostic purposes in accordance with the farmer's fish disease management protocols. The sampling was carried out with the permission of the University of Udine's ethical committee (n. 03/2022 OPBA UNIUD) and animal handling was performed following the European/Italian guidelines on animal welfare norms (L.D. No. 26/2014, implementation of the European Directive 2010/63/EU).

2.3 | Leukocyte differential count and bacterial detection on blood smears

Blood smears, prepared only for unvaccinated asymptomatic and symptomatic groups, were air-dried at RT, fixed with absolute methanol for 5 min (Merck Life Science s.r.l.) and stained with May-Grünwald Giemsa solutions according to manufacturer instructions (Merck Life Science s.r.l.). They were observed under an oil immersion objective (×1000) with an optical microscope (Leica DMRB). One hundred leukocytes/fish were counted and morphologically differentiated in three randomly selected zones. Then, the relative percentage of each cell population was calculated. Moreover, each blood smear was accurately examined to detect the presence of circulating bacteria (*L. garvieae*). Digital images of representative optical fields were captured by a digital camera (Nikon Fi3) equipped with the imaging software NIS-Elements BR (Nikon Instruments).

2.4 | Non-specific immune parameters

2.4.1 | Serum lysozyme activity

The serum lysozyme activity was quantified according to the method of Parry et al. (1965). Briefly, 10 µL of serum (in triplicate) was incubated in a 96-well microplate (Sarstedt) with 200 µL of lyophilized *Micrococcus lysodeikticus* (0.2 mg/mL, Merck Life Science s.r.l.) in sodium phosphate buffer 0.04 M (Na_2HPO_4, Merck Life Science s.r.l.). The reduction in absorbance due to bacterial cell lysis was measured at 450 nm every 10 min for 1 h using a microplate reader (Sunrise, Tecan s.r.l.). The activity of lysozyme (U/mL) in serum was calculated using a reference standard curve prepared with serial dilutions of lysozyme from chicken egg white (Merck Life Science s.r.l.).

2.4.2 | Serum bactericidal activity

The bactericidal activity was evaluated according to the microplate-based method proposed by Budiño et al. (2006), with some modifications. Briefly, 50 µL/well of viable *L. garvieae* (041, IZSVe) suspension in TSB (1×10^6 cfu/mL) was added to 25 µL/well of serum and 25 µL/well of sterile PBS. After 6 h incubation at RT, 50 µL/well of 2 mg/mL MTT (dimethylthiazol-diphenyl tetrazolium bromide) (Sigma, M5655) was added and incubated at RT for 15 min. Then, 50 µL/well of DMSO was used to dissolve the formazan crystals. The amount of viable bacteria (formazan positive) was assayed spectrophotometrically at 600 nm by a microplate reader (Sunrise, Tecan s.r.l.). All samples were analysed in duplicate.

2.4.3 | Serum peroxidase activity

The peroxidase activity in serum was measured according to Quade and Roth (1997) by oxidation of 3,3′,5,5′-Tetramethylbenzidine (TMB). Briefly, 15 µL/well of serum was diluted with Hanks's buffer (HBSS) without Ca^{+2} or Mg^{+2} to a final volume of 150 µL in a flat-bottomed 96-well plate. Then, 50 µL/well of 10 mM TMB with 0.025 of 30% H_2O_2 was added as substrate, and the colour change reaction was stopped after 3 min by adding 50 µL 2 M H_2SO_4. The OD was read at 450 nm in a microplate reader (Sunrise, Tecan s.r.l.). Samples without serum were used as blanks and the OD values were subtracted for each sample value. One unit was defined as the amount producing an absorbance change of 1 while the activity was expressed as U/mL for the serum samples. All samples were analysed in triplicate.

2.4.4 | Serum antiprotease activity

Antiprotease activity was determined based on the capacity of serum to inhibit trypsin activity, according to Bowden et al. (1997). Briefly, 10 µL of serum was incubated for 5 min at 22°C with 10 µL of trypsin solution (bovine pancreas Type 1, prepared at 0.3% in Tris–HCl 0.01 M pH 8.2, Sigma-Aldrich) in sterile vials. Subsequently, 500 µL of BAPNA (Na-benzoyl-DL-p-nitroanilide HCl, prepared 5 mM in DMSO and H_2O, Sigma-Aldrich) and 480 µL of Tris–HCl 0.1 M pH 8.2 were added to reach the final volume of 1 mL. Samples were incubated for 25 min of incubation at 22°C with constant shaking, the reaction was stopped by adding 150 µL of 30% acetic acid. The samples were then centrifuged (400 *g*, 5 min) and 200 µL of the supernatants was transferred to a flat-bottomed 96-well plate. The OD was read at 410 nm using a plate

reader (Sunrise, Tecan s.r.l.). As a positive control (100% trypsin inhibition) a combination of Tris–HCl 0.01 M pH 8.2 and trypsin solution without serum was used. As a negative control (0% trypsin inhibition), Tris–HCl 0.01 M pH 8.2, with no serum or trypsin, was used. The activity for each sample was expressed as a percentage of trypsin inhibition in relation to the controls and calculated according to Zuo and Woo (1997). All samples were analysed in triplicate.

2.4.5 | Serum total proteins and total immunoglobulins

Serum proteins were determined by the Bradford (1976) method. Forty μL/well of serially diluted serum was distributed in 96-well microtiter plates, and 200 μL/well of Bradford solution (B6916, Sigma) was added. The absorbance was read at 595 nm by a microplate reader (Sunrise, Tecan s.r.l.). The immunoglobulin concentration was measured by the method of Klein and Siminovitch (1986) modified as follows. Immunoglobulins were precipitated with 10,000 kDa polyethylene glycol (PEG, P-6667, Sigma). Serum (100 μL) was mixed with an equal volume of 12% PEG solution and shaken for 2 h at RT. After centrifuging at 5000 *g* for 15 min, the immunoglobulin-deprived supernatant was collected and the concentration of proteins was determined as described above. The total immunoglobulin concentration in each serum sample was calculated by subtracting this value from the serum's total protein concentration.

2.5 | Specific immune parameters: serum anti-*L. garvieae* antibody titre

2.5.1 | Antigen

Inactivated bacteria (bacterin) for agglutination and ELISA were produced and kindly provided by Dr. Prearo (IZS Torino). Briefly, a recently isolated *L. garvieae* strain was cultivated in an appropriate medium until the log phase and then treated with 40% formaldehyde (Merck Life Science s.r.l.) at 4% v/v under continuous agitation at RT for 24 h. The complete inactivation of bacteria was determined on blood agar plates after 72 h of incubation at 37 ± 2°C. Thereafter, the culture medium and formaldehyde were discarded by centrifugation at 3875 *g* for 15 min. Subsequently, three serial washes were performed with sterile PBS. The obtained *L. garvieae* bacterin pellet was weighed and re-suspended in sterile PBS at a concentration of 60 g/L, then preserved at 4°C until use.

2.5.2 | Bacterial agglutination assay

The serum agglutination titre was determined according to the method proposed by Barnes and Ellis (2004), partially modified. Initially, the sera were heated for 30 min at 45°C to inactivate complement proteins. Then, serial two-fold dilutions (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 and 1/256) of test sera in sterile PBS (50 μL/well) were incubated in a U-bottom 96-well microtiter plate (Sarstedt) with 50 μL/well of *L. garvieae* bacterin in sterile PBS (OD at 610 nm = 0.3) at 22 ± 2°C for 24 h. Wells were also tested without serum (blank), wells with a pool of sera collected from *naïve* trout (negative control) and with a reference positive trout serum to *L. garvieae*. After incubation, the agglutination response was recorded by naked-eye observation with the presence of a defined whitish button on the bottom of the well (bacterial precipitate) considered a negative reaction and the presence of diffused turbidity on the bottom of the well-considered a positive reaction. The agglutination titre was expressed as log2 of the reciprocal of the highest serum dilution that resulted in visible bacterial agglutination, as described by Yarahmadi et al. (2014).

2.5.3 | Indirect ELISA

The serum-specific antibody titre against *L. garvieae* was determined by using a partially modified indirect ELISA (Bakopoulos et al., 1997). Ninety-six well microplates (Nunc, Merck Life Science s.r.l.) were coated with 100 μL/well of 0.001% poli-L-lysine (Merck Life Science s.r.l.) in carbonate/bicarbonate buffer 0.05 M, pH 9.6 (Na_2CO_3 and $NaHCO_3$, Merck Life Science s.r.l.) at RT. After three washes with LSWB pH 7.3, 100 μL/well of *L. garvieae* bacterin (O.D. = 1 at 610 nm) was added. The plates were centrifuged at 210 *g* for 10 min at RT and incubated for 1 h. After three washes with LSWB, the plates were treated with 1% gelatin (Merck Life Science s.r.l.) in LSWB (200 μL/well) at RT for 3 h and with 5% goat serum (Merck Life Science s.r.l.) in LSWB (200 μL/well) at 4°C overnight. After three washes with HSWB pH 7.7, 100 μL/well of heat treated (30 min at 45°C) rainbow trout serum diluted (1:25, in duplicate) in PBS with 0.1% Tween 20 (Merck Life Science s.r.l.) was added and incubated at RT for 2 h and 30 min. After five washes with HSWB, 100 μL/well of monoclonal antibody anti rainbow trout IgM (5F12, Biorad) diluted 1:800 in PBS with 1% BSA (Merck Life Science s.r.l.) were incubated at RT for 1 h. After five washes with HSWB, 100 μL/well of goat polyclonal antibody against mouse immunoglobulins (A4416, Sigma-Aldrich) conjugated with peroxidase (1:3000 in PBS with 1% BSA) were added. After 1 h incubation at RT, the plates were washed with HSWB and incubated with 0.42 mM TMB (Merck Life Science s.r.l.) in substrate buffer pH 5.4 ($C_6H_8O_7$ and $C_2H_3O_2Na$, Merck Life Science s.r.l.) with 5 mM H_2O_2 (100 μL/well, Merck Life Science s.r.l.). The colorimetric reaction was stopped after 5 min by adding 50 μL/well of 2 M H_2SO_4 (Merck Life Science s.r.l.). The OD was read at 450 nm using a microplate reader (Sunrise, Tecan s.r.l.). On each plate, the following reference controls were included: absence of rainbow trout serum (blank); positive sera referencing to *L. garvieae* (positive controls); pool of sera collected from *naïve* rainbow trout (negative control).

2.6 | Statistical analyses

The results are expressed as arithmetic mean ± SD. Statistical analyses were performed using SPSS version 22.0. Data were tested for normality using the Kolmogorov–Smirnov test. The one-way ANOVA was applied for the normally distributed data. If the variances were not normally distributed, the Mann–Whitney U post hoc test was applied. Significance level was set to $p \leq .05$.

3 | RESULTS

3.1 | Clinical diagnosis

Rainbow trout with symptoms were lethargic, displayed erratic swimming and were easily recognizable from the healthy/asymptomatic fish. They also showed uni- or bi-lateral exophthalmia, swollen abdomen, anal prolapses and haemorrhages in the periorbital and intraocular area, at the base of fins, opercula and the buccal region. Relevant external gross lesions detected in symptomatic fish are illustrated in Figure 1.

3.2 | Leukocyte differential count and bacterial detection on blood smears

Lymphocytes were the most abundant blood leukocytes detected in the investigated rainbow trout (Table 2), displaying a round shape with a round purple nucleus and a narrow rim of basophilic cytoplasm (Figure 2a). Neutrophils closely resembled their mammalian counterparts, being round and having a nucleus with 2–5 lobes connected with thin threads of nuclear material and granules in the cytoplasm (Figure 2b). The morphology of thrombocytes was oval or elongated, with a purple nucleus and a small rim of pale cytoplasm (Figure 2c). Eosinophil and basophil granulocytes were not identifiable in any of the evaluated blood smears. Monocytes showed an abundant cytoplasm containing evident vacuoles and a bean-shaped nucleus (Figure 2d).

Blood leukocyte differential count of unvaccinated asymptomatic and symptomatic groups is reported in Table 2. The relative percentages of neutrophils and monocytes were not statistically different between asymptomatic and symptomatic rainbow trout ($p \leq .05$). However, symptomatic fish had a statistically significantly lower percentage of blood lymphocytes than asymptomatic fish ($p \leq .05$). Simultaneously, the percentage of thrombocytes in symptomatic fish was significantly higher than in asymptomatic fish ($p \leq .05$). Seven out of 10 (70%) symptomatic fish had bacteria in the peripheral blood (Figure 2e), but no bacteria were detected in the peripheral blood of asymptomatic fish (Table 2).

3.3 | Non-specific immune parameters

In all three groups, serum lysozyme (Figure 3a) and serum bactericidal activity (Figure 3b) revealed a similar pattern. The values for vaccinated rainbow trout were slightly higher than those for unvaccinated (asymptomatic and symptomatic) fish, although no statistically significant differences were found among the groups ($p \leq .05$).

Serum antiprotease activity (% of trypsin inhibition) and peroxidase activity (U/mL) (Figure 4A,B) were significantly higher in asymptomatic fish (vaccinated and unvaccinated) compared with symptomatic-unvaccinated fish ($p \leq .05$).

The results of the evaluation of serum total proteins and total immunoglobulins (IgM) are summarized in Figure 5a,b. Both assays revealed significant differences among groups. Protein concentration (mg/mL) in the vaccinated group was significantly higher compared with both unvaccinated asymptomatic and symptomatic groups ($p \leq .05$). There were no significant differences between the unvaccinated asymptomatic and symptomatic fish ($p \leq .05$). Immunoglobulin concentration (mg/mL) showed a similar pattern. In fact, this parameter was significantly higher in the vaccinated group compared with the unvaccinated asymptomatic and symptomatic groups ($p \leq .05$). There were no significant differences between unvaccinated asymptomatic and symptomatic groups ($p \leq .05$).

3.4 | Specific immune parameters: serum anti-*L. garvieae* antibody titre

The agglutination test did not indicate serum-specific activity against *L. garvieae* in any of the three target groups' analysed samples (data not shown). Figure 6 shows the specific antibody titers detected in unvaccinated asymptomatic, unvaccinated symptomatic and vaccinated fish, measured by ELISA. Vaccinated fish had a significantly higher level of serum conversion compared with the other two groups (asymptomatic and symptomatic) ($p \leq .05$). There were no significant differences between unvaccinated asymptomatic and symptomatic groups were recorded ($p \leq .05$).

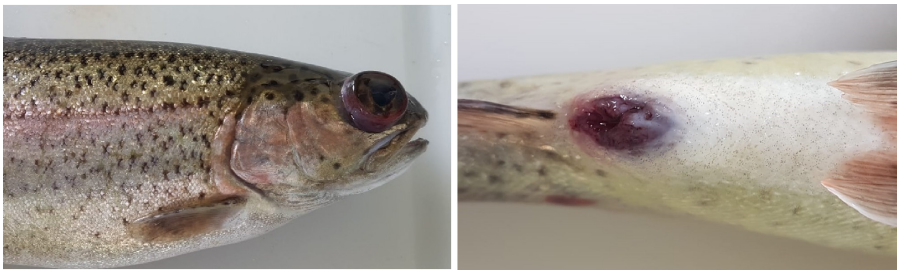

FIGURE 1 Exophthalmia, haemorrhages in the periorbital area and anal prolapse in a symptomatic rainbow trout.

TABLE 2 Leukocyte differential count in unvaccinated asymptomatic and symptomatic rainbow trout.

Group	Leukocyte population				Bacteria detection
	Lymphocyte	Neutrophil granulocyte	Monocyte	Thrombocyte	
Asymptomatic unvaccinated	64.67 ± 5.31[a]	9.11 ± 3.87[a]	4.44 ± 2.36[a]	21.78 ± 3.43[b]	0% (0/10)
Symptomatic unvaccinated	45.78 ± 4.94[b]	9.44 ± 2.27[a]	4.67 ± 1.63[a]	40.11 ± 3.84[a]	70% (7/10)

Note: Data (%) are expressed as mean ± SD ($n = 10$). Different letters in each column indicate significant differences between asymptomatic and symptomatic groups ($p \leq .05$).

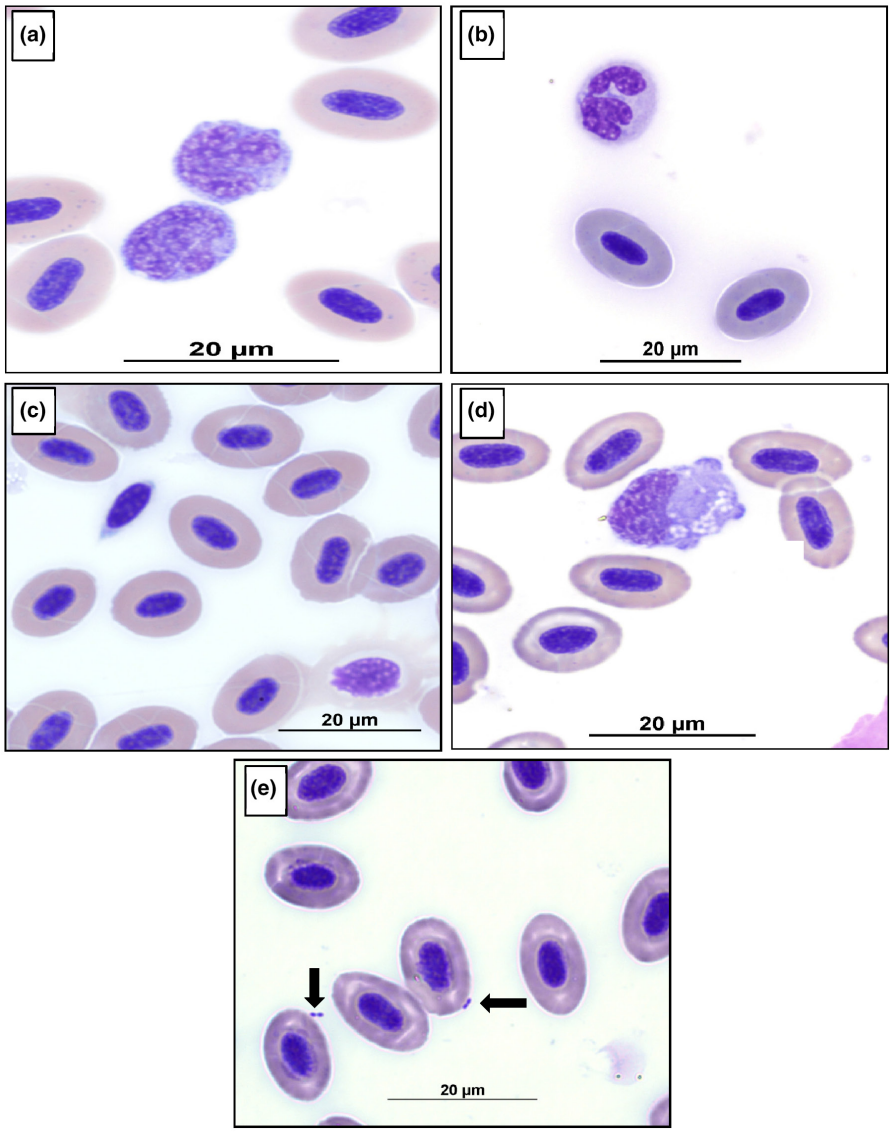

FIGURE 2 Rainbow trout peripheral blood smears stained with May-Grünwald Giemsa. Morphological details of the specific cell populations submitted to differential count: (a) lymphocyte; (b) neutrophil granulocyte; (c) thrombocyte; (d) monocyte. The image also illustrates the morphological aspect of circulating bacteria, in the proximity of two red blood cells, (e) black arrows.

4 | DISCUSSION

In the present study, three groups of rainbow trout—unvaccinated asymptomatic and symptomatic, and vaccinated asymptomatic—were monitored within a commercial farm periodically affected by lactococcosis outbreaks to highlight differences in the immunological profiles of fish displaying a different reactivity to the bacterial exposure, in terms of absence/presence of clinical signs. Several variations in innate and adaptive immunological parameters were detected in rainbow trout populations.

Symptomatic fish had a significantly lower relative percentage of peripheral blood lymphocytes than asymptomatic fish, suggesting that the recruitment/activation of specific immune cells during the systemic defensive response against *L. garvieae* occurred more

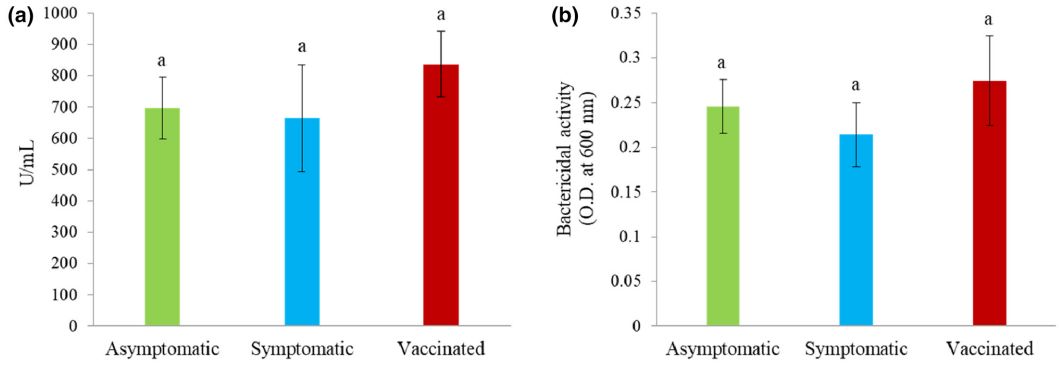

FIGURE 3 Serum lysozyme activity (U/mL) (a) and serum bactericidal activity (O.D. at 600 nm) (b) in unvaccinated asymptomatic, unvaccinated symptomatic and vaccinated rainbow trout. Data are expressed as mean ± SD ($n = 10$ for symptomatic and vaccinated; $n = 20$ for asymptomatic). Different letters indicate significant differences among experimental groups ($p \leq .05$).

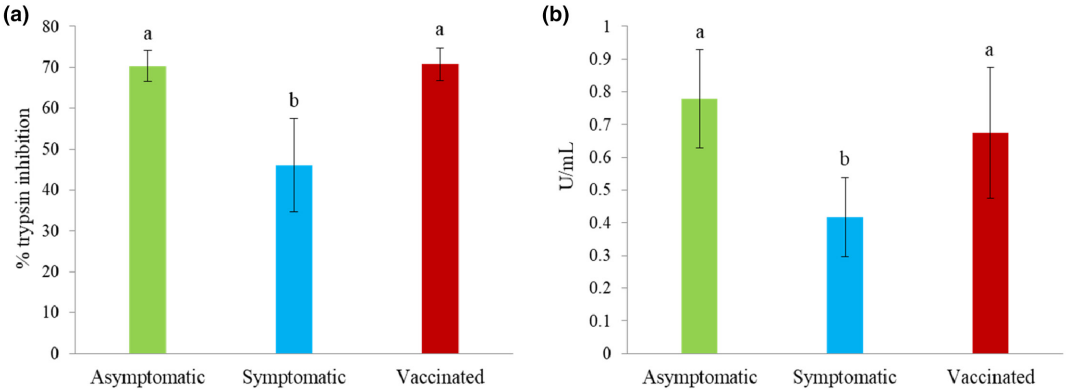

FIGURE 4 Serum antiprotease activity (% trypsin inhibition) (a) and peroxidase activity (U/mL) (b) in unvaccinated asymptomatic, unvaccinated symptomatic and vaccinated rainbow trout. Data are expressed as mean ± SD ($n = 10$ for symptomatic and vaccinated; $n = 20$ for asymptomatic). Different letters indicate significant differences among experimental groups ($p \leq .05$).

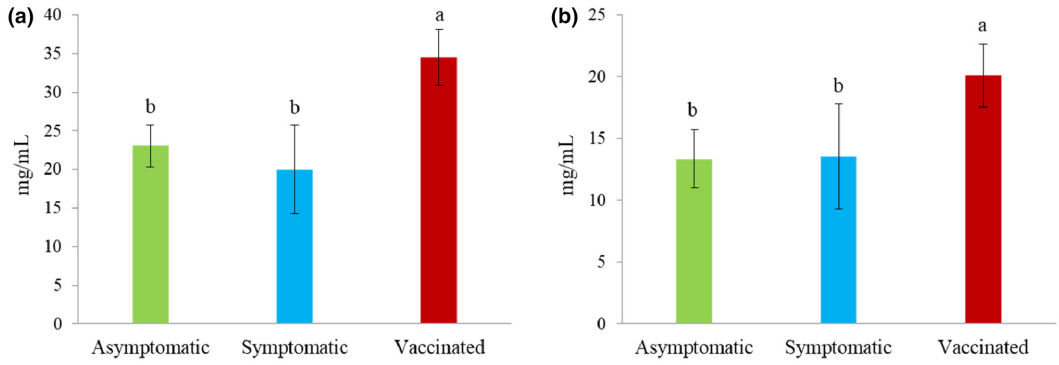

FIGURE 5 Serum total proteins (mg/mL) (a) and immunoglobulins (mg/mL) (b) in unvaccinated asymptomatic, unvaccinated symptomatic and vaccinated rainbow trout. Data are expressed as mean ± SD ($n = 10$ for symptomatic and vaccinated; $n = 20$ for asymptomatic). Different letters indicate significant differences among experimental groups ($p \leq .05$).

effectively in the asymptomatic group. Simultaneously, circulating thrombocytes were significantly higher in symptomatic fish compared with asymptomatic ones. These fish blood cells are analogous to anucleate mammalian platelets and have extra immune functions including phagocytosis (Nagasawa et al., 2014). In mammals, it is known that platelets express various immune receptors that enable them to act as sentinels to recognize intravascular pathogens. Upon activation, platelets directly restrict pathogen proliferation through the release of AMPs (antimicrobial peptides) (Portier & Campbell, 2021). Their role in protecting rainbow trout against lactococcosis has not been investigated extensively. The only study we can mention is the one published by Bulfon et al. (2020), who

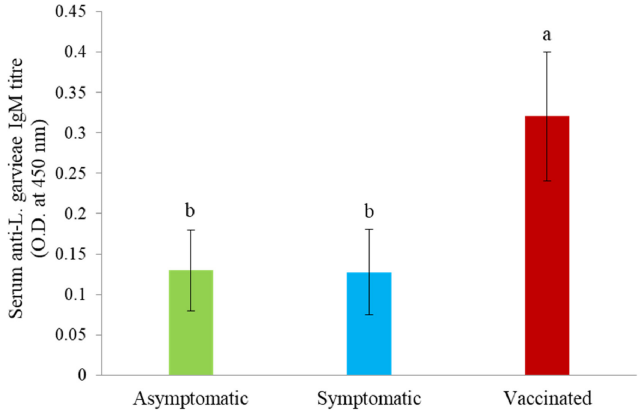

FIGURE 6 Serum IgM titres against *Lactococcus garvieae* detected by ELISA (O.D. at 450 nm) in unvaccinated asymptomatic, unvaccinated symptomatic and vaccinated rainbow trout. Data are expressed as mean ± SD ($n = 10$ for symptomatic and vaccinated; $n = 20$ for asymptomatic). Different letters indicate significant differences among groups ($p \leq .05$).

found an increase in lymphocytes and a decrease in thrombocytes in resistant trout compared with susceptible fish exposed to *L. garvieae*, which is in agreement with our results. Otherwise, the relative percentages of neutrophils and monocytes in peripheral blood were similar in asymptomatic and symptomatic fish.

Lysozyme is a bactericidal enzyme found in lysosomes that catalyses the bacterial cell wall peptidoglycan degradation, thereby causing bacteriolysis and preventing bacteria proliferation. Therefore, it is considered a protective factor against infections, especially those determined by Gram-positive bacteria (Saurabh & Sahoo, 2008). The present study did not reveal significant differences in serum lysozyme levels among groups. In the study carried out by Halimi et al. (2018), the vaccination of rainbow trout with inactivated *L. garvieae* significantly increased the serum lysozyme activity suggesting that the administration of bacterial antigens can also stimulate the non-specific immune response of rainbow trout. The increased lysozyme activity has also been reported in uninfected fish compared with infected fish with *A. hydrophila* (Yarahmadi et al., 2016).

Serum bactericidal ability against *L. garvieae* that was evaluated in unvaccinated asymptomatic, unvaccinated symptomatic and vaccinated rainbow trout exhibited no significant differences among groups. It is reasonable to hypothesize that vaccinated fish might possess a stronger bactericidal ability to inhibit pathogen colonization/proliferation, than unvaccinated fish. In fact, vaccination might trigger bactericidal activity through specific IgM-based complement activation on target bacteria. There were no significant differences among groups, regardless of their profile (vaccinated, unvaccinated, with or without clinical signs) as observed by Yarahmadi et al. (2016), which did not find any differences between uninfected and infected rainbow trout with *A. hydrophila*. In contrast with our results, several studies (Halimi et al., 2018, 2019, 2020) using the same methodology indicated an increase in bactericidal activity in the serum of fish vaccinated with *L. garvieae* compared with unvaccinated fish.

Protease inhibitors are detectable in serum and other body fluids and they can be involved in the defence against pathogens, that secrete proteolytic enzymes. These metabolites contribute to the innate immunity of fish by restricting the ability of bacteria to invade and proliferate in them (Ellis, 2001). In our analysis, asymptomatic (unvaccinated and vaccinated) fish showed a significantly higher antiprotease activity with respect to the symptomatic-unvaccinated fish; however, no comparative studies evaluating this parameter in fish during *L. garvieae* infections were reported. In line with our results, Yarahmadi et al. (2016) found significantly higher antiprotease activity in uninfected rainbow trout compared to those infected with *A. hydrophila*. In this regard, our hypothesis is that in general, asymptomatic trout, whether unvaccinated or vaccinated, have serum antiprotease activity that is more potent than that of fish with clinically evident lactococcosis.

The peroxidase activity of asymptomatic-unvaccinated and -vaccinated fish was significantly higher than that of symptomatic fish. A reduced peroxidase activity may negatively affect the ability to neutralize pathogens (Klebanoff, 1998), suggesting that in our study, the unvaccinated symptomatic rainbow trout may show impaired defence capacity against bacteria. On the contrary, vaccinated rainbow trout or rainbow trout without clinical signs, which revealed higher levels of peroxidase activity, could be more efficient in preventing lactococcosis. Previous studies have also shown that vaccinated fish have higher levels of peroxidase activity than unvaccinated fish. For instance, Kong et al. (2022) found a significantly increased peroxidase activity in serum and skin mucus of yellow catfish (*Pelteobagrus fulvidraco*) following a vaccination against *Aeromonas veronii* and *Edwardsiella tarda*.

The serum total proteins and immunoglobulins are an important part of the humoral immune system of vertebrates. In our study, both parameters were significantly higher in vaccinated fish than in unvaccinated asymptomatic and symptomatic fish, suggesting that the vaccination stimulates the synthesis of specific antibodies to the pathogen and consequently, it positively affects also the concentration of circulating total IgM/total proteins, as already reported by Halimi et al. (2018) for rainbow trout orally vaccinated against *L. garvieae*. On the contrary, Halimi et al. (2020) reported no significant changes in serum total immunoglobulin and total protein after oral vaccination against *L. garvieae* in rainbow trout. These discrepancies may be attributable to the different routes of vaccination and the final effectiveness of the vaccine. The increase in serum total IgM/total proteins can be alternatively achieved by adding immunostimulants to aquafeeds addressed to rainbow trout (Dorucu et al., 2009; Tahmasebi-Kohyani et al., 2012; Yousefi et al., 2016). For example, Dorucu et al. (2009), found improved serum proteins and immunoglobulins in rainbow trout after feeding them a supplement of black cumin seeds (2.5%) for 21 days.

Antibody synthesis is of paramount importance in the humoral immune response of bony fish. This defence is particularly important when dealing with extracellular threats, as is the case with most bacterial pathogens. Antibodies prevent the growth and colonization of bacterial pathogens by neutralization, complement

activation and/or opsonization to enhance phagocytosis. By far the most frequent immunoglobulin in teleost serum is IgM, which is responsible for systemic immunity in bony fishes (Semple & Dixon, 2020). In the present study, serum anti-*L. garvieae* antibodies were detected and found to be statistically higher in vaccinated fish compared with both unvaccinated asymptomatic and symptomatic fish, deeming this an important parameter to support a vaccination campaign. In a previous study published by Halimi et al. (2018), the specific IgM titers against *L. garvieae* resulted in higher levels in rainbow trout after oral vaccination which is in accordance with our study. In addition, even other studies reported that vaccinated rainbow trout against lactococcosis synthesized detectable amounts of specific IgM compared with unvaccinated groups (Bastardo et al., 2012; Halimi et al., 2019). Concerning the other two groups under investigation (unvaccinated with or without clinical signs of lactococcosis) the ELISA revealed a negligible presence of specific antibodies in the serum (recorded O.D. lower than 0.2). This was an expected outcome in the case of asymptomatic fish, but we found a similar response also in symptomatic fish, displaying in most cases a septicemic condition (detection of bacteria in blood smears in 7 out of 10 fish). They later encountered the pathogen in the environment and, in most cases, it reached a spreading phase within the fish body, but the duration of host–pathogen interaction apparently was not sufficient to promote a reliable/detectable synthesis of specific antibodies to *L. garvieae*. Based on these observations, we can assume that the proposed ELISA allows discrimination between vaccinated and unvaccinated fish but is not suitable for discriminating between uninfected and infected fish. Unfortunately, because our study was conducted within a commercial facility, it was difficult to establish the precise moment of the lactococcosis outbreak rising, as well as to discriminate between fish surely not exposed to *L. garvieae* and asymptomatic fish recovering from a previous infection state.

5 | CONCLUSION

In summary, this study established that vaccination helps trout fight against lactococcosis by boosting immunity, as higher levels of innate immune parameters (like antiprotease, peroxidase activity, total protein and total IgM) and specific immune parameters (IgM to *L. garvieae*) were observed in vaccinated fish than in unvaccinated ones (asymptomatic and symptomatic). Moreover, the proposed analytical approach can be useful to evaluate the immune modulation of unvaccinated rainbow trout upon *L. garvieae* infection, since we detected differences in antiprotease and peroxidase activity between fish apparently susceptible and resistant to the outbreak, hosted in the same basin. These selected parameters could be eventually proposed as phenotypical biomarkers of resistance in this fish species.

AUTHOR CONTRIBUTIONS

All authors have confirmed to submit the manuscript.

ACKNOWLEDGEMENTS

We thank Dr. Massimiliano Bruno and Mr. Pierluigi Bagatella (Di4A, University of Udine, Italy) for the help during infield sampling. Open Access Funding provided by Universita degli Studi di Udine within the CRUI-CARE Agreement.

FUNDING INFORMATION

This work was supported by the SUPERTROUT PRIMA project: Improving SUstainability and PERformance of aquaculture farming system: breeding for lactococcosis resistance in rainbow TROUT with reference 'Research and Innovation Action approved under the call Section 2 – Multi-Topics 2019, "Farming System"'.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Khalil, S. M. I., Bulfon, C., Galeotti, M., Acutis, P. L., Altinok, I., Kotzamanidis, C., Vela, A. I., Fariano, L., Prearo, M., Colussi, S., & Volpatti, D. (2023). Immune profiling of rainbow trout (*Oncorhynchus mykiss*) exposed to *Lactococcus garvieae*: Evidence in asymptomatic versus symptomatic or vaccinated fish. *Journal of Fish Diseases*, *00*, 1–11. https://doi.org/10.1111/jfd.13782