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Aeromonas salmonicida infects Atlantic salmon (*Salmo salar*) erythrocytes

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Abstract

Aeromonas salmonicida subsp. *salmonicida* (hereafter *A. salmonicida*) is the aetiological agent of furunculosis in marine and freshwater fish. Once *A. salmonicida* invade the fish host through skin, gut or gills, it spreads and colonizes the head kidney, liver, spleen and brain. *A. salmonicida* infects leucocytes and exhibits an extracellular phase in the blood of the host; however, it is unknown whether *A. salmonicida* have an intraerythrocytic phase. Here, we evaluate whether *A. salmonicida* infects Atlantic salmon (*Salmo salar*) erythrocytes in vitro and in vivo. *A. salmonicida* did not kill primary *S. salar* erythrocytes, even in the presence of high bacterial loads, but *A. salmonicida* invaded the *S. salar* erythrocytes in the absence of evident haemolysis. Naïve Atlantic salmon smolts intraperitoneally infected with *A. salmonicida* showed bacteraemia 5 days post-infection and the presence of intraerythrocytic *A. salmonicida*. Our results reveal a novel intraerythrocytic phase during *A. salmonicida* infection.

KEYWORDS
Aeromonas salmonicida, erythrocytes, furunculosis, *Salmo salar*

1 | INTRODUCTION

Aeromonas salmonicida subspecies *salmonicida* (hereafter *A. salmonicida*) is a Gram-negative bacterial pathogen of fish and the aetiological agent of furunculosis. Although its natural route of infection is through skin lesions and mucosal epithelia (e.g. gut and gills), intraperitoneal experimental doses of 30 bacterial cells can result in a lethal infection (Valderrama, Saravia, & Santander, 2017).

The virulence of *A. salmonicida* is associated with its ability to replicate in phagocytic cells, such as macrophages (Daly, Kew, Moore, & Olivier, 1996; Garduño & Kay, 1992; Vanya Ewart et al., 2008). In addition, *A. salmonicida* can invade and replicate in non-phagocytic epithelial cells (Garduño et al., 2000; Valderrama et al., 2017). However, the network of host–microbe interactions that contribute to *A. salmonicida* pathogenesis is complex and not fully understood. For instance, it is known that *A. salmonicida* uses the A-layer to resist the complement (Munn, Ishiguro, Kay, & Trust, 1982) during a short phase in the blood (Effendi & Austin, 1995; Svendsen, Dalmo, & Bogwald, 1999), but invasion and replication in fish erythrocytes is unknown.

In contrast to mammalian erythrocytes, fish erythrocytes are nucleated cells (Clauss, Dove, & Arnold, 2008; Farrel, 2011; Witeska, 2013) and there are no reports of intracellular bacterial infection in these teleost cells. *Francisella tularensis* (Horzempa et al., 2011), *Mycoplasma suis* (Groebel, Hoelzle, Wittenbrink, Ziegler, & Hoelzle, 2009), *Mycoplasma gallisepticum* (Vogl et al., 2008) and *Bartonella* spp. (Dehio, 2008) invade mammalian erythrocytes. *Haemobartonella* sp. and *Eperythrozoon perekropovi* colonize or attach to the fish erythrocytes externally (Davies & Johnston, 2000; Morera & MacKenzie, 2011), but they do not intracellularly invade fish erythrocytes. Intracellular infection of erythrocytes could help the bacteria to bypass host defence mechanisms and to get easy access to necessary nutrients, such as iron. Additionally, the ability to remain inside the erythrocytes would allow the bacterium to disperse within the host. In this study, we provide evidence of a novel intraerythrocytic phase of *A. salmonicida* infection.

2 | MATERIAL AND METHODS

2.1 | Bacterial strains, media and reagents

Virulent *A. salmonicida* J223 (NZ_LSGV00000000) (Valderrama et al., 2017) was utilized in this study. Bacteriological media and components were from Difco. Tryptic Soy Broth (TSB) was used routinely. The media were supplemented with 1.5% agar when required. *A. salmonicida* was routinely incubated at 15°C aerobically (180 rpm). Bacterial growth was monitored spectrophotometrically and/or by plating (Valderrama et al., 2017). A single colony of *A. salmonicida* J223 was grown routinely in 3 ml of Trypticase Soy Broth (TSB, Difco) at 15°C in a 16-mm diameter glass tube and placed in a roller for 24 hr. After growth, 300 µl of the overnight culture was added in 30 ml of TSB media using a 250-ml flask and incubated for 24 hr at 15°C with aeration (180 rpm). The bacterial growth was monitored spectrophotometrically until optical density (O.D.) at 600 nm ~ 0.7 (1×10^8 CFU/ml) using the Genesys 10 UV spectrophotometer (Thermo Spectronic, Thermo Fisher Scientific Inc.). Then, the bacterial culture was centrifuged at 4,302 *g* at room temperature for 10 min. The pellet was washed twice with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4; pH 7.4 (Sambrook & Russell, 2001)) and centrifuged at 6,000 rpm at room temperature for 5 min, and finally resuspended in 300 µl of PBS (~5 × 1,010 CFU/ml). The concentrated bacterial inoculum was serial diluted and quantified by plating onto TSA supplemented with Congo red (50 µg/ml).

2.2 | *Salmo salar* holding

All the fish procedures were performed at the Dr. Joe Brown Aquatic Research Building (JBARB) at the Department of Ocean Sciences, Memorial University of Newfoundland, Canada. The animal protocols were approved by the Animal Care Committee at Memorial University protocols #17-01-JS; #17-02-JS), based on the guidelines of the Canadian Council of Animal Care. Atlantic salmon (0.8 ± 0.1 kg) was reared under optimal conditions in 3,800-L tanks with 95%–100% oxygen saturation, ambient photoperiod, flow-through seawater system using seawater filtered, UV-treated, and heated or chilled to maintain water optimal temperature (10–12°C). The biomass density was maintained in a range of 5–30 kg per cubic metre. The fish were fed 3 days per week at a level of 1% body weight per feeding time, using a commercial dry pellet (Skretting, BC, Canada; 50% protein, 18% fat, 1.5% carbohydrate, 3% calcium, 1.4% phosphorus).

2.3 | Primary *S. salar* erythrocyte isolation

Primary *Salmo salar* erythrocytes or red blood cells (RBC) were isolated from head kidney as previously described (Valderrama et al., 2017). Briefly, fish were killed with an overdose of MS222 (400 mg/L; Syndel Laboratories). The head kidney tissue from individual fish was aseptically removed and individually minced through 100 µm nylon sterile cell strainers (Fisher Scientific, Thermo Fisher Scientific) in isolation media ((Leibovitz-15 (Gibco®) supplemented with 2 mM L-glutamine, 4.2 mM $NaHCO_3$, 25 mM HEPES, 1.8 mM glucose, 20 U/ml heparin, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.1% foetal bovine serum (FBS; Gibco)). After this period, 3 ml of cell suspension was centrifuged (400 × g at 4°C) for 40 min in a 34/51% Percoll gradient (GE Healthcare). Following centrifugation, the sedimented RBC were washed twice in isolation media at 400 × g for 5 min at 4°C. The cells were resuspended in isolation media, and viable cells were determined using the Countess™ cell counter (Invitrogen), and trypan blue stain (Invitrogen). The numbers of alive and dead cells were determined at 12 hr and 48 hr post-infection using trypan blue. The cells were stained with trypan blue (0,4%; Invitrogen) in a ratio of 1:1 (10 µl:10 µl) and quantified using Countess™ Cell Counting Chamber Slides (Invitrogen) and Countess® Automated Cell Counter (Invitrogen) according to the manufacturer's instructions. After determining the numbers of cells from each sample, the primary RBC were seeded in 1.5-ml centrifuge tubes at a concentration of 1×10^7 cells/ml and incubated at 15˚C overnight (16 hr). Following incubation, the RBC were washed with PBS twice and incubated in culture media (Leibovitz-15 (Gibco®), supplemented with 2 mM L-glutamine, 4.2 mM $NaHCO_3$, 25 mM HEPES, 1.8 mM glucose, and 5% FBS) until the infection assays.

2.4 | Viability of primary *S. salar* erythrocytes infected with *A. salmonicida*

Viability of primary *S. salar* RBC infected with *A. salmonicida* at different multiplicity of infection (MOI) was determined at 15°C. Isolated primary RBC (1×10^7 cell/ml) were seeded in 1.5-ml centrifuge tubes and incubated at 15°C overnight (16 hr) in culture media without antibiotics. The *S. salar* RBC were infected with 10 µl of *A. salmonicida* at MOI 10:1, 1:1, 1:10; 1:100 and 1:1,000 (erythrocyte:bacteria). The numbers of alive and dead cells were determined at 12 hr and 48 hr post-infection using trypan blue. The cells were stained with trypan blue (0,4%; Invitrogen) in a ratio of 1:1 (10 µl:10 µl) and quantified using Countess™ Cell Counting Chamber Slides (Invitrogen) and Countess® Automated Cell Counter (Invitrogen) according to the manufacturer's instructions. The assays were done by triplicate with cells isolated from independent fishes.

2.5 | Gentamicin exclusion assay

To determine the number of viable *A. salmonicida* that attach and invade the *S. salar* primary RBC, the gentamicin exclusion assay was utilized. The *S. salar* RBC were infected with 10 µl of *A. salmonicida* at different multiplicity of infection (MOI) raging from 10:1 to 1:1,000 (erythrocyte: bacteria). For attachment assays, the RBC were infected with a MOI of 1. The infected RBC were incubated for 1 hr post-infection, then washed 3 times with PBS, and lysed with 400 µl of Triton X-100 (0.01%; Sigma) for 10 min (Sung, Khan, Nawaz, & Khan, 2003), and then, 600 µl of PBS was added to a final volume of 1 ml of lysed RBC suspension. Then, the lysed cells were serially

diluted (1:10) and plated on TSA plates to determine the number of viable bacteria. The TSA plates were incubated at 15°C for 5 days to determine the CFU of *A. salmonicida* per well.

For invasion assay and exclude the extracellular bacteria, the RBC were infected for 1 hr, washed 3 times with PBS, and 1 ml of fresh culture media supplemented with gentamicin (10 μg/ml, a higher concentration than the minimal inhibitory concentration) was added. This treatment is utilized to kill extracellular bacteria. Samples were taken at 2 and 3 hr post-infection for intracellular bacterial counting. Each time, the infected RBC were washed 3 times with PBS, and lysed with 400 μl of Triton X-100 (0.01%; Sigma) for 10 min (Sung et al., 2003) and then 600 μl of PBS was added to complete 1 ml of lysed RBC suspension. Then, the lysed cells were serially diluted (1:10) and plated on TSA plates to determine the number of viable bacteria (Leboffe & Pierce, 2015). The plates were incubated at 15°C for 5 days. Additionally, *S. salar* RBC viability was determined for each time point as described previously.

2.6 | *A. salmonicida* fluorescent labelling

Aeromonas salmonicida was grown in TSB until O.D. 600 nm ~ 0.7 (~1 × 10^8 CFU/ml), centrifuged (6,000 rpm, 10 min) and washed with PBS 3 times. The bacterial pellet was resuspended in 950 μl of bicarbonate buffer (0.1 M, pH 9) according to the dye manufacturer's protocol and mixed with 50 μl of 5-([4,6-dichlorotriazinyl] amino) fluorescein hydrochloride (DTAF) solution (100 μg in dimethyl sulfoxide (DMSO); Sigma). The bacteria were stained for 2 hr at 15°C in darkness. After this period, the cells were washed three times with bicarbonate buffer, resuspended in PBS, and counted for viability.

2.7 | Flow cytometry

To verify the results obtained in the gentamicin exclusion assays, flow cytometry was utilized. *S. salar* RBC were washed with PBS and resuspended in fresh culture media without antibiotics. *S. salar* RBC were infected with 10 μl of *A. salmonicida* suspension (~1 × 10^7 cells; MOI 1:1) or with 10 μl of DTAF-labelled *A. salmonicida* suspension (~1 × 10^7 cells; MOI 1:1) for 1 hr at 15°C, washed 3 times with PBS, and resuspended in 1 ml of fresh culture media supplemented with gentamicin (10 μg/ml). One-hour post-gentamicin, the cells were washed with PBS and resuspended in 500 μl of FACS buffer (1% FBS in PBS). Control consisted in RBC inoculated with formalin-killed *A. salmonicida*, DTAF-labelled formalin-killed *A. salmonicida*, and 1 μm of diameter FITC-labelled latex beads (Polysciences, PA, USA). Negative control consisted in formalin-killed *A. salmonicida* labelled and not labelled, and FITC-labelled latex beads.

Fluorescence was detected from 20,000 cells using a BD FACS Aria II flow cytometer and analysed using BD FACS Diva v7.0 software (BD Biosciences, San Jose, CA, USA). FITC-positive cells, based on gating using the negative-unstained cells, stained *A. salmonicida*, and labelled beads, were identified and the mean fluorescence intensity and percentage of cells of FITC-positive cells determined for each condition. The assays were done by triplicate from 3 independent fish samples.

2.8 | Confocal microscopy imaging of erythrocytes and *A. salmonicida*

Aeromonas salmonicida DTAF labelled and erythrocytes infected were visualized with a Nikon AR1 laser scanning confocal microscope. The infected erythrocytes were stained with 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher) according to the manufacturer's instructions.

2.9 | *S. salar* infection by *A. salmonicida*

Thirty-six naïve Atlantic salmon smolts of 200 g were intraperitoneally injected with 10^4 CFU per 100 g^{-1} of fish in 100 μl of PBS. The infection procedures were done at the AQ3 biocontainment Cold-Ocean Deep-Sea Research Facility (CDRF) under Institutional Animal Care Committee approved protocols (protocol #18-01-JS; #18-02-JS). Mortality was monitored daily for 30 days. Samples of blood were taken at 5 and 10 days post-infection from alive animals. The fish were anaesthetized using MS-222 (50 mg/L) before sampling. The bacterial load in the blood samples was quantified by serial dilution and plating onto TSA. Sample of blood was air dry fixed, stained with Giemsa, and visualized under 1000x oil immersion objective using light microscopy (Olympus CX21).

2.10 | Statistical analysis

All data are shown as the mean ± standard error (SE). Assumptions of normality and homogeneity were tested for the detected variances. A Kruskal–Wallis nonparametric test was performed for gentamicin exclusion assay results. Differences were considered significant at $p < .05$. All statistical analyses were performed using GraphPad Prism (GraphPad Software; www.graphpad.com). The assays were done by triplicate with cells isolated from independent fish.

3 | RESULTS

3.1 | Viability of primary *S. salar* erythrocytes infected with *A. salmonicida*

To determinate the viability of erythrocytes during *A. salmonicida* infection, *S. salar* RBC were infected with different MOIs. The viability was determined at 12 and 48 hr post-infection. *S. salar* RBC showed a high resistance to *A. salmonicida* infection, with a cell viability over 80% at 48 hr in MOIs up to 1:100 (erythrocyte: bacteria) (Figure 1a). No significant differences were detected between MOI 10:1 and 1:100. In contrast, significant differences were detected in MOI 1:1,000. The RBC viability drops under 20% 48 hr post-infection in this treatment (Figure 1a). These results indicate that *A. salmonicida* do not kill RBC in physiological relevant MOIs (1:1).

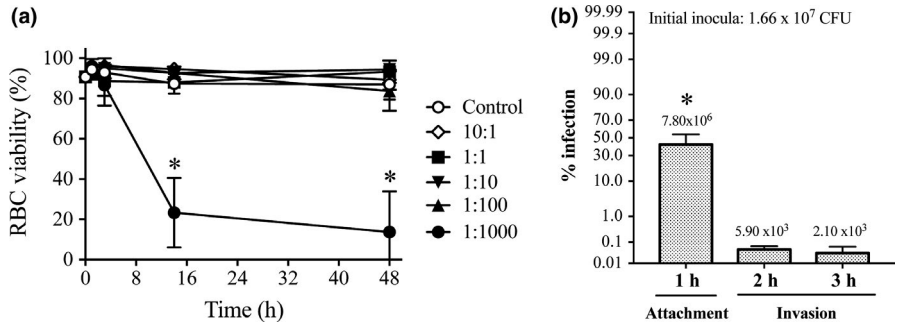

FIGURE 1 Infection of primary *Salmo salar* erythrocytes by *Aeromonas salmonicida*. (a) Survival of primary *S. salar* erythrocytes or red blood cells (RBC) infected with different doses of *A. salmonicida* J223 during 48 hr at 15°C. The RBC cells were infected with different MOIs (erythrocyte: bacteria); (b) Gentamicin exclusion assay in *S. salar* primary erythrocytes. *A. salmonicida* J223 attachment (1 hr post-infection), and invasion (2 hr and 3 hr post-infection) in primary *Salmo salar* erythrocytes at 15°C. The percentages of *A. salmonicida* are respected to the initial inoculum. The experiment was repeated three times in three independent fish each time. The percentage showed above bars indicate the total % of attachment (1 hr post-infection) and invasion (2, and 3 hr post-infection) of *A. salmonicida* in Atlantic salmon RBC, $p < .05$. Each value represents the ± S.E.*M* ($n = 3$). Symbol (*) indicates statistical differences between each time post-infection

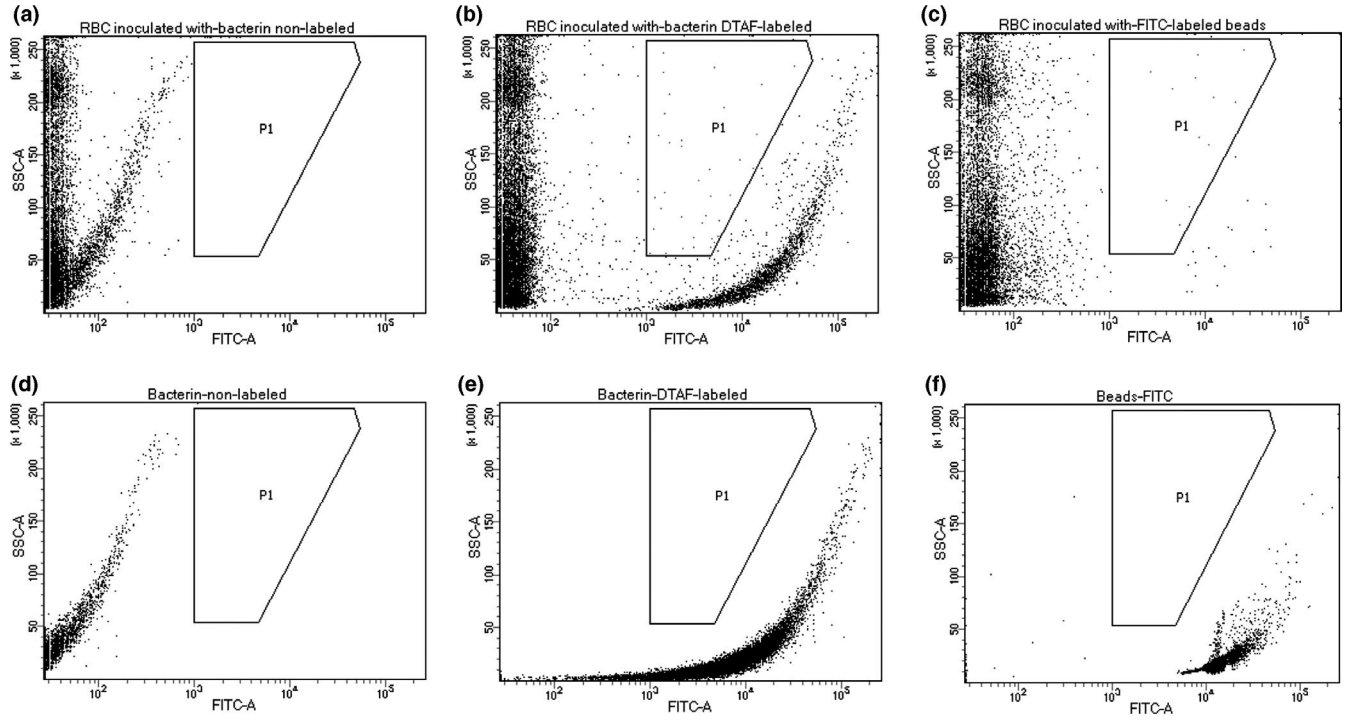

FIGURE 2 *Aeromonas salmonicida* J223 infection of *Salmo salar* primary red blood cells (RBC) evaluated by fluorescence-activated cell sorting (FACS). (a) Control, non-infected erythrocytes; (b) Control, erythrocytes infected with non-labelled *A. salmonicida* 2 hr post-infections and 1 hr post-gentamicin treatment; (c) Erythrocytes infected with DTAF-labelled *A. salmonicida* 2 hr post-infections and 1 hr post-gentamicin treatment; (d) Control, non-labelled *A. salmonicida*; (e). Control, DTAF-labelled *A. salmonicida*. The assays were done at 15°C

3.2 | Gentamicin exclusion assay with *A. salmonicida* in *S. salar* erythrocytes

A. salmonicida has the ability to infect non-phagocytic cells (Valderrama et al., 2017) and phagocytic cells (Soto-Dávila, Hossain, Chakraborty, Rise, & Santander, 2019). Using the gentamicin exclusion assay, we evaluated whether *A. salmonicida* has the ability to infect and survive in primary *S. salar* RBC. First, we evaluate the ability of *A. salmonicida* to attach to RBC in 12-well plates. The mean percentage of *A. salmonicida* attached to RBC was 42.27 ± 11% (7.8 x 10^6 CFU) compared to the initial bacteria inoculate (1.66 x 10^7 CFU) (Figure 1b). After 1 hr post-infection, the cells were treated with gentamicin to exclude extracellular *A. salmonicida*. In contrast to the attached bacteria to RBC, the intracellular *A. salmonicida* was 0.047 ± 0.018% (5.90 x 10^3 CFU) 2 hr post-infection and 0.032 ± 0.031% (2.10 x 10^3 CFU) 2 hr post-infection compared to the initial bacteria inoculate, respectively (Figure 1b). There were no significant differences between 2 hr and 3 hr post-infection.

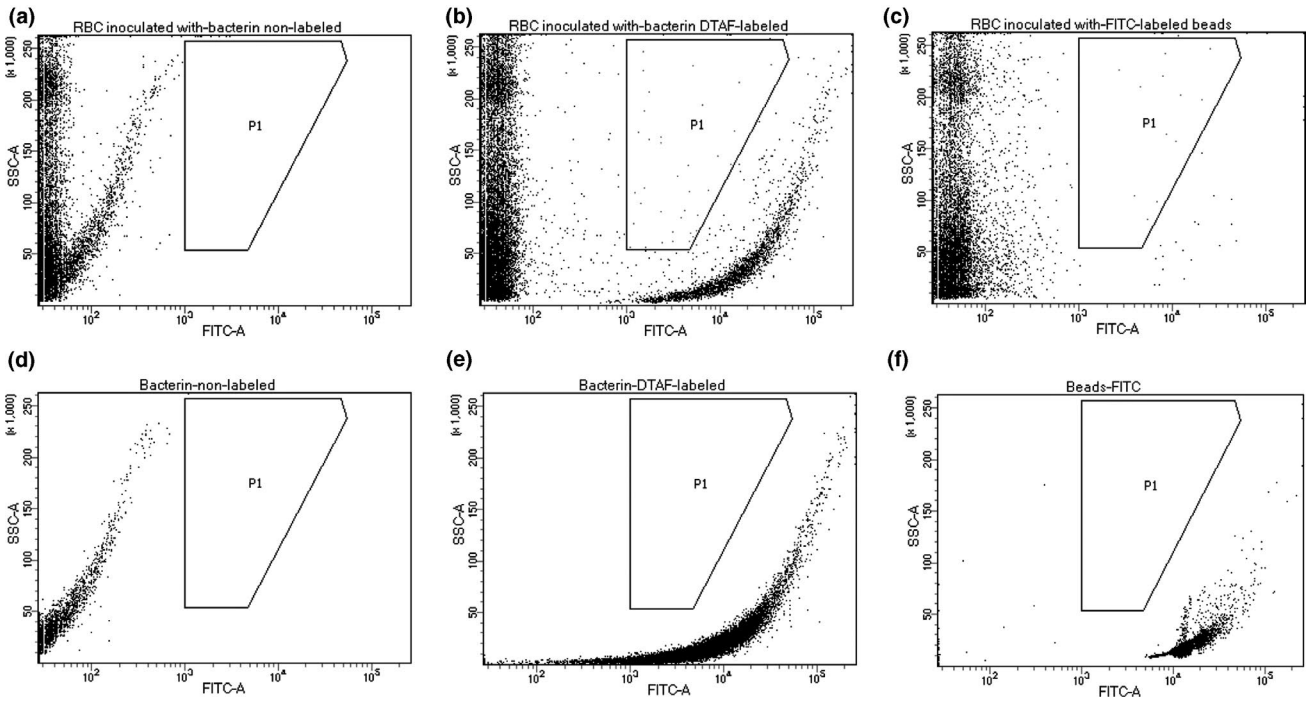

FIGURE 3 Phagocytosis of *Salmo salar* primary red blood cells (RBC) evaluated by fluorescence-activated cell sorting (FACS). (a) Erythrocytes inoculated with non-labelled inactivated *A. salmonicida*; (b) Erythrocytes inoculated with DTAF-labelled inactivated *A. salmonicida*; (c) Erythrocytes inoculated with FITC-labelled latex beads; (d) Control, non-labelled inactivated *A. salmonicida;* (e) Control, DTAF-labelled inactivated *A. salmonicida.* (f) FITC-labelled latex beads. The assays were done at 15°C

3.3 | Flow cytometry

We verified the results obtained by the gentamicin exclusion assay at 2 hr post-infection and 2 hr post-gentamicin treatment using flow cytometry. The flow cytometry data of three independent experiments of infected erythrocytes with DTAF-labelled *A. salmonicida* showed a mean percentage of infection of 0.192 ± 0,074 (Figure 2) after 2 hr post-infection (1 hr post-gentamicin treatment). Additionally, we determined that formalin-inactivated DTAF-labelled *A. salmonicida* and FITC-labelled latex beads are phagocyted by *S. salar* primary RBC, with a mean percentage of 0.140 ± 0.064 and 0.043 ± 0.025, respectively (Figure 3).

3.4 | Confocal microscopy of infected erythrocytes

To determine the presence of intracellular bacteria in primary *S. salar* RBC, *A. salmonicida* labelled with DTAF (Figure 4a) was utilized to infect RBC. The confocal microscopy showed that *A. salmonicida* (Figure 4a) invade primary *S. salar* erythrocytes. *A. salmonicida* was located in the cytoplasm of the erythrocytes (Figure 4b-d). No lysis of the RBC was observed. 3D imaging confirms that *A. salmonicida* was located intracellularly in primary *S. salar* RBC (Figure 4d).

3.5 | *S. salar* infection and erythrocytes colonization by *A. salmonicida*

To determine whether the in vitro results have a biological significance, naïve *S. salar* smolts were infected, and the presence of intraerythrocytic bacteria was evaluated in peripherical RBC. *S. salar* smolts i.p. injected with *A. salmonicida* J223 showed signs of furunculosis 5–8 days post-infection (dpi). The first mortality occurred at 8 dpi and mortality continued through 13 dpi. The per cent survival at 30 dpi was 8.6% (Figure 5a). Bacteraemia was observed at 5 and 10 dpi (Figure 5b). Intraerythrocytic *A. salmonicida* was observed at 5 and 10 dpi (Figure 6).

4 | DISCUSSION

In contrast to the mammalian erythrocytes, piscine erythrocytes are nucleated (Figure 3) and contain the transcriptional and translational machinery necessary to mount an immune response against pathogens (Nombela & Ortega-Villaizan, 2018). Indeed, it was shown that Atlantic salmon erythrocyte infected with infectious salmon anaemia virus (ISAv) resulted in an up-regulation of the *IFN-α* gene (Workenhe et al., 2008). Rainbow trout (*Oncorhynchus mykiss*) erythrocytes have a specific transcriptional responses upon stimulation with poly (I:C), secreting thermo-labile molecules that can modulate the anti-viral response in macrophages, indicating that erythrocytes can trigger a PAMP-mediated response in leucocytes (Morera et al., 2011). Erythrocytes of Nile tilapia (*Oreochromis niloticus*) challenged with poly (I:C) up-regulated several genes related with anti-viral immunity (Shen, Wang, Zhao, & Chen, 2018). Similar results were obtained with erythrocytes infected with piscine orthoreovirus (PRV), showing that infected erythrocytes have the capability of response to viral infection (Dahle et al., 2015;

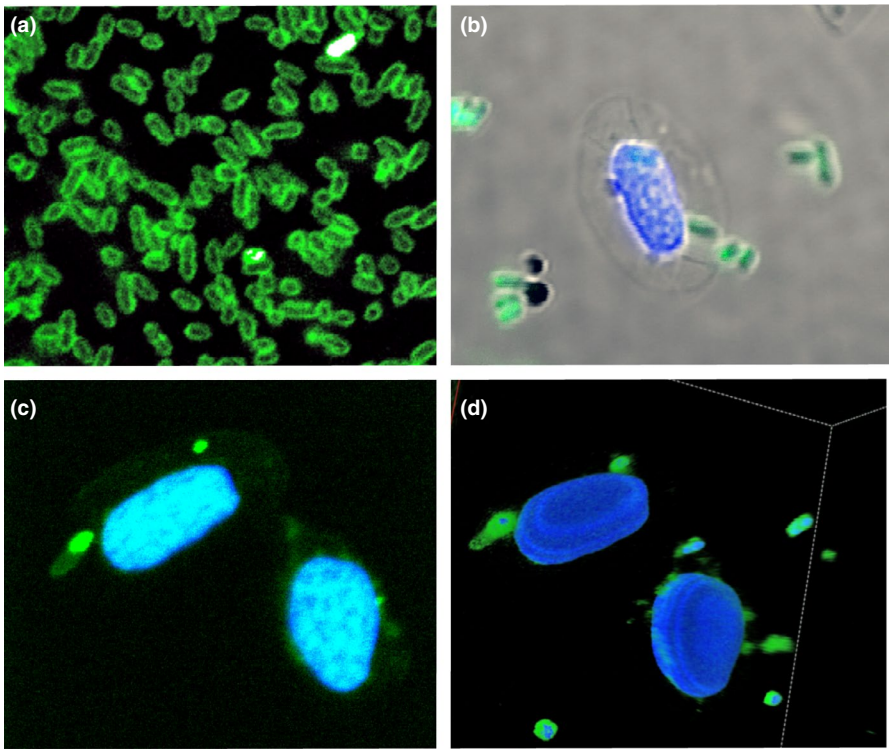

FIGURE 4 *Aeromonas salmonicida* subsp. *salmonicida* infection of *Salmo salar* primary erythrocytes evaluated by confocal microscopy. (a) DTAF-labelled *A. salmonicida* (1000x); (b) Erythrocytes stain with DAPI and infected with DTAF-labelled *A. salmonicida* 2 hr post-infections and 1 hr post-gentamicin treatment (600x); (c) Erythrocytes stained with DAPI and infected with DTAF-labelled *A. salmonicida* after 2 hr post-infection and 1 hr post-gentamicin treatment (600x; dark-field); (d) 3D imaging of erythrocytes stain with DAPI and infected with DTAF-labelled *A. salmonicida* after 2 hr post-infections and 1 hr post-gentamicin treatment (600x). The assays were done at 15°C

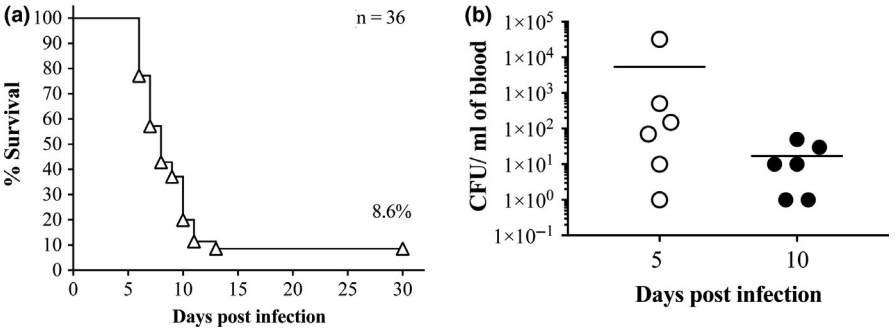

FIGURE 5 Survival and bacterial blood quantification of *Salmo salar* infected with *A. salmonicida*. (a) Survival of *Salmo salar* intraperitoneal infected with *A. salmonicida* J223 (10^4 CFU 100/g) at 10°C; (b) Detection of viable *A. salmonicida* J223 in infected *Salmo salar* blood 5 and 10 days post-infection

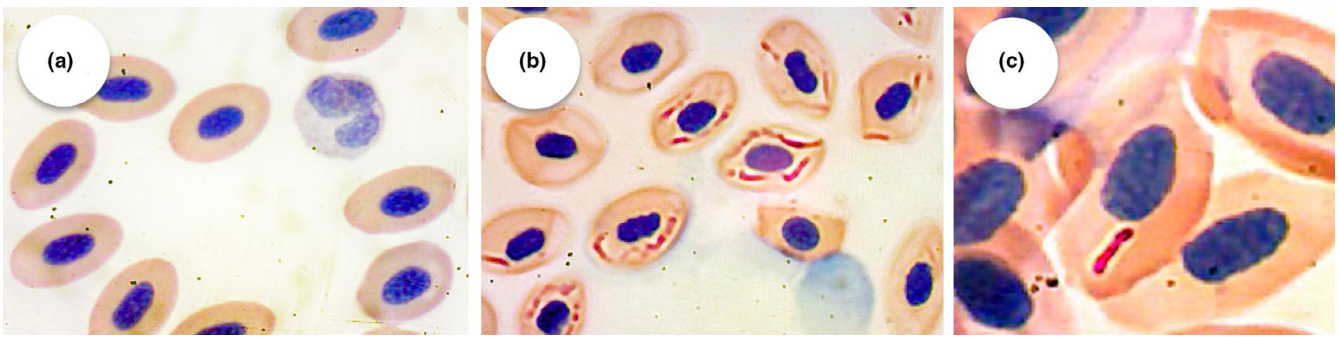

FIGURE 6 Detection of *Aeromonas salmonicida* subsp. *salmonicida* in vivo infected *Salmo salar* erythrocytes. Peripherical blood smear. (a). Erythrocytes from non-infected *S. salar*; (b) *S. salar* erythrocytes 5 days post-infection with *A. salmonicida* (1000x); (c) Zoom of *S. salar* erythrocyte infected with *A. salmonicida* localized intracellularly. All the cells were air dry fixed for 2 hr and stained with Giemsa

Wessel, Krasnov, Timmerhaus, Rimstad, & Dahle, 2018). Also, rainbow trout erythrocytes have the ability to phagocyte *Candida albicans* and form rosettes to facilitate the clearance of pathogens by the fish macrophages (Passantino et al., 2002). In addition, an in silico analysis revealed that rainbow trout RBC may act as professional APCs (antigen-presenting cells) and may participate in the immunological synapse with T and NK cells (Puente-Marin et al., 2018). This suggests that fish erythrocytes have an active participation of the fish immune defence and could be a potential target for pathogens like *A. salmonicida*.

According to Davies & Johnston (Davies & Johnston, 2000) and Morera & Mackenzie (Morera & MacKenzie, 2011), only two

bacterial species have been described to infect fish erythrocytes. *Haemobarthonella* sp and *Eperythrozoon perekropovi*, α-proteobacteria members of the family *Anaplasmataceae*, infect lamprey (*Petromyzon marinus*) and pike (*Esox lucius*) erythrocytes, respectively. Bacteria of this family form cytoplasmatic inclusion in red blood cells, but apparently do not affect the health of the host (Morera & MacKenzie, 2011). Here, using in vivo and in vitro approaches, we present evidence that *A. salmonicida* invades *S. salar* erythrocytes, which is a novel feature for furunculosis that could benefit the bacterial dispersion throughout the host tissues. Additionally, we observed that *S. salar* erythrocytes have a minor phagocytic activity compared to primary macrophages of Atlantic salmon and Atlantic cod (Soto-Davila et al., 2019). These results are coincident with previous studies that showed that *O. mykiss* RBC have phagocytic activity (Passantino et al., 2002).

Aeromonas salmonicida have the capability to infect non-phagocytic cells (Garduño et al., 2000; Valderrama et al., 2017) and phagocytic cells (Soto-Dávila et al., 2019). In vitro tests indicate that the initial infection percentages in RBC are around 42% (Figure 1b; attachment), and similar values were founded in non-phagocytic cells, (Valderrama et al., 2017). However, the attachment in phagocytic cells, like macrophages of Atlantic salmon and Atlantic cod, is around 10% (Soto-Dávila et al., 2019). The number of intraerythrocytic *A. salmonicida* at 2 hr (0.047%; 5.90×10^3 CFU) and 3 hr (0.032%; 2.10×10^3 CFU) post-infection was significantly lower than the intracellular *A. salmonicida* founded in different type of cells at the same infection times. For instance, the number of intracellular *A. salmonicida* founded at 2 hr and 4 hr post-infection in CHSE-214 cells was 0.47% and 0.29%, respectively (Valderrama et al., 2017). The number of intracellular *A. salmonicida* founded at 2 hr and 3 hr post-infection in primary Atlantic salmon cells was 0.42% and 0.26%, respectively, and in Atlantic cod primary macrophages, the number of intracellular *A. salmonicida* founded at 2 hr and 3 hr post-infection was 0.42% and 0.37%, respectively (Soto-Dávila et al., 2019). The results observed in RBC infected with *A. salmonicida* were verified by the FACS results (Figure 2). Using FACS, we determined that 2 hr post-infection (1 hr after-gentamicin treatment) the intracellualr bacteria was ~0,192 ± 0,074. Although the percentage of intracellular bacteria founded in the FACS assays was higher compared to the gentamicin exclusion assay (Figure 1b), these differences are due to that the FACS assay detects the total number of cells, including death and alive. Contrary, the gentamicin exclusion assay detects only viable cultivable bacteria. Phagocytosis of FITC-labelled latex beads was lower than phagocytosis of inactivated bacterin, 0.140 ± 0.064 and 0.043 ± 0.025, respectively (Figure 4). This could be due to the biological nature of the bacterin (e.g. presence of proteins, lipopolysaccharide, etc) that enhance the uptake by the RBC in contrast to a latex particle.

In vivo assays showed that *A. salmonicida* have an infection phase in smolt Atlantic salmon peripherical RBC (Figure 4). The range of bacteria per RBC was variable during the first 5 days post-infection, perhaps product of the fish innate immune response. At 10 days post-infection, total viable bacterial counts were more stable. Mortality was initiated at 6 days post-infection coinciding with the presence of bacteria in the blood. Only intraerythrocytic bacteria was observed at 5 d and 10 d post-infection. Extracellular *A. salmonicida* was not observed in the blood smears samples analysed (Figure 6). However, in vivo certain RBC showed more than one intracellular bacteria per cell (Figure 6b). Also, it was possible to observe an *A. salmonicida*-containing vesicle (Figure 6c). This last result was not observed in vitro, suggesting that infection of RBC by *A. salmonicida* is part of the infection process in vivo and could be more pronounced in peripherical RBC.

In summary, we found that *Salmo salar* erythrocytes have phagocytic capabilities (Figures 1–3), but lower than primary macrophages (Soto-Davila et al., 2019). Although the *S. salar* erythrocytes have the ability to phagocyte bacteria and particles (Figures 2–4), *A. salmonicida* remains in a viable cultivable state in erythrocytes and blood of infected animals (Figure 1b). Coincident, all the infected fish that presented bacteraemia die during the next 3 days (Figure 5a). The presence of intracellular *A. salmonicida* in the erythrocytes was observed in all fish sampled (Figure 6b,c), concurring with an infection process. These results describe a novel infection phase of *A. salmonicida*.

5 | CONCLUSION

Our data showed that *A. salmonicida* J223 does not kill *S. salar* primary erythrocytes (Figure 1a) but invades them intracellularly (Figures 1–6). These results suggest that *A. salmonicida* might use the erythrocytes to bypass host defence mechanisms, such as complement lysis, circulating phagocytes, antibodies, or antibacterial peptides, and to gain access to nutrients sequestered by the host, such as iron. Also, the intraerythrocytic phase of *A. salmonicida* could benefit the bacterial dispersion throughout the host tissues.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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REFERENCES

Clauss, T. M., Dove, A. D., & Arnold, J. E. (2008). Hematologic disorders of fish. *Veterinary Clinics of North America Exotic Animal Practice*, *11*(3), 445–462. https://doi.org/10.1016/j.cvex.2008.03.007

Dahle, M. K., Wessel, O., Timmerhaus, G., Nyman, I. B., Jorgensen, S. M., Rimstad, E., & Krasnov, A. (2015). Transcriptome analyses of Atlantic salmon (*Salmo salar* L.) erythrocytes infected with piscine orthoreovirus (PRV). *Fish & Shellfish Immunology*, *45*(2), 780–790. https://doi.org/10.1016/j.fsi.2015.05.049

Daly, J., Kew, A., Moore, A., & Olivier, G. (1996). The cell surface of *Aeromonas salmonicida* determines *in vitro* survival in cultured brook trout (*Salvelinus fontinalis*) peritoneal macrophages. *Microbial Pathogenesis*, *21*, 447–461. https://doi.org/10.1006/mpat.1996.0075

Davies, A. J., & Johnston, M. R. L. (2000). The biology of some intraerythrocytic parasites of fishes, amphibia and reptiles. *Advances in Parasitology*, *45*, 1–107.

Dehio, C. (2008). Infection-associated type IV secretion systems of *Bartonella* and their diverse roles in host cell interaction. *Cellular Microbiology*, *10*(8), 1591–1598. https://doi.org/10.1111/j.1462-5822.2008.01171.x

Effendi, I., & Austin, B. (1995). Uptake of *Aeromonas salmonicida* by Atlantic salmon (*Salmo salar* L.). *Bulletin of the European Association of Fish Pathologists*, *15*(4), 115–118.

Farrel, A. P. (2011). Cellular composition of the blood. In A. P. Farrel (Ed.), *Encyclopedia of fish physiology: From genome to environment* (Volume *2*: Gas exchange, internal homeostasis, and food uptake, pp. 984–991). Cambridge, US: Elsevier.

Garduño, R., & Kay, W. (1992). Interaction of the fish pathogen *Aeromonas salmonicida* with rainbow trout macrophages. *Infection and Immunity*, *60*(11), 4612–4620.

Garduño, R., Moore, A., Olivier, G., Lizama, A., Garduño, E., & Kay, W. (2000). Host cell invasion and intracellular residence by *Aeromonas salmonicida*: Role of the S-layer. *Canadian Journal of Microbiology*, *46*, 660–668.

Groebel, K., Hoelzle, K., Wittenbrink, M. M., Ziegler, U., & Hoelzle, L. E. (2009). *Mycoplasma suis* invades porcine erythrocytes. *Infection and Immunity*, *77*(2), 576–584. https://doi.org/10.1128/IAI.00773-08

Horzempa, J., O'Dee, D. M., Stolz, D. B., Franks, J. M., Clay, D., & Nau, G. J. (2011). Invasion of erythrocytes by *Francisella tularensis*. *Journal of Infectious Diseases*, *204*(1), 51–59. https://doi.org/10.1093/infdis/jir221

Leboffe, M. J., & Pierce, B. E. (2015). *Microbiology: Laboratory theory & application*, (4th edn). Englewood, CO: Morton Publishing.

Morera, D., & MacKenzie, S. A. (2011). Is there a direct role for erythrocytes in the immune response? *Veterinary Research*, *42*, 1–8. https://doi.org/10.1186/1297-9716-42-89

Morera, D., Roher, N., Ribas, L., Balasch, J. C., Doñate, C., Callol, A., … MacKenzie, S. A. (2011). RNA-Seq reveals an integrated immune response in nucleated erythrocytes. *PLoS ONE*, *6*(10), 1–9. https://doi.org/10.1371/journal.pone.0026998

Munn, C., Ishiguro, E., Kay, W., & Trust, T. (1982). Role of surface components in serum resistance of virulent *Aeromonas salmonicida*. *Infection and Immunity*, *36*(3), 1069–1075.

Nombela, I., & Ortega-Villaizan, M. D. M. (2018). Nucleated red blood cells: Immune cell mediators of the antiviral response. *PLoS Path*, *14*(4), 1–6. https://doi.org/10.1371/journal.ppat.1006910

Passantino, L., Altamura, M., Cianciotta, A., Patruno, R., Tafaro, A., Jirillo, E., & Passantino, G. (2002). Fish Immunology. I. Binding and engulfment of *Candida albicans* by erythrocytes of rainbow trout (*Salmo gairdneri* Richardson). *Immunopharmacology and Immunotoxicology*, *24*(4), 665–678.

Puente-Marin, S., Nombela, I., Ciordia, S., Mena, M. C., Chico, V., Coll, J., & Ortega-Villaizan, M. D. M. (2018). In Silico functional networks identified in fish nucleated red blood cells by means of transcriptomic and proteomic profiling. *Genes (Basel)*, *9*(4), 1–11. https://doi.org/10.3390/genes9040202

Sambrook, J., & Russell, D. (2001). Molecular Cloning. A laboratory manual. In J. Sambrook & D. Russell (Eds.), *Molecular Cloning. A laboratory manual*, (Vol. *3*, 3rd ed). New York, NY: Cold Spring Harbor.

Shen, Y., Wang, D., Zhao, J., & Chen, X. (2018). Fish red blood cells express immune genes and responses. *Aquaculture and Fisheries*, *3*(1), 14–21. https://doi.org/10.1016/j.aaf.2018.01.001

Soto-Dávila, M., Hossain, A., Chakraborty, S., Rise, M. L., & Santander, J. (2019). *Aeromonas salmonicida* subsp. *salmonicida* Early Infection in Atlantic Cod (*Gadus morhua*) Primary Macrophages. *Frontiers in Immunology*, *10*(1237), https://doi.org/10.3389/fimmu.2019.01237

Sung, K., Khan, S. A., Nawaz, M. S., & Khan, A. A. (2003). A simple and efficient Triton X-100 boiling and chloroform extraction method of RNA isolation from Gram-positive and Gram-negative bacteria. *FEMS Microbiology Letters*, *229*(1), 97–101. https://doi.org/10.1016/s0378-1097(03)00791-2

Svendsen, Y., Dalmo, R. A., & Bogwald, J. (1999). Tissue localization of *Aeromonas salmonicida* in Atlantic salmon, *Salmo salar* L., following experimental challenge. *Journal of Fish Diseases*, *22*, 125–131.

Valderrama, K., Saravia, M., & Santander, J. (2017). Phenotype of *Aeromonas salmonicida* sp. *salmonicida* cyclic adenosine 3',5'-monophosphate receptor protein (Crp) mutants and its virulence in rainbow trout (*Oncorhynchus mykiss*). *Journal of Fish Diseases*, *40*(12), 1849–1856. https://doi.org/10.1111/jfd.12658

Vanya Ewart, K., Williams, J., Richards, R. C., Gallant, J. W., Melville, K., & Douglas, S. E. (2008). The early response of Atlantic salmon (*Salmo salar*) macrophages exposed *in vitro* to *Aeromonas salmonicida* cultured in broth and in fish. *Developmental & Comparative Immunology*, *32*(4), 380–390. https://doi.org/10.1016/j.dci.2007.07.005

Vogl, G., Plaickner, A., Szathmary, S., Stipkovits, L., Rosengarten, R., & Szostak, M. P. (2008). *Mycoplasma gallisepticum* invades chicken erythrocytes during infection. *Infection and Immunity*, *76*(1), 71–77. https://doi.org/10.1128/IAI.00871-07

Wessel, O., Krasnov, A., Timmerhaus, G., Rimstad, E., & Dahle, M. K. (2018). Antiviral responses and biological consequences of piscine orthoreovirus infection in salmonid erythrocytes. *Frontiers in Immunology*, *9*, 1–6. https://doi.org/10.3389/fimmu.2018.03182

Witeska, M. (2013). Erythrocytes in teleost fishes: A review. *Zoology and Ecology*, *23*(4), 1–8. https://doi.org/10.1080/21658005.2013.846963

Workenhe, S. T., Kibenge, M. J., Wright, G. M., Wadowska, D. W., Groman, D. B., & Kibenge, F. S. (2008). Infectious salmon anaemia virus replication and induction of alpha interferon in Atlantic salmon erythrocytes. *Virology Journal*, *5*, 1–12. https://doi.org/10.1186/1743-422X-5-36

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