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Common carp (*Cyprinus carpio*) blood cells are modulated by a parasitic antimicrobial peptide, A-2S and *Edwardsiella* spp. strains

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ABSTRACT

Aquaculture industry faces significant challenges due to edwardsiellosis, a bacterial disease caused by *Edwardsiella* species, particularly *E. tarda*, which leads to high mortality in farmed fish and consequent financial losses. This pathogen, opportunistic in nature, becomes virulent under unsuitable conditions for fish, causing severe tissues damage in the host. Antibiotic treatments are increasingly ineffective due to *Edwardsiella* antibiotic resistance, necessitating alternative solutions. This for, we tested the antimicrobial efficiency of the cecropin anisaxin-2S (A-2S), an antimicrobial peptide (AMP) isolated from marine nematodes *Anisakis simplex* and *A. pegreffii* against six *Edwardsiella* spp. and their strains (in total 11). Based on the significant differences in the observed antimicrobial activity, *E. tarda* strain ET20 (fish isolate, A-2S resistant) and DSM 30052 (human isolate, A-2S sensitive) were further *in vitro* tested in the common carp (*Cyprinus carpio*) white and red blood cells treated with anisaxin-2S. A-2S significantly stimulated the production of ROS by erythrocytes and leukocytes challenged with both strains, contributing to a higher cell survival, compared to A-2S non-treated blood cells exposed to bacteria. Pro-inflammatory response was equally expressed in both cell types upon stimulation with *E. tarda* strains but has been further amplified upon A-2S treatment. A-2S alone induced significant upregulation of *il-1β* in both cell types and time points. The strong antimicrobial activity and immunostimulatory properties of this anisaxin justify further trials against edwardsiellosis.

1. Introduction

The global aquaculture industry has continued to face the growing challenges of edwardsiellosis over the years, a common bacterial disease with zoonotic character, affecting different classes of animals, including freshwater and marine fish [1–4]. The causative agent is Gram-negative bacteria of the genus *Edwardsiella*, including *E. anguillarum*, *E. piscicida*, *E. ictaluri, E. hoshinae,* and *E. tarda* [5]. The latter has been identified as a highly contagious and virulent species responsible for the high mortality of farmed fish, associated with large financial losses [1]. Namely, over 31 cultured fish species have been reported to be affected by *E. tarda* [6, 7], whose incidence in the Mediterranean Sea is starting to emerge as a new threat [8] since the first pathogen identification in 1962 [9,10].

Infected fish rarely survive *E. tarda* infection because of the pathogen's rapid and severe damage to kidney, liver, and spleen [8,9,11–13]. Due to *E. tarda* pronounced virulence in fish compared to other susceptible animals [7], the application of antibiotics in managing the infection in aquaculture settings is required. However, one of the adaptive strategies of *E. tarda* in evading the effect of antibiotics is through the expression of antibiotic-resistant genes in their plasmid and chromosomes [14–16]. Consequently, several classes of antibiotics including penicillin, quinolone, amphenicol, beta-lactam, tetracyclines,

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lincosamide, macrolide, sulfonamides, and fluoroquinolone have been reported to be ineffective in the treatment of the infection [16–20]. Despite this, their persistent application keeps facilitating the emergence and spread of multiple-antibiotic resistant genes of *E. tarda*, also among other pathogenic strains in the aquatic ecosystems [21–23].

In the search for sustainable alternatives to antibiotic treatment of *E. tarda* infection, and mitigation of antimicrobial resistance (AMR) in the aquaculture industry, antimicrobial peptides (AMPs) have been identified as attractive candidates due to their ability to unselectively damage pathogen membrane, modulate the host immune system, and activate wound healing mechanisms [24–26]. For example, the bacteriostatic and bactericidal properties of amidated AMP from marine lugworm *Arenicola marina* against *E. tarda* demonstrated greater efficiency compared to conventionally used norfloxacin [25].

AMPs, also known as host defense peptides (HDPs), are short-chained peptides of 10–60 amino acids [26] synthesized mainly by ribosomes in the phagocytic and epithelial cells of single-celled to complex organisms, including fungi, insects, plants, and animals with characteristic immunomodulatory properties [23]. Their evolutionary role in acting as the first defense responder against invading pathogens in the host system has made them a key component of the innate immune system. The ability of AMPs to interact with immune cells through activation of receptors that lead to cascades of intracellular reactions resulting in the production of cytokines and chemokineshas been well reported [27]. For instance, defensin, an AMP in vertebrates, is known to enhance the activity of macrophages and mast cells through the production of proinflammatory molecules such as interleukin-1β and histamine [28,29]. However, further assessment of AMP toxicity on host cells and genome, and their stability in the host serum are required for the practical application of AMPs in the treatment of infections [30].

Anisaxins, a group of cecropin-like AMPs from the zoonotic marine nematodes *Anisakis simplex* and *A. pegreffii*, have been highlighted to exhibit potency against human multidrug-resistant (MDR) Gram-negative bacteria [30]. Among the characterized peptides, anisaxin-2S (A-2S) from *A. simplex* is recognized for its exceptional bactericidal effect and no cyto- and genotoxicity towards host cells [30], suggesting A-2S as an alternative to control *E. tarda* infection in cultured fish. Majstorović et al. [31] confirmed the ability of A-2S to modulate the expression of proinflammatory cytokines in common carp fish blood cells *in vitro* and *in vivo* against bacterial (*Aeromonas hydrophila*) and parasitic infections (*Sphaerospora molnari*).

To understand better the potential of anisaxin-2S use in aquaculture, this study firstly aimed to assess the antimicrobial efficacy of A-2S against different *Edwardsiella* spp. Secondly, to address the interaction between the innate immunity of the host, the bacterium, and anisaxin, the immunomodulatory properties of A-2S were tested *in vitro* in fish erythrocytes and leukocytes challenged with two *E. tarda* strains – the isolate ET20 (from fish) that was shown to be resistant to A-2S, and the isolate DSM 30052 (from humans), shown to be susceptible to A-2S. Expression of innate immunity genes in blood cells and the measurement of reactive oxygen species (ROS) were performed to evaluate the level of inflammatory environment, while the effect of A-2S on the cell wall of *E. tarda* was evaluated by transmission electron microscopy.

2. Materials and methods

2.1. Ethics statement

All experimental protocols were approved by the Resort Professional Commission of the Czech Academy of Sciences (CAS) for Approval of Projects of Experiments on Animals. The manipulation and sampling protocols were executed with a consistent approach and in strict adherence to the provisions of the Czech legislation governing the welfare of animals, as set forth in the Protection of Animals Against Cruelty Act No. 246/1992. All procedures were authorized by the Czech Ministry of Agriculture. The study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org).

2.2. Testing of antimicrobial activity of anisaxin-2S

Antibacterial capacity was tested against 11 *Edwardsiella* spp. strains, including *E. anguillarum*, *E. hoshinae*, *E. ictaluri*, *E. piscicida*, and *E. tarda* from the inhouse collection of the Hellenic Centre for Marine Research (HCMR). The minimum inhibitory concentrations (MIC10, MIC50, MIC90), minimum bactericidal concentrations (MBC10, MBC50, MBC90), and non-biocidal concentration (NBC) were calculated. The mean inoculation titer was 9.5×10^5 cultured in Mueller-Hinton broth with addition of 0.5 % NaCl. The assays were performed using the broth microdilution method described previously [32]. In brief, each MIC measurement included the addition of 100 μL of an anisaxin-2S (A-2S) standard solution (200 μM in sterile medium; 688 μg/mL) in a single well of a 384-well polystyrene microplate, the addition of 50 μL of sterile medium in the following 10 wells, and the implementation of serial two-fold dilutions by transferring 50 μL from well to well. Then, 50 μL of medium containing the bacterial strain under investigation were added in all 11 wells (final total volume: 100 μL per well) to achieve A-2S concentrations ranging from 0.098 to 100 μM (i.e., 0.34–344 μg/mL). To avoid the "edge-effect", all peripheral wells of the microplates were not used for microcultures (i.e., they were filled with 100 μL of sterile water), while all measurements were carried out in triplicate for each bacterial strain. In addition, Growth Controls (i.e., no-A-2S cell culture) and sterility controls (i.e., no-cell growth medium) were also included in every microplate that was assayed. Loading of the assay components (i.e., A-2S solution, bacterial suspension) on the microplate and the preparation of serial dilutions were performed using an automated liquid handling system (Biomek 2000; Beckman Coulter, Fullerton, CA, USA). Microplates were incubated at 25 °C for 22 h and bacterial growth in each microculture was monitored by measuring optical density at 600 nm (OD600) every 20 min using a microplate reader (Infinite F200 PRO, Tecan GmbH, Grödig, Austria). The area under the growth curve (i.e., OD600 vs. time) was integrated for each microdilution assay and the data were used to determine MIC and non-inhibitory concentration (NIC) in GraphPad Prism v9.0 software (www.graphpad.com). After the incubation, 5 μL from each microculture was immediately transferred into a fresh microplate containing 100 μL of sterile medium in each well (without A-2S), and bacterial growth was monitored for another 22 h to determine MBC and NBC.

2.3. Experimental animals

Specific pathogen-free (SPF) common carp (*Cyprius carpio*) was reared from peroxide-treated fertilized eggs (700 mg/L for 15 min) in an experimental recirculating system in the animal facility of the Institute of Parasitology, Biology Centre CAS (BCAS). Fish were housed in separate tanks with UV-irradiated and ozonized water at 21 ± 1 °C, with water quality parameters (oxygen, pH, ammonia, nitrite, and nitrates) monitored daily using probes and titration tests. Ammonia levels were kept at <0.02 mg/L. During the experiment, fish (weight 75 g ± 5 g, length 14 cm ± 2 cm) were selected and fed twice a day with a commercial carp diet (Skretting) at a daily rate of 1.5 % of their body weight.

2.4. Edwardsiella tarda culture

Two strains of *E. tarda*, deposited in the collection of the Aquaculture Microbiology Laboratory of the Institute of Marine Biology, Biotechnology and Aquaculture of the Hellenic Center of Marine Research (HCMR) that have shown contrasting levels of resistance against A-2S were used for the *in vitro* assays. *E. tarda* isolate ET20 (fish) was shown to be resistant to A-2S (MIC = 134.53 μM), while the isolate DSM 30052 (human) was shown to be susceptible to A-2S (MIC = 0.424 μM). Cultures of both strains in Brain Heart Infusion soft agar supplemented with 0.5 % NaCl were shipped to BCAS. Bacteria were grown in the laboratory

in Luria-Bertani (LB) agar and LB broth at 37 °C for 24 h. Next, bacteria were centrifuged at 1600 g for 10 min, and the bacterial pellet was reconstituted in sterile PBS (280 mOsM). Optical density was measured at 625 nm, and data were aligned with a previously derived McFarland scale to determine the bacterial concentration.

Additionally, the PCR was performed with *E. tarda* strains using specific primers for 16S rRNA for additional confirmation of the bacterial species and the purity of the strain used for the downstream experiments. Primers were custom designed using the software Geneious Prime (Supplementary Table 1.)

2.5. Blood preparation

In total 500 μL of whole blood was obtained from each of four individual fish using syringes rinsed with heparin solution at a concentration of 5000 IU/mL. Prior to this, the fish had been anesthetized with MS-222. The blood samples were then mixed with cell culture medium RPMI 1640 (from Gibco, USA) at a 1:4 ratio for dilution. Next, the diluted blood was carefully layered on top of Ficoll-Paque PREMIUM medium (density 1.077 g/mL, from Cytiva, Sweden) for density centrifugation, which allowed for the separation of red blood cells (RBCs) from white blood cells (WBCs). The centrifugation process was set at 500 g for 10 min, with minimal acceleration and deceleration. Following centrifugation, the pellet containing the erythrocyte fraction, or the buffy coat layer enriched with leukocytes was collected. To ensure the purity of both the WBC and RBC suspensions, a second round of density centrifugation was performed. For further verification of purity, the samples underwent additional assessment using both flow cytometry and light microscopy.

2.6. Flow cytometry

All changes were captured using FACSCanto II (BD Biosciences, USA). To assess the susceptibility of *E. tarda* to A-2S, ET20 and DSM 30052 were incubated at a concentration of 5×10^4 CFU/mL with the following concentrations of A-2S for 1 h: 25 ng/mL, 50 ng/mL, and 100 ng/mL and measured by flow cytometry. Selected concentrations represent the sub-MIC concentrations intending not to damage and kill the bacterium irreversibly but to exert stress on the bacterial cell wall over experimental time. For evaluating the purity of the isolated blood cells and monitoring changes in their number and activity, 2 μL of whole blood was suspended in 200 μL of RPMI-1640 and each sample was analyzed for 20 s at a flow rate of 60 μL/min by flow cytometry. RBC and WBC were distinguished based on forward scatter width (FSC-W) and side scatter area (SSC-A).

To measure *in vitro* detection of reactive oxygen species (ROS), RBCs and WBCs at a concentration of 1×10^6 cells/mL were stimulated with ET20 and DSM 30052 strains at 5×10^4 CFU/mL. The cells were resuspended in 1 mL of RPMI-1640 and incubated for 1 h. ROS detection was carried out by incubating the cells for 15 min at 26 °C with DHR123.

2.7. In vitro stimulation assay of blood cells with E. tarda

Freshly isolated RBCs and WBCs were counted in Bürker chambers using the Olympus BX51 light microscope and their concentrations were adjusted to 1×10^6 per mL of RPMI-1640. These cells were then added individually to a 24-well plate; each cell type was placed in separate wells. *E. tarda* strains, maintained in LB broth, were pelleted, resuspended in RPMI-1640, and quantified. A concentration of 1×10^4 CFU/mL was added to all cell suspensions, except for the negative control. The experimental setup included two conditions: i) RBCs or WBCs cultured in RPMI-1640 alone, and ii) RBCs or WBCs challenged with live bacterial strains. Each biological replicate was plated in its corresponding well without mixing. The cells were incubated at 26 °C with 5 % CO_2 for 1 and 6 h. Following incubation, the cells were harvested, centrifuged at 500 g for 5 min at 4 °C, and pellets were collected for immediate RNA isolation.

2.8. qPCR and cytokines gene expression analysis in E. tarda-stimulated blood cells

To evaluate inflammatory responses following *in vitro* stimulation, we measured the expression of cytokines *tnfα* (tumor necrosis factor alpha), *il-1β* (interleukin 1 beta), *il-6* (interleukin 6), and *ifnγ* (interferon gamma) in RBC and WBC using quantitative reverse transcription polymerase chain reaction (qRT-PCR). The expression levels of these target genes were normalized to the housekeeping gene elongation factor 1a (*ef-1a*). Primer details are provided in Supplementary Table 1.

RNA was freshly extracted from RBCs and WBCs using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The integrity of the RNA was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). For cDNA synthesis, 100 ng of RNA per specimen was used with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Germany), following the manufacturer's recommendations and the prescribed thermocycler program. Non-template controls (NTC) and non-reverse transcriptase controls (RT-) were included.

Technical duplicate measurements were performed using the QuantStudio 6 (Applied Biosystems, USA). PCR reactions consisted of 2 μL of 10–20-fold diluted cDNA, 10 μL of Fast SYBR Green Master Mix, and 0.4 μM of each specific primer set in 20 μL mixtures. If discrepancies of more than half a cycle were observed between technical duplicates, the specimens were reanalyzed in a new reaction. Data analysis was conducted using the Pfaffl method [33].

$$Ratio = \frac{\left(E_{target}\right)^{\Delta Ct} Target^{(control-sample)}}{\left(E_{reference}\right)^{\Delta Ct} Reference^{(control-sample)}}$$

where E is the efficiency of the primers for each gene, *Target* represents the gene of interest and *Reference* is the housekeeping gene elongation factor 1a (*ef-1a*).

2.9. Transmission electron microscopy (TEM)

For TEM, 1×10^5 bacterial cells of both *E. tarda* strains were incubated with 10 μM A-2S separately for 1 h. Additionally. 1×10^6 blood cells were incubated with 1×10^4 of both bacterial strains for 1 h and centrifuged at 1800 g. Live cells were then frozen with a Leica EM PACT2 high-pressure freezer (Leica Microsystems) and subsequently freeze-substituted in 100 % acetone containing 2 % OsO_4 for 96 h at −90 °C using a Leica AFS (Leica Microsystems). The temperature was raised 5 °C/h to −20 °C and after 24 h, samples were rinsed in acetone and infiltrated in graded series of resin (EMBed 812, EMS) solutions (25 %, 50 %, 75 % in acetone) 1 h each. Cells were infiltrated in pure resin overnight, embedded in fresh resin, and polymerized at 60 °C for 48 h. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by JEOL JEM-1400 microscope (JEOL, Akishima, Tokyo, Japan) operating at an accelerating voltage of 120 kV. The XAROSA 20-megapixel CMOS camera (EMSIS GmbH) was used for imaging.

3. Results

3.1. Edwardsiella spp. show different in vitro susceptibility to anisaxin-2S

The results of the antimicrobial capacity of A-2S against the 11 strains of *Edwardsiella* spp., as presented in Table 1, indicated a remarkable variation in MIC, MBC, NBC, and NIC values depending on *Edwardsiella* spp., ranging between <0.10 μM to >100 μM of A-2S (over the upper reading threshold).

A-2S had the highest MIC and MBC readings of 134.53 ± 10.32 μM and >100 μM, respectively, against the ET20 strain of *E. tarda*; meanwhile, for the DSM 30052 strain of the same bacterial species, much

Table 1
Antimicrobial activity of cecropin-like peptide, anisaxin-2S, against strains of *Edwardsiella* spp.

Edwardsiella spp.	Strains	Anisaxin-2S Antimicrobial Activity (μM)									
		MIC	NIC	MIC10	MIC50	MIC90	MBC	NBC	MBC10	MBC50	MBC90
E. tarda	ET20	134.53 ± 10.32	14.59 ± 3.79	13.84 ± 4.06	44.78 ± 3.85	149.4 ± 21.13	>100.00	>100.00	>100.00	>100.00	>100.00
	DSM 30052	0.42 ± 0.06	0.21 ± 0.01	0.18 ± 0.02	0.27 ± 0.02	0.42 ± 0.10	3.36 ± 0.90	0.48 ± 0.01	0.40 ± 0.00	1.25 ± 0.19	3.98 ± 1.16
E. anguillarum	EA011113	>100.00	>100.00	>100.00	>100.00	>100.00	>100.00	>100.00	>100.00	>100.00	>100.00
	51+C	93.28 ± 16.39	7.15 ± 1.15	6.07 ± 1.11	25.17 ± 0.28	107.02 ± 21.32	>100	>100	>100	>100	>100
	DSM 27202	41.53 ± 6.16	31.13 ± 1.84	27.37 ± 0.55	31.22 ± 2.48	35.42 ± 5.26	45.78 ± 11.18	44.90 ± 2.68	39.64 ± 5.88	43.92 ± 8.38	47.20 ± 11.02
	Edw 7.6	60.11 ± 4.37	50.35 ± 3.15	48.67 ± 3.31	54.59 ± 3.55	60.69 ± 2.90	93.07 ± 6.96	74.21 ± 12.08	64.82 ± 13.52	72.25 ± 14.69	81.22 ± 16.99
E. piscicida	DSM 104083	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
	Edw new	>100.00	>100.00	>100.00	>100.00	>100.00	>100.00	>100.00	>100.00	>100.00	>100.00
Edwardsiella sp.	markellos	12.08 ± 1.04	0.59 ± 0.05	0.46 ± 0.04	2.65 ± 0.03	15.45 ± 1.74	144.40 ± 10.21	58.85 ± 9.49	52.71 ± 8.82	91.21 ± 4.37	159.30 ± 11.25
E. ictaluri	DSM 13697	0.10 ± 0.00	0.09 ± 0.00	0.07 ± 0.02	0.09 ± 0.00	0.12 ± 0.03	6.96 ± 0.93	0.17 ± 0.03	0.17 ± 0.02	1.13 ± 0.01	7.38 ± 0.79
E. hoshinae	DSM 13771	2.84 ± 0.31	0.53 ± 0.01	0.46 ± 0.01	1.18 ± 0.06	3.04 ± 0.38	7.14 ± 0.05	4.28 ± 0.62	3.75 ± 0.62	5.30 ± 0.46	7.52 ± 0.07

NOTE: Mean value ± Standard deviation; MIC = Minimum Inhibitory Concentration; NIC = Non-Inhibitory Concentration; MIC10, MIC50, MIC90 – Minimum Inhibitory Concentrations of A-2S at which 10 %, 50 % and 90 %, respectively of the bacterial cells were inhibited; MBC = Minimum Bactericidal Concentration; NBC = Non-Bactericidal Concentration; MBC10, MBC50, & MBC90 – Minimum Bactericidal Concentrations of A-2S at which 10 %, 50 % and 90 % respectively of the bacterial cells were killed.

lower MIC and MBC of A-2S were recorded; 0.42 ± 0.06 μM and 3.36 ± 0.09 μM, respectively.

3.2. *In vitro* production of reactive oxygen species in blood cells upon stimulation with *E. tarda* isolates differs depending on anisaxin-2S presence

To investigate whether WBCs and RBCs respond differently to bacterial strains with and without A-2S co-stimulation, flow cytometry was performed. A significant production of ROS was observed at 1 h for both

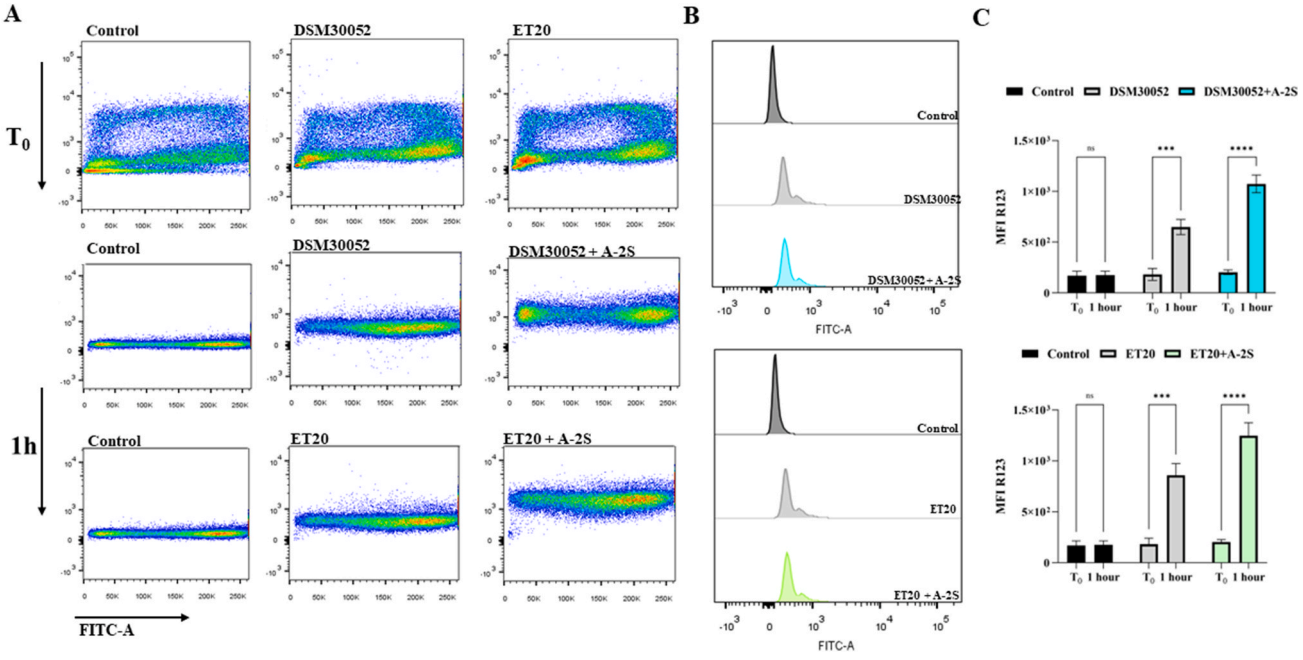

Fig. 1. Production of ROS in fish red blood cells (RBC) exposed to *Edwardsiella tarda* strains and anisaxin-2S. (A) Rhodamine 123-positive (R123-positive) RBCs analyzed by flow cytometry and presented as plots of R123 fluorescence (y-axis) versus forward scatter area (FITC-A). The samples include: the control group (RPMI-1640-treated and incubated with DHR only otherwise), the DSM 30052-stimulated group after 1 h, DSM30052 + A-2S stimulated group, ET20-stimulated groups after 1 h, and ET20 + A-2S stimulated group. (B) Representative histograms of the flow cytometry data are presented in (A). (C) Summary of the data presented as box plots of median fluorescent intensity (MFI) for all experimental groups at the tested time points. A two-way ANOVA was performed with Dunnett's multiple comparisons post hoc test to compare each experimental condition to their respective non-stimulated group (t0) at each time point. ns (not significant); ***$p < 0.001$. n = 4 biological replicates.

strains and both cell types, as indicated by the production of fluorescent rhodamine 123 (R123). RBCs showed significant ROS production in the presence of DSM 30052 and ET20 strains. Additionally, the presence of A-2S further increased ROS production in RBCs. Representative data are shown as density plots (Fig. 1A) and histograms (Fig. 1B), with a summary provided in Fig. 1C.

WBCs exposed to ET20 alone did not show significant ROS production after 1 h, in contrast to ROS production when co-stimulated with A-2S. When WBCs were stimulated with DSM 30052 alone, they produced a significant amount of ROS compared to the control, and the addition of A-2S led to similar results as observed in RBCs. Representative data are shown as density plots (Fig. 2A) and histograms (Fig. 2B), with a summary provided in Fig. 2C.

3.3. Sub-MIC concentrations of anisaxin 2S limit DSM 30052 proliferation already at 1 h

To examine the sensitivity of both *E. tarda* strains to A-2S, we incubated the bacteria with various sub-MIC concentrations of A-2S (25 ng/mL, 50 ng/mL, and 100 ng/mL) for 1 h. Flow cytometry analysis revealed that the DSM 50032 (human) isolate exhibited higher sensitivity to all tested concentrations of A-2S as confirmed by the antimicrobial assay, with the bacterial count decreasing as the concentration of A-2S increased. Expectedly, the ET20 (fish) strain remained resistant to all A-2S concentration treatments. The live bacterial cell numbers in the ET20 groups were unchanged compared to the control groups (Fig. 3A–B).

3.4. The early inflammatory response of common carp RBCs and WBs against E. tarda strains is pro-inflammatory

Given ROS production in blood cells, the changes in the expression of selected cytokines that orchestrate an early inflammatory response were analyzed and compared between the cytokine signatures of blood cells during the first 6 h of stimulation.

No clear pattern was discerned in the gene expression of WBCs (Fig. 4, right column), except for *il-1β*, while the expression of three other cytokines did not display significant upregulation in any of the experimental groups. Among all genes tested, the differential expression of *tnfα* was weakest in the WBCs, although the approximately 5-fold log2 changes in expression were statistically significant in the DSM 30052-stimulated group. In comparison, the expression of *il-6*, *il-1β*, and *ifnγ* were three orders of magnitude higher than *tnfα* at multiple time points.

On the other hand, in the RBCs, gene expression analysis unveiled a significant upregulation of most of the tested cytokines (Fig. 4, left column) with a limited increase in *ifnγ* expression. A significant upregulation of *tnfα* was observed at all sampling time points in all experimental groups, while expression of *ifnγ* and *il-6* was observed in only ET20-stimulated groups. The expression of *tnfα* was >5 log2-fold change higher relative to the control group. Furthermore, *il-6* expression in ET20-stimulated groups ranged from about 7 to 9 log2 fold change compared to the control group.

3.5. A-2S damages cell wall of both E. tarda strains

Transmission Electron Microscopy (TEM) analysis demonstrated that the cell wall of both the DSM 30052 and ET20 is damaged by 10 μM of A-2S. In the control groups of both strains, consisting of bacterial cells in RPMI-1640, the cells remained intact (Fig. 5A and D). In contrast, the

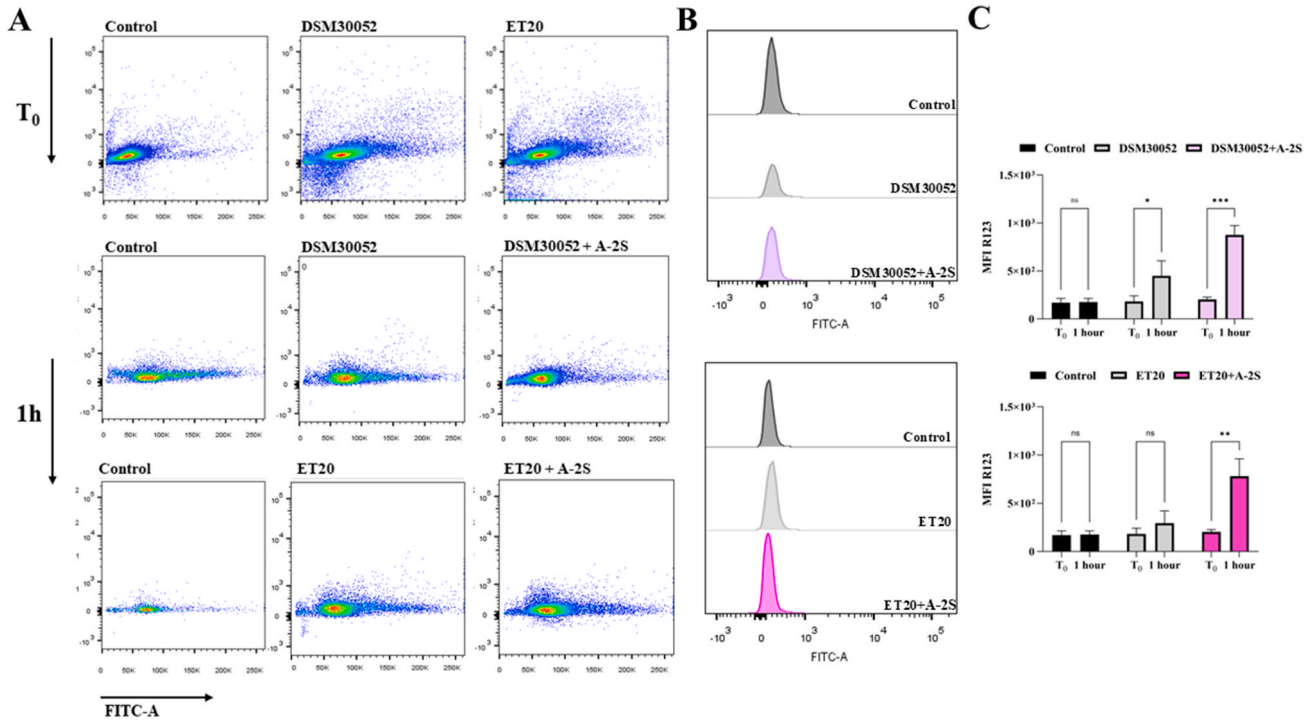

Fig. 2. Production of ROS in fish white blood cells (WBC) exposed to *Edwardsiella tarda* strains and anisaxin-2S. (A) Rhodamine 123-positive (R123-positive) WBCs analyzed by flow cytometry and presented as plots of R123 fluorescence (y-axis) versus forward scatter area (FSC-A). The samples include: the control group (RPMI-1640-treated and incubated with DHR only otherwise), the DSM 30052-stimulated groups after 1 h, DSM 30052 + A-2S stimulated group, the ET20-stimulated groups after 1 h, and ET20 + A-2S stimulated group. (B) Representative histograms of the flow cytometry data are presented in (A). (C) The summary of the data is presented as a box plot of median fluorescent intensity (MFI) for all experimental groups at the tested time points. A two-way ANOVA was performed with Dunnett's multiple comparisons post hoc test to compare each experimental condition to their respective t0 group at each time point. ns (not significant); ***p < 0.001. n = 4 biological replicates.

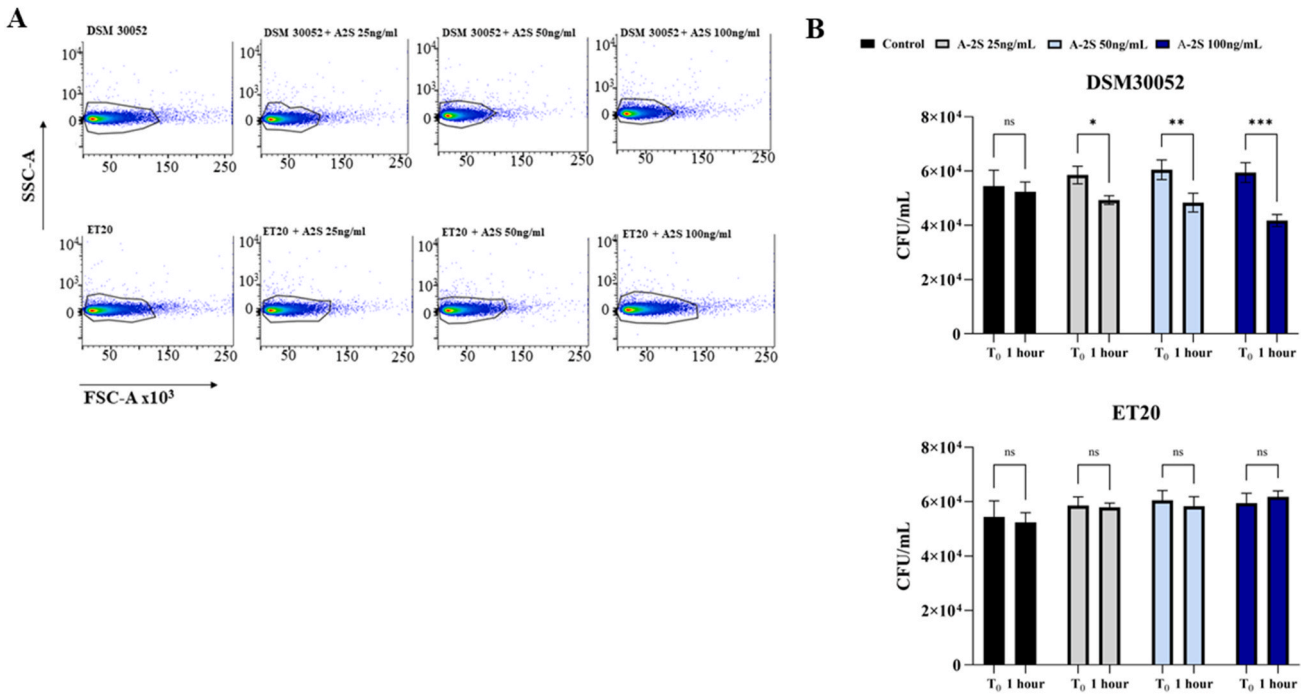

Fig. 3. Flow cytometry analysis of *E. tarda* strains incubated with different concentrations of A-2S (25 ng/mL, 50 ng/mL, 100 ng/mL). Distinct populations of both bacterial strains were identified based on their side scatter area (SSC-A) versus forward scatter area (FSC-A) profiles, as shown in the top row. The two-way ANOVA was performed with Dunnett's multiple comparisons post hoc test to compare each experimental condition to the respective RPMI-1640 control group at the same corresponding time point. Statistical significance is indicated as follows: ns (not significant); *p < 0.05; **p < 0.01; ***p < 0.001.

experimental groups treated with A-2S showed a visible number of dead bacterial cells (Fig. 5B–C and 5E-F). Replicating and damaged bacterial cells were observed for the A-2S-treated DSM 30052 isolate.

3.6. Interaction of blood cells with E. tarda strains

RBCs and WBCs display interactions with both strains of *E. tarda*. RBCs did not show the tendency to engulf bacteria after 1 h of contact with DSM 30052 (Fig. 6B), unlike WBC. ET20 caused considerable cell death in both cell lineages (Fig. 6C and F). WBCs showed phagocytic activity towards DSM 30052 by lymphocyte-like cells, as well as the protrusion of phagocytic pseudopodia towards ET20 (Fig. 6E).

4. Discussion

The 11 strains of *Edwardsiella* spp. tested showed a considerable variation in the response to anisaxin-2S, similar to what has been reported for *Edwardsiella* spp. treated with tetracyclines and aminoglycosides [34]. Despite the unspecific A-2S mode of action against Gram-negative bacteria that facilitates a broad-range bacterial response [30], from the inhibition of cell proliferation to cell elimination, some *Edwardsiella* spp. and strains appear resilient. This has been previously attributed to the modification of proteins on the outer membrane of *E. tarda* [35] and the lipopolysaccharide structure of *E. ictaluri* [36], which play a major role in adaptability against antimicrobial substances. This suggests that some strains within *Edwardsiella* species modulate their outer membrane to prevent interaction with the A-2S, as a strategy to develop resistance against the AMP treatment. It is therefore not surprising that the strain isolated from fish (ET20) showed resistance to anisaxin-2S, potentially induced by previous interaction within fish hosts.

Fish blood cells exposed to A-2S and *E. tarda* strains differ in the production of reactive oxygen species (ROS). Within cells, the levels of ROS undergo fluctuations due to various factors [37], including the response to diverse pathogens [38–40], being consistently produced as byproducts of oxidative metabolic processes [41]. Given the abundance of RBC [42] and their sentinel role in first encounters with pathogens, it is unsurprising that their ROS levels elevate upon stimulation by *E. tarda* strains. This may be due to ET20 strain virulence factors, such as *ethA* and *ethB* haemolysis genes of *E. tarda*, which protein exerts high haemolytic activities [43]. Despite lacking nuclei and organelles, mammalian RBCs are known to generate antimicrobial ROS as part of their host defense mechanism [44]. Our data indicated a similar phenomenon in the RBC of teleosts. Both mammalian and teleost RBCs employ hemoglobin to produce ROS in response to bacterial infection [44]. By expressing a strong affinity for oxygen to oxidize hemoglobin, leading to increased ROS production during infections, RBCs contribute to observed higher ROS levels compared to WBCs. The difference in ROS production between RBCs and WBCs can also be attributed to hemoglobin, the most abundant protein in RBCs [45] that serves as an antimicrobial protein against pathogens, primarily through ROS production during pathogenesis [45]. In humans, lysed erythrocytes can release heme and hemoglobin into the extracellular environment in the vicinity of invading microbes, where hemoglobin can bind LPS and generate toxic ROS that function in host defense [44]. In contrast, the lower WBC count, along with fewer specialized phagocytic cells like macrophages and neutrophils, limits ROS release during pathogen invasion.

RBCs exposed to the A-2S-resistant *E. tarda* isolate (ET20, fish) produced more ROS after 1 h compared to the susceptible strain (DSM 30052, human), potentially due to differences in virulence factors, particularly hemolysins that release iron from hemoglobin, which can then oxidize through the Fenton reaction, increasing ROS. Other studies have shown that mutated *E. tarda* strains exhibited lower virulence and reduced siderophore activity, which affected ROS production. Interestingly, the susceptible human strain DSM 30052 triggered higher ROS production in WBCs after 1 h vs the resistant fish strain ET20, suggesting that the latter can evade phagocytic cells more effectively than DSM 30052. This observation aligns with previous findings that virulent *E. tarda* can suppress ROS production in macrophages, facilitating the evasion of the host's immune response [46]. Similarly, only virulent

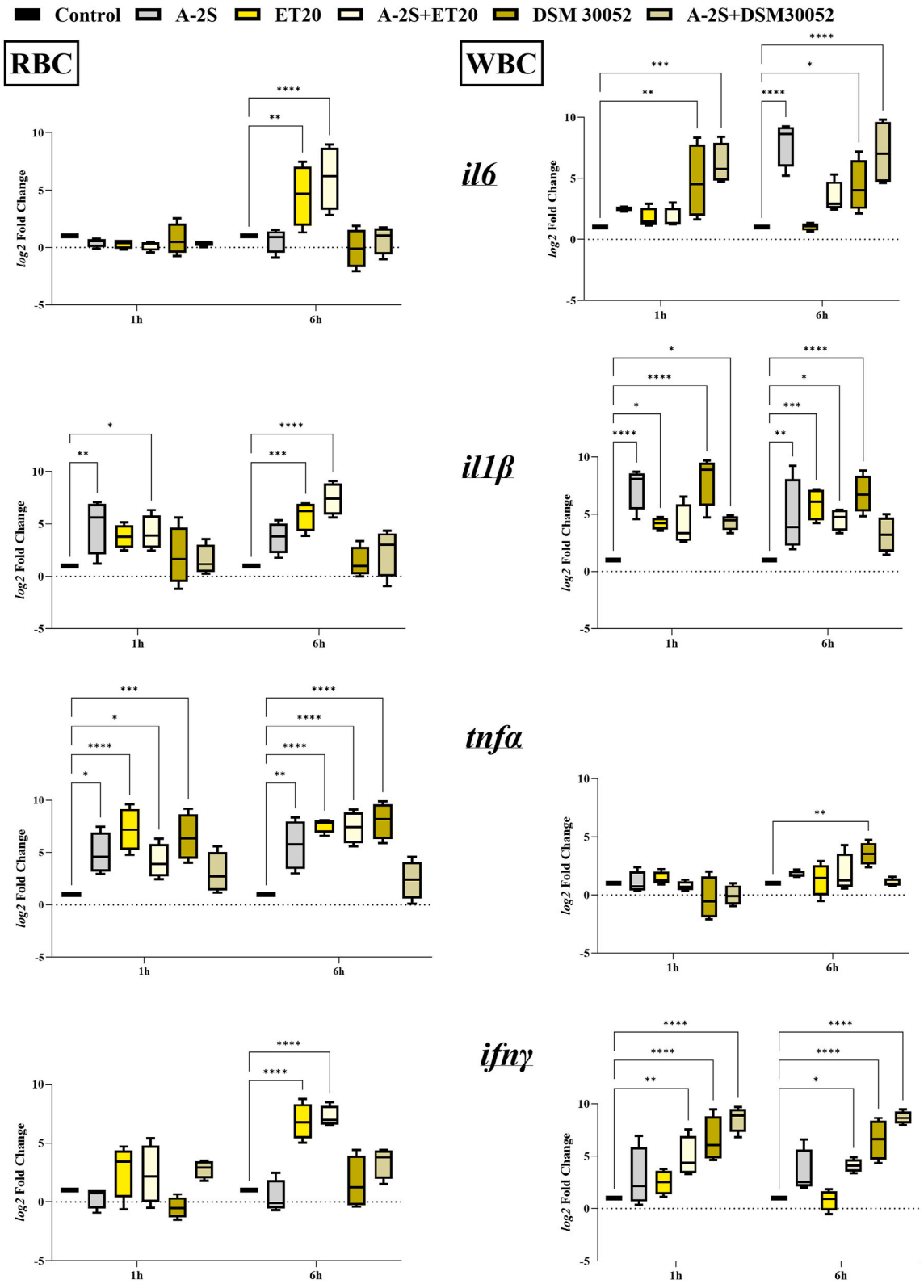

Fig. 4. | Cytokine gene expression profiles of red blood cells (RBCs, left column) and white blood cells (WBCs, right column) of seven fish (biological replicates) stimulated with *E. tarda* strains ET20 (fish, A-2S-resistant) and DSM 30052 (human, A-2S-susceptible). A pair of graphs were created for each target gene (*il-6, il-1β, tnfα, ifnγ*). The units of measure are fold changes of the target gene relative to the housekeeping gene *(ef-1a)* (ΔCt) relative to the corresponding baseline control (t0) (ΔΔCt). Data are depicted as box-and-whisker plots, where the whiskers extend to the smallest and largest values, and the midline represents the median. A two-way ANOVA was performed with Dunnett's multiple comparisons post hoc test to compare each experimental group to their respective RPMI group at each time point. Annotations indicate statistical significance: ns (not significant); *p < 0.05; **p < 0.01.

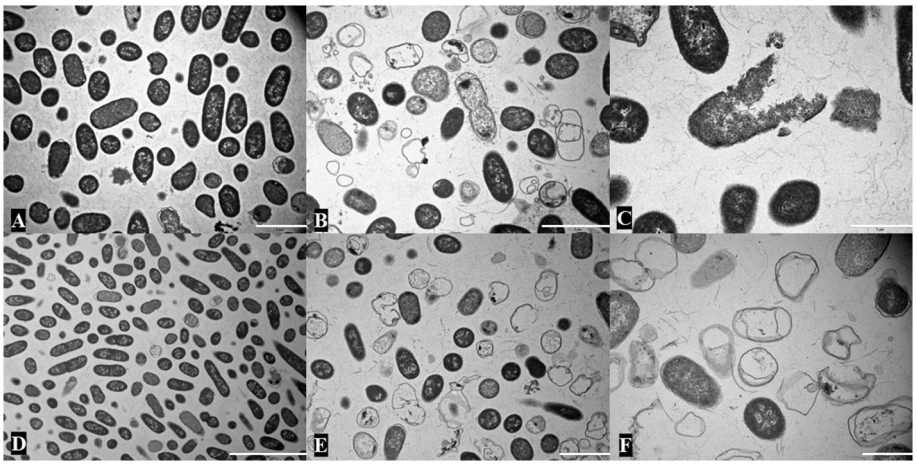

Fig. 5. | Representative pictures of the transmission electron microscopy of the DSM 30052 (B–C) and ET20 (E–F) incubated with 10 μM anisaxin-2S for 1 h. Images A and D represent the untreated negative control of the bacterial strains in RPMI-1640. The electron micrographs show increased cell death in both bacterial strains. Scale bars: 5 μm (1, 2 and 5), 2 μm (4), 1 μm (3 and 6).

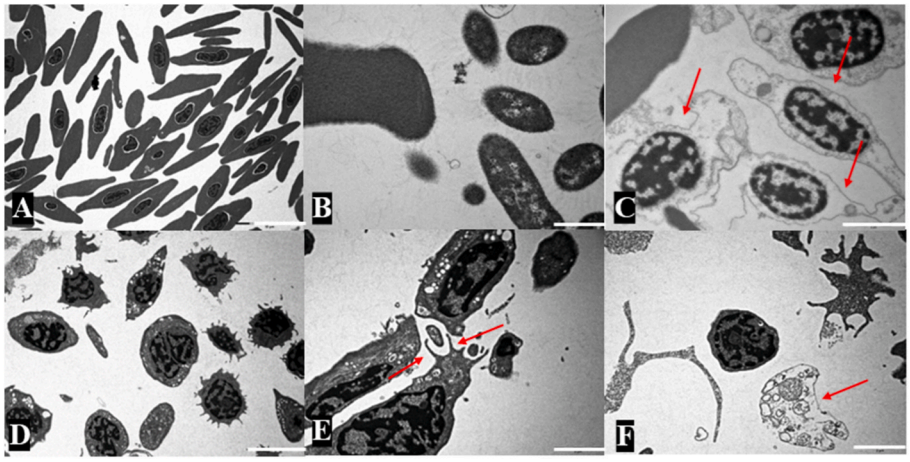

Fig. 6. | Representative pictures of the transmission electron microscopy of RBCs (row above) and WBCs (row below) interactions with DSM 30052 (B–E) and ET20 (C–F) isolates. The red arrows in (C) and (F) point to the cell death caused by the bacterium whereas the suspected phagocytosis by WBC is observed in (E). The red blood cells (B) show no phagocytic activity after 1 h of incubation with live bacterium. Scale bars: 2 μm.

strains of *E. tarda* can evade the bactericidal activity of phagosome-associated reactive oxygen intermediates (ROIs) when opsonized by teleost phagocytes, while the non-virulent strains cannot resist the elevated toxic ROIs of the phagocytes upon infection [47]. However, the interpretation of virulence and ROS levels in our study needs caution as previous studies compared the immune interaction of *Edwardsiella* strains originating from the same host, while we tested an interaction between fish blood cells and a human strain (DSM 30052) for which the data on virulence in this host are lacking.

In all the treatments, A-2S significantly stimulated the production of ROS by both RBCs and WBCs when challenged with DSM 30052 and ET20, supporting a higher number of blood cells surviving the challenge after 1 h of treatment, compared to the blood cells that were not treated with A-2S after the bacterial exposure. Foremost, the discovery of A-2S potential in enhancing the robustness of the fish blood cells to withstand high levels of ROS could be attributed to its effectiveness in promoting the activities of enzymatic antioxidants to offer self-protection of the blood cells against oxidative stress, while making the microenvironment highly toxic for the pathogen's survival and evasion of the immune response. Secondly, a higher ROS could also be due to the oxidation of iron released from the lysed bacterial cells by the AMP thereby resulting in oxidative stress in the pathogens. Rowe-Magnus et al. [48] proposed that interruption of respiratory electron transport in Gram-negative bacteria by cathelicidin led to the release of superoxide, which further reacted with the bacteria enzyme to release its Fe^{2+} content and trigger oxidative stress with eventual damage to the bacterial system. In addition, overexpression of AMP diptericin in *Drosophila* fly, enhances the host cells' resistance to oxidative stress by maintaining a balance between ROS and antioxidant production under stress [49]. Whether this could be also attributed to A-2S still remains to be tested. Overall, the amount and the kinetics of the produced ROS differ in RBCs and WBCs, and while being elevated under the addition of A-2S, both cell types exhibit antimicrobial properties against *E. tarda* in the absence of the AMP.

The result of the experimental antimicrobial activity against the *E. tarda* strains indicated that A-2S exerted a gradual declining effect on the population of DSM 30052 cells, as the pathogen's ability to undergo binary fission appeared to be completely inhibited within the first 1 h of A-2S treatment. However, ET20 seemed to be unaffected by the A-2S treatment, even though cell deaths were evident in the electron microscopy result. Possibly, a continuous bacterial cell division was still ongoing at a low rate to balance up for the dead cells in the toxic microenvironment, thereby masking the bactericidal effect of A-2S on ET20. Bacterial species such as *Escherichia coli* were observed to develop a "behaviour" described as a "bust and boom" strategy [50] to maintain equilibrium between cell division and death with a relatively stable

population under a stressful condition [51]. Antibiotic-resistant bacterial strains already at the steady stage of their growth curve before exposure to antibiotics tend to delay cell regrowth to maintain cell dormancy to evade the toxicity of the antimicrobial agent [52] and potentially against AMPs [53].

The difference in the MIC and MBC of A-2S against ET20 and DSM 30052 is reflected in the observed cytometric data. It is tempting to speculate whether the resistance of ET20 isolated from fish implied a developed resistance against an AMP that the bacterium has been sharing the host with. Nevertheless, the fish-originating isolate ET20 could have evolved a natural resistance towards A-2S, an AMP amply produced in *Anisakis*-infected marine fish [54], in contrast to the human *E. tarda* isolate (DSM 30052) that likely lacked any previous contact with the marine AMP. However, since other *Edwardsiella* spp. isolated from fish show a wide range of resistance to A-2S (MIC range from <0.1 to >100), it is plausible that other factors play a role in the mechanism.

Beyond the production of reactive oxygen species (ROS), we also noticed robust pro-inflammatory responses in both cell types upon the *in vitro* stimulation with *E. tarda* strains. The fish RBC exhibited a pro-inflammatory response equal to that of WBC, contrasting the conventional belief that the latter are the only carriers of antibacterial immunity across vertebrate species. The immune properties of fish's RBC against virulent pathogens were evident in the production of *ifnγ* and expression of ROS [55]. However, RBCs and WBCs express different cytokine patterns and kinetics depending on the *E. tarda* strain. RBCs are more reactive to ET20, while WBCs showed higher sensitivity to DSM 30052, which also matches their ROS production profile obtained by flow cytometry. The addition of A-2S alone induced significant upregulation of *il-1β* in both cell types and time points. A-2S alone has upregulated only *il-6* in WBCs, with increased *tnfα* expression in RBCs. Similar to *A. hydrophila* infection [56], *tnfα* seems to be the most abundant cytokine in the RBCs, while WBCs mostly upregulate *il-1β* and *ifnγ*. Across various vertebrate species, similar cytokine expression patterns in the RBCs have been observed. For example, Rohu fish RBCs, cultured both *in vivo* and *in vitro*, expressed *TLR4*, *NOD1*, and *NOD2* genes, as well as *il-8*, *il-1β*, and *ifnγ1* genes following LPS stimulation [57]. Moreover, human and murine red blood cells enhance the procoagulant and proinflammatory responses of white blood cells (WBCs) upon exposure to LPS, likely through RBC-DARC-mediated interactions in microenvironments where monocytes and RBCs are in proximity [58]. Finally, transcriptome analysis of the Japanese flounder, *Paralichthys olivaceus* RBC reveals upregulation of the genes involved in *NOD*-like receptors (NLRs)-mediated pathogen recognition and downstream *NF-κB* activation induced by *in vivo E. tarda* infection [59]. However, *il-10* and *SOCS1*, key anti-inflammatory regulators, along with other anti-inflammatory genes (*PTGS2*, *TGFBR2*, and *CYLD*) were also highly upregulated, indicative of RBC anti-inflammatory response accompanying the bacterial evasion mechanisms. The discrepancy of data with this study may be due to contrasting experimental design, i.e., the immune response of spleen RBC vs. peripheral RBC, 24 h vs. 1 and 6 h infection, as well as *in vivo* vs. *in vitro* setup.

Similar to *E. tarda* infection in ginbuna crucian carp, *Carassius auratus langsdorfii* [60], the common carp WBC mostly upregulated *ifnγ*. The significant upregulation of *ifnγ* of WBC against DSM 30052 and ET20 strains of *E. tarda* upon treatment with A-2S suggests the ability of the AMP to enhance the capacity of the fish leukocytes against intracellular pathogens. AMPs-like NK-lysin have been reported to upregulate the expression of *ifnγ* in Atlantic salmon leukocytes [61]. In teleost fish, *ifnγ* promotes cell-mediated immunity in a similar manner to that of mammals [62]. Along with the stimulation of both innate and adaptive immune cells, *ifnγ* activity against intracellular disease-causing agents typical of viral and bacterial infections [63–66] upon activation by A-2S could be a sign of its broader spectrum of cell activation against pathogens.

The transmission electron micrographs confirmed the cytometric data indicating that A-2S induced higher cell death in susceptible DSM 30052, while ET20 strain did not display high sensitivity to A-2S. The structural changes observed in DSM 30052 correspond to an unspecific A-2S effect on the cell wall and the cell membrane, respectively, leading to plasmolysis. The electron microscopy analysis showed that sub-MIC of A-2S interacted with DSM 30052 cell wall with disturbance in permeability and disruption of the wall, leading to eventual bacterial death, as previously described [67].

The interactions between RBC and WBC with *E. tarda* strains inferred by TEM showed that while RBC expressed a higher proinflammatory response to ET20, these cells had a higher incidence of cell death. RBCs incubated with DSM 30052 show no morphological changes. Phagocytosis by the fish RBC and their lysosomal activity against bacteria was not observed, despite the previous reports [56,67,68]. It is unclear whether this was due to potent haemolytic properties in the case of ET20, or in the case of DSM 30052, no fish susceptibility to human *E. tarda* strain or more time needed for pathogen engulfment. Since RBCs are not primarily phagocytic cells, they may require more time, approximately four to 5 h, for attachment and engulfment of the pathogen [56].

WBC, in contrast, showed no marked cell death upon the incubation with both strains. The formation of pseudopodia surrounding the bacterial cell was observed in lymphocyte-like cells, potentially preparing for phagocytosis. Teleost B cells, traditionally known for their role in adaptive immunity, have also been shown to possess phagocytic and microbicidal abilities. Their strong antimicrobial and antigen-presenting functions, along with their abundance in blood and mucosal tissues, highlight their significant role in fish immune defense [69–71].

5. Conclusions

The research indicates a dynamic immune response from fish RBCs and WBCs when in contact with *E. tarda*, highlighting the unique roles of these cells in mediating oxidative stress and inflammation during bacterial infections. A-2S enhances ROS production and exhibits differential antimicrobial effectiveness, suggesting potential for therapeutic applications against some *Edwardsiella* spp. infections in fish. These findings contribute to the understanding of the role of anisaxin in fish immune response and its potential use in fish health management.

6. Statistical analysis

The data were analyzed using the software Prism 10 (GraphPad Software, USA). In the gene expression graphs, the data are presented as mean values ± standard deviation (SD). Statistical analyses were conducted on fold change gene expression data calculated by the Pfaffl method. Gene expression data for the *in vitro* experiment was analyzed using two-way ANOVA with Dunnett's multiple comparisons post hoc test to compare each experimental condition to the corresponding uninfected group at each time point. The statistical test applied for each assay is indicated in their respective figure legends.

CRediT author statement

Oluwabusayo Israel Okeleye and Jovana Majstorovic: Conceptualized the study, designed the methodologies to answer the research questions, performed experiments, analyzed the data, and wrote the original draft of the article. **Anupa Sudharaka**: Performed the bacterial identification assay. **Adriana Triga, Manolis Mandalakis, Pantelis Katharios, Ivona Mladineo:** Performed the bacterial cultivation and provided the bacteria for the experiment. **Ivona Mladineo:** Acquired funding and supervised the research. All authors reviewed and edited the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2025.110427.

Data availability

Data will be made available on request.

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