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Mucosal and systemic immune responses in Senegalese sole (*Solea senegalensis* Kaup) bath challenged with *Tenacibaculum maritimum*: A time-course study

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ABSTRACT

Tenacibaculosis, caused by *Tenacibaculum maritimum*, continues to inflict substantial losses among cultured marine species, particularly in the Senegalese sole. However, the immune mechanisms in fish involved in fighting against this disease are still poorly understood. Thus, the present study aimed to investigate the skin mucus's terminal carbohydrate composition, several immune-related enzymes (i.e. lysozyme, peroxidase, proteases and antiproteases), the haemolytic activity of complement and the bactericidal activity in the skin mucus and plasma of the Senegalese sole in a time-course study following a bath challenge with *T. maritimum*. The haematological profile and the kinetics of cell migration post-infection were also considered. The bath challenge induced slight variations in the terminal carbohydrate composition of Senegalese sole skin mucus. In general, results from this study showed a delay in the mucosal immune response compared to that found at the systemic level (i.e. blood and plasma). For instance, a significant increase in the skin mucus's lysozyme, complement, protease and antiprotease activities were observed at the end of the experiment (14 d post-challenge). Interestingly, the higher activity of these enzymes could be related to the skin mucus's bactericidal capacity and haemolytic complement activity, suggesting that these enzymes play an important role in the defence against Gram-negative bacteria. The haematological profile revealed a significant increase in circulating neutrophils in challenged fish after 48 and 72 h, which was positively correlated to the increments observed in peroxidase and lysozyme activities, respectively, in the plasma of challenged fish at the same time. Although the route of entry and the survival strategy of *T. maritimum* are still not fully elucidated, results from the present study will contribute to this endeavour through the study of the mucosal immune responses of fish against this particular pathogen.

1. Introduction

Aquaculture is an emerging industry responsible for supplying fish as food and its importance has been increasing during recent years. Nevertheless, this sector is challenged by several daunting issues about sustainability from biological, environmental and socio-economic points of view [1]. Among these challenges are the introduction of new cultured species for the purpose of diversification, the emergence of diseases, which constitutes one of the main causes of economic losses, and the problems associated with intensive farming.

According to Food and Agriculture Organization (FAO) [2], following recent scientific research and technological development, the

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culture of flatfish species constitutes one of the most promising areas in the future of aquaculture. In Europe, flatfish cultivation is dominated by the turbot (*Scophthalmus maximus*) and the Atlantic halibut (*Hippoglossus hippoglossus*). However, the production of Senegalese sole (*Solea senegalensis*) has recently increased. Consequently, Senegalese sole is a high-value flatfish with great potential for future farming at a commercial scale and has also been considered since the 1990s to be a promising flatfish species for diversifying European marine aquaculture [3]. Nonetheless, disease outbreaks, mainly those caused by bacteria, seem to be the most critical impediment to the advancement of the commercial production of Senegalese sole [4]. One of the most serious pathogens that threatens Senegalese sole aquaculture is the Gram-negative bacterium *Tenacibaculum maritimum*, the aetiological agent for marine tenacibaculosis (formerly flexibacteriosis) [5], which causes severe economic losses in aquaculture.

Tenacibaculosis is among the most threatening bacterial infections limiting the culture of many marine fish species of commercial value worldwide, including the turbot and Senegalese sole. Although this disease has a great impact upon aquaculture, relatively little is known about its pathogenesis and routes of infection. The pathology of tenacibalulosis has been associated with characteristic gross lesions on the body surface of fish, including ulcers, necrosis, eroded mouth, frayed fins and tail rots, and sometimes necrosis on the gills and eyes [5–7]. To date, some studies on disease transmission support the theory that *T. maritimum* is an opportunistic pathogen that primarily causes extensive skin damage and gill abrasion with consequent systemic infection [8]. Fish skin and gills act as physical barriers that are considered to be the first line of defence against microbial infections [9–11]. Teleost skin is unique and histologically diverse [12]; one of the most distinctive features of this tissue is the presence of external mucus that serves as a repository of numerous immune components of both the innate and acquired immune system [13–15]. The mucus forms a biofilm insulating layer that protects the underlying epithelium from damage, and therefore the interactions between the mucus layer and bacteria are very important in disease progression.

The adherence of *T. maritimum* to host tissues depends directly on its ability to neutralize or evade the immune system of the fish, such as the bactericidal activity of skin mucus, and also on its ability to accumulate the nutrients required for its growth [8,16]. Avendaño-Herrera et al. [17] suggested that the presence of the *T. maritimum* within the skin mucus layer might mean that this bacterium is part of the autochthonous populations in fish and, therefore, the bacterium can remain in the aquatic environment utilizing fish mucus as a reservoir. Nevertheless, little is known regarding the ability of Senegalese sole to cope with this pathogen and information on the interactions between *T. maritimum* and the host's humoral immune response is still scarce at systemic and mucosal levels. Taking into account all these considerations, the aim of the present study was to evaluate both the systemic and mucosal immune responses of Senegalese sole that were bath challenged with *T. maritimum*. Moreover, the modulation in the skin mucus glycosylation patterns in Senegalese sole exposed to this particular pathogen will also be assessed using a lectin-binding assay.

2. Material and methods

2.1. Fish care and maintenance

Healthy specimens of Senegalese sole (25.5 ± 5.9 g mean body weight) were obtained from a commercial fish farm, located in northwest Portugal, with no history of tenacibaculosis. Prior to the trials, the fish were maintained in a recirculating aerated seawater (33‰ salinity) system with mechanical and biological filtration where dissolved oxygen was maintained around 90%, water temperature at 21 ± 1 °C, and a 12 h light/12 h dark photoperiod was adopted. The fish were fed to apparent satiety with commercial pellets (Skretting LE-2 ELITE, Spain). Ammonia and nitrite levels in the water were measured twice a week using commercial kits and never exceeded 0.025 and 0.3 mg L^{-1}, respectively.

2.2. Bacterial culture conditions and inoculum preparation

The *T. maritimum* strain ACC6.1, which was isolated from Senegalese sole in a local fish farm (Póvoa de Varzim, Portugal), was kindly provided by Professor Alicia E. Toranzo (*Departamento de Microbiología y Parasitología, Facultad de Biología,* University of Santiago de Compostela, Spain). The bacteria were kept frozen at −80 °C until being used. The recovery of the bacteria was achieved using marine agar (CONDA, Spain) at 25 °C for 48 h. For the inoculum preparation, the bacteria were harvested and inoculated into 50 ml of marine broth (MB) for an additional 48 h at the same temperature with continuous shaking (140 rpm). Afterwards, the density of the exponentially growing bacteria was measured according to the McFarland standard to find out the volume of the culture of a 2.7×10^8 colony-forming unit (CFU) L^{-1}. This volume was transferred to a tube to collect the bacteria by centrifugation (4,000 × *g* for 30 min at 4 °C) and then re-suspended in the minimum volume of sterile MB to obtained the final concentration of 2.7×10^5 CFU ml^{-1} (2.7×10^8 CFU L^{-1}) according to Mabrok et al. [18].

2.3. Experimental design and sampling

Experiments were performed by trained scientists and following the European Directive 2010/63/EU of the European Parliament and the Council of the European Union on the protection of animals used for scientific purposes. Fish were randomly distributed in 2 identical recirculated seawater systems composed of 6 tanks (n = 42) filled with 8 L (0.05 m^2) of aerated seawater at flow rate 900 L h^{-1}. Each aquarium contained groups of 7 fish and those fish were left to acclimate for fifteen days prior to bacterial challenge.

Bacterial inoculum was prepared with sterile MB according to the method described in Section 2.2. Subsequently, one of those systems (6 tanks, 7 fish/tank) were bath challenged with a sub-lethal final bacterial concentration of 2.7×10^5 CFU mL^{-1} in 1 L (2.7×10^8 CFU) of sea water at 23 ± 1 °C with strong aeration for 24 h. For this, the recirculation system was stopped and the water volume in each aquarium was lowered to 1 L. Afterwards, the rearing water in each tank was changed three times and the recirculation system was re-established. The fish in the remaining system (6 tanks, 7 fish/tank) were challenged under the same conditions with sterile physiological saline solution instead of bacteria and served as controls (unchallenged group). One fish was then removed from each tank at the following times after bacterial challenge: 0 h, 4 h, 24 h, 48 h, 72 h, 7 days and 14 days, and skin mucus, blood and plasma were collected as described below. The fish were fed daily at a ratio of 1% of total fish biomass. In addition, ammonia and nitrite levels were assessed daily and kept below 0.025 and 0.3 mg L^{-1}, respectively.

2.4. Samples collection

Prior to each sampling point, one fish from each tank was caught and anesthetized with 2-phenoxyethanol (1 ml L^{-1}; Sigma) in different buckets. Skin mucus was aseptically collected from specimens using the method described by Guardiola et al. [19] with slight modifications. Briefly, the ocular and blind sides of both control and challenged fish were gently scraped by using a cell scraper with enough care to avoid contamination with urogenital and/or intestinal excretions. Collected mucus samples were then centrifuged at 2,000 × *g* and 4 °C for 10 min. The supernatant was then filtrated (0.2 μm pore size; Sarstedt), aliquoted and stored at −80 °C until further analyses. Following mucus collection, blood withdrawal was performed from the caudal vein using heparinized syringes. An aliquot of fresh blood was used for the assessment of the haematological profile according to Machado and

colleagues [20], whereas the remaining blood was centrifuged at 10,000 × *g* during 10 min at 4 °C and the resulting plasma stored at −80 °C until assayed.

2.5. Haematological procedures

The haematological profile consisted of total red (RBC) and white (WBC) blood cells counts, as well as haemoglobin evaluation. Immediately after blood collection, blood smears were performed from fresh blood, air dried, after fixation with formol-ethanol (10% of 37% formaldehyde in absolute ethanol) detection of peroxidase activity was conducted as described by Afonso et al. [21] in order to facilitate detection of neutrophils. Blood smears were then stained with Wright's stain (Haemacolor; Merck). The slides were examined (1,000 x), and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes, and neutrophils. The absolute value ($\times 10^4$ ml^{-1}) of each cell type was then calculated. Haemoglobin was measured (Hb; SPINREACT kit, ref. 1001230, Spain) and mean corpuscular haemoglobin (MCH) was calculated as follows:

$$\text{- MCH (pg cell}^{-1}\text{)} = \text{(Hb/RBC) x} \qquad 10$$

2.6. Terminal glycosylation pattern determination

Glycosylation pattern in the skin mucus was determined by lectin ELISA as described previously [22]. Briefly, 100 µl of skin mucus (diluted 1:5 with 50 mM carbonate-bicarbonate buffer, pH 9.6) were placed in triplicate wells of flat-bottomed 96-well plates and coated overnight at 4 °C. Plates were rinsed 3 times with PBS-T [20 mM phosphate buffer (PBS) and 0.05% Tween 20, pH 7.3], blocked for 2 h at room temperature with blocking buffer (PBS-T containing 3% bovine serum albumin BSA) and rinsed again. Samples were then incubated for 1 h with 100 µl of each biotinylated lectin at 20 µg ml^{-1} (Table 1, Sigma), washed 3 times and incubated with streptavidin horseradish-peroxidase (1:1000 in PBS-T; Life Technologies) for 1 h. After 3 washes with PBS-T the samples were developed using 100 µl of a 0.42 mM solution of 3,3,5,5 - tetramethyl benzidine hydrochloride (TMB, Sigma), prepared daily in a 100 mM citric acid/sodium acetate buffer (pH 5.4) containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min, stopped by the addition of 50 µl of 2M H_2SO_4 and the plates read at 450 nm in a plate reader (FLUO star Omega, BMG Labtech). Negative controls consisted of samples without skin mucus or without lectins, whose optical density (OD) values were subtracted for from each sample value. Data are presented as the OD at 450 nm for each lectin used.

2.7. Innate humoral parameters

2.7.1. Natural haemolytic complement activity

Natural haemolytic complement activity was measured in skin mucus according to Sunyer and Tort [23] with some modifications. The following buffers were used: GVB (Isotonic veronal buffered saline), pH 7.3, containing 0.1% gelatin; EDTA-GVB, pH 7.3, containing 0.1% gelatin with 20 mM of EDTA; and Mg-EGTA-GVB, which is GVB with 10 mM Mg^{+2} and 10 mM EGTA. Rabbit red blood cells (RaRBC; Probiologica Lda, Portugal) were used for natural haemolytic complement determination. RaRBC were washed four times in GVB and resuspended in GVB to a concentration of 2.5 × 10^8 cells ml^{-1}. Twenty µl of RaRBC suspension were then added to 40 µl of serially diluted skin mucus in Mg-EGTA-GVB buffer. The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 40 µl of distilled water or Mg-EGTA-GVB buffer to 20 µl samples of RaRBC, respectively. Samples were incubated at room temperature for 100 min with regular shaking every 20 min. The reaction was stopped by adding 150 µl of cold EDTA-GVB. Samples were then centrifuged and the extent of haemolysis was estimated by measuring the optical density of the supernatant at 414 nm in a microplate reader (Synergy HT). The degree of haemolysis (Y) was estimated and the lysis curve for each specimen was obtained by plotting Y $(1\text{-}Y)^{-1}$ against the volume of skin mucus added (µml) on a log-log scaled graph. The volume of skin mucus producing 50% haemolysis (ACH_{50}) was determined and the number of ACH_{50} units ml^{-1} obtained for each experimental fish. In the case of plasma samples, the haemolytic complement activity was not assessed due to technical constraints.

Table 1
Lectins used in this study, their acronym and sugar-binding specificities.

Acronym	Lectin source	Sugar binding specificity
Con A	*Canavalia ensiformis*	α-D-mannose, α-D-glucose
SNA	*Sambucus nigra*	*N*-acetylneuraminic acid
WFA	*Wisteria floribunda*	*N*-acetyl-D-galactosamine
UEA I	*Ulex europeaus*	α-L-Fucose
LEA	*Lycopersicon esculentum*	*N*-acetyl-β-D-glucosamine

2.7.2. Lysozyme activity

Lysozyme activity was measured according to the turbidimetric method described by Welker et al. [24] with some modifications. Briefly, 20 µl of skin mucus or plasma were placed in flat-bottomed 96-well plates. To each well, 180 µl of freeze-dried *Micrococcus lysodeikticus* (0.2 mg ml^{-1}, Sigma) in 40 mM sodium phosphate (pH 6.2) was added as lysozyme substrate. As blanks, 20 µl of skin mucus or plasma were added to 180 µl of sodium phosphate buffer. The absorbance at 450 nm was measured after 20 min (endpoint measurement) at 35 °C in a microplate reader (Synergy HT). The amounts of lysozyme present in skin mucus and plasma were obtained from a standard curve made with hen egg white lysozyme (HEWL, Sigma) through serial dilutions in the above buffer. Skin mucus and plasma lysozyme values are expressed as µg ml^{-1} equivalent of HEWL activity.

2.7.3. Peroxidase activity

The peroxidase activity in skin mucus or plasma was measured according to Quade and Roth [25] with some modifications. Briefly, 30 µl of skin mucus or 15 µl of plasma were diluted with 120 µl or 135 µl of Hank's buffer (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates, respectively. Fifty µl of 20 mM TMB and 5 mM H_2O_2 were then added to each well and serves as substrates. After 2 min the reaction was secured by adding 50 µl of 2 M sulphuric acid and the OD was measured at 450 nm in a microplate reader (Synergy HT). Samples without skin mucus or plasma, respectively, were used as blanks. Absorbance variation more than 1 can be defined as one unit of peroxidase. The final results were expressed as units ml^{-1}.

2.7.4. Esterase activity

Esterase activity in skin mucus was determined according to the method of Guardiola et al. [26] with some modifications. An equal volume of skin mucus samples was mixed with 0.4 mM *p*-nitrophenyl myristate as substrate in 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100 (pH 7.8, 30 °C). The OD was continuously measured at 1 min intervals over 1 h at 405 nm in a microplate reader (Synergy HT). Standard samples without skin mucus were used as blanks. The initial rate of the reaction was used to calculate the activity because the reaction follows a linear correlation. The activity was expressed as units ml^{-1}, which was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenyl myristate product in 1 min. In the case of plasma samples, the esterase activity was not assessed due to technical constraints.

2.7.5. Protease activity

Protease activity was measured in skin mucus samples using the azocasein hydrolysis assay according to Guardiola et al. [27] with some

modifications. Briefly, 100 µl of skin mucus was incubated with equal volume of 115 mM phosphate buffered saline (PBS, pH 7.0) containing 2% azocasein (Sigma) for 24 h at 22 °C. The reaction was stopped by adding 10% trichloro acetic acid (TCA) and the mixture centrifuged (10,000 × *g*, 10 min). The supernatants were transferred to a 96-well plate in triplicate containing 100 µl well^{-1} of 1 N NaOH, and the OD read at 450 nm using a microplate reader (Synergy HT). Skin mucus was replaced by trypsin (5 mg ml^{-1}, Sigma), as positive control (100% of protease activity), or by buffer, as negative controls (0% activity). The percentage of trypsin activity compared to the positive control was calculated. In the case of plasma samples, the protease activity was not assessed due to technical constraints.

2.7.6. Antiprotease activity

Total antiprotease activity was determined by the ability of skin mucus or plasma to inhibit trypsin activity with some modifications [27]. Briefly, 50 µl of skin mucus or 10 µl of plasma were incubated for 10 min at 22 °C with 10 µl of standard trypsin solution (5 mg ml^{-1}, in 60 mM sodium bicarbonate, pH 8.3). Afterwards, 60 µl or 100 µl of 115 mM PBS (pH 7.0) for skin mucus and plasma samples, respectively, and 125 µl of 2% azocasein (in 60 mM sodium bicarbonate, pH 8.3) were added and the samples incubated for 60 min at 22 °C. Finally, 250 µl of 10% of TCA (trichloroacetic acid) were added and a new incubation for 30 min at 22 °C was done. The mixture was then centrifuged (10,000 × *g*, 5 min) being the supernatants transferred to a 96-well plate in triplicate containing 100 µl well^{-1} of 1 N NaOH, and the OD read at 450 nm using a microplate reader (Synergy HT). PBS in place of skin mucus, plasma and trypsin served as blank whereas the reference sample was PBS in place of skin mucus and plasma. The percentage inhibition of trypsin activity compared to the reference sample was calculated.

2.8. Bactericidal activity

Bactericidal activity was determined using two opportunist marine pathogenic bacteria: *Photobacterium damselae* subsp. *piscicida* (*Pdp*) and *Tenacibaculum maritimum* (strains PP3 and ACC6.1, respectively). Strain PP3 was kindly provided by Dr. Ana do Vale (Institute for Molecular and Cell Biology, University of Porto, Portugal) and isolated from yellowtail (*Seriola quinqueradiata*; Japan) by Dr Andrew C. Barnes (Marine Laboratory, Aberdeen, UK). Bacteria were grown in agar plates at 26 °C in the adequate media for 24 h. Tryptic soy (TSA, BD) was used for *Pdp* and marine agar (MA, Conda) for *T. maritimum*. Afterwards, fresh single colonies of 1–2 mm were transferred to appropriate liquid culture medium and cultured as described above on an orbital incubator (250 rpm) until exponential growing (20 h), at which point bacteria were resuspended in adequate media and adjusted to 1×10^6 and 1×10^8 CFU ml^{-1} for *Pdp* and *T. maritimum*, respectively.

Skin mucus and plasma bactericidal activity was then determined according to Machado et al. [20] with some modifications. Briefly, 20 µl of skin mucus or plasma were added to duplicate wells of a U-shaped 96-well plate. Hank's balanced salt solution was added to some wells instead of sample and served as positive control. To each well, 20 µl of each bacterium were added and plates were incubated for 2.5 h at 25 °C. To each well, 25 µl of 3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 1 mg ml^{-1}; Sigma-Aldrich) were added and incubated for 10 min to allow the formation of formazan. Plates were then centrifuged at 2,000 × *g* for 10 min and the precipitate was dissolved in 200 µl of dimethyl sulfoxide (DMSO, Sigma-Aldrich). The absorbance of the dissolved formazan resulting from the reduction of MTT in direct proportion to the number of viable bacteria present was measured at 560 nm. Viable bacteria were expressed as percentage, calculated from the difference between the dissolved formazan in samples and the one formed in the positive controls (100% of live bacteria). The bactericidal activity was calculated as the percentage of non-viable bacteria.

2.9. Statistical analyses

All analyses were conducted in triplicates and the results are expressed as mean ± standard error of the mean (SEM). Data were analysed by *t*-test student and One way ANOVA (followed by Tukey tests) to determine differences between experimental groups and each group respect to time, respectively. Normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using the Levene test. Non-normally distributed data were log-transformed to perform parametric tests whilst non-parametric Kruskal-Wallis test, followed by a Dunn's multiple comparison test, was used when data did not meet parametric assumptions. In addition, parametric Pearson correlation tests were applied to test correlations between related parameters that varied significantly. All statistical analyses were conducted using the computer package STATISTICA 12 software for WINDOWS. The level of significance used was $P < 0.05$ for all statistical tests.

3. Results

3.1. Pathogenicity assay

No mortalities were recorded among the specimens of Senegalese sole bath challenged with the sub-lethal *T. maritimum* dose assayed. However, some specimens showed caudal fin erosions, red mouth, detached skin and pale gills (results not shown) as previously reported by our research group [18].

3.2. Haematological profile

The haematological profile was calculated and tabulated in the control and challenged fish (Tables 2 and 3). Both the haemoglobin concentration and MCH showed an increase in the challenged fish at 24 h post-challenge compared to the control group. The number of circulating total WBC decreased in the challenged fish at the first experimental time post-challenge (0 h) compared to the unchallenged fish whilst the RBC remained unaltered. The total concentration of peripheral thrombocytes, lymphocytes and monocytes did not change among the experimental groups, whereas the number of circulating neutrophils increased after 48 and 72 h in the challenged fish compared to the control group.

The bath challenged fish showed an increase in haemoglobin concentration and MCH at 24 h compared to other sampling points. In contrast, total peripheral WBC numbers decreased in the control fish after 7 d compared to 0 and 4 h, whilst those numbers increased in the challenged fish at the end of the experiment (14 d) compared to the values found at 0, 48, 72 h and 7 d. The differential counting revealed that the number of circulating thrombocytes decreased in the control group at 7 d post-challenge compared to the first sampling time at 0 h. Similarly, the number of neutrophils decreased at 48 and 72 h in fish from the unchallenged group compared with the values obtained at 0 h. In the challenged group, the number of neutrophils decreased at 7 d compared to the results observed at 4 h.

3.3. Glycosylation of skin mucus carbohydrates

All the tested terminal monosaccharide residues were present in the skin mucus samples of the unchallenged and challenged fish with variations among both the sampling time and lectins used (Fig. 1). In general, the lectin binding to the Senegalese sole skin mucus was SNA > Con A > WFA > UEA I > LEA as evidenced by the OD readings. This binding pattern suggests that the terminal carbohydrates abundant in the skin mucus were *N*-acetylneuraminic acid, mannose, glucose, *N*-acetyl-galactosamine, fucose and *N*-acetyl-glucosamine residues in descendent order. Comparing both experimental groups, a decrease of binding to UEA I, LEA and WFA was observed in the

Table 2
Haemoglobin (Hb), mean corpuscular haemoglobin (MCH), red blood cells (RBC) and total white blood cells (WBC) in control (unchallenged) Senegalese sole juveniles and challenged specimens with *T. maritimum* for 24 h after 0, 4, 24, 48, 72 h, 7 and 14 days.

Experimental times	Experimental groups	Parameters			
		Hb (g dl^{-1})	MCH[a] (pg $cell^{-1}$)	RBC (× 10^6 μl)	WBC (× 10^4 μl)
0 h	Control	0.3 ± 0.0	2.9 ± 0.4	1.1 ± 0.1	13.0 ± 0.6a
	Challenged	0.4 ± 0.0AB	2.8 ± 1.0A	0.9 ± 0.1	8.6 ± 0.7*A
4 h	Control	0.3 ± 0.0	4.0 ± 0.3	0.8 ± 0.0	15.3 ± 0.8a
	Challenged	0.5 ± 0.1B	6.6 ± 1.1B	0.8 ± 0.1	13.6 ± 0.8BC
24 h	Control	0.3 ± 0.1	2.5 ± 0.5	0.8 ± 0.1	11.5 ± 1.3 ab
	Challenged	0.9 ± 0.1*C	10.6 ± 1.5*C	0.8 ± 0.1	10.3 ± 1.1AC
48 h	Control	0.3 ± 0.0	2.4 ± 0.2	1.1 ± 0.0	11.8 ± 0.9 ab
	Challenged	0.4 ± 0.0ABD	3.1 ± 0.3A	0.9 ± 0.1	9.5 ± 0.8A
72 h	Control	0.3 ± 0.0	3.4 ± 0.5	1.0 ± 0.1	11.8 ± 1.1 ab
	Challenged	0.3 ± 0.0ABD	2.5 ± 0.3A	1.0 ± 0.1	8.6 ± 0.6A
7 days	Control	0.4 ± 0.0	3.6 ± 0.3	1.1 ± 0.1	9.3 ± 0.7b
	Challenged	0.4 ± 0.0AB	4.1 ± 0.3AB	0.8 ± 0.1	9.3 ± 1.4A
14 days	Control	0.2 ± 0.0	1.4 ± 0.5	1.0 ± 0.0	10.0 ± 2.4 ab
	Challenged	0.1 ± 0.0D	0.6 ± 0.1A	1.0 ± 0.0	14.3 ± 1.0B

Values are expressed as mean ± SEM (n = 6). Asterisks denote significant differences between experimental groups (T-test; $p < 0.05$), whilst small (unchallenged) and capital letters (challenged) denote significant variations between experimental groups regarding time (ANOVA; $p < 0.05$).
[a] **MCH (pg $cell^{-1}$)** = (Hb/RBC) × 10.

challenged fish after 48 h, 7 d and 14 d, respectively, compared to values found in the control group. An increase of binding to SNA was observed in challenged fish after 72 h compared to 4 h. Binding to LEA increased in the control group after 7 d compared to values found at 4 h.

3.4. Humoral factors measured in skin mucus

The haemolytic complement activity showed a reduction in the challenged fish after 4 and 24 h compared to the values found in the control group, whilst this activity increased at the end of the experiment (14 d; Fig. 2). Lysozyme (Fig. 3A) and peroxidase (Fig. 3B) activities increased in the skin mucus of challenged fish after 14 d compared to the unchallenged fish. However, a reduction of peroxidase activity was observed in the challenged fish at 24 h compared to the unchallenged group. Regarding the esterase activity (Fig. 3C), no variations were observed in the unchallenged and challenged fish throughout the experimental trial. Protease (Fig. 3D) and antiprotease (Fig. 3E) activities augmented in the challenged fish after 4 h and 14 d compared to the unchallenged specimens.

Comparing sampling times within each experimental groups, the haemolytic complement activity did not change in any experimental group (Fig. 2). Peroxidase activity increased in the control fish at 24 h compared to all experimental times except to the values observed at 0 h (Fig. 3B). Protease activity decreased in the control specimens from 48 h after the challenge until the end of the experiment at all sampling points compared to the values obtained at 0 h (Fig. 3D). Antiprotease activity in the skin mucus from the unchallenged fish showed a reduction after 4 h compared to the rest of experimental times, except at 48 h (Fig. 3E). In the challenged fish, lysozyme and peroxidase activities increased at the end of the experiment compared to the rest of the sampling points, except at 0 h in the case of peroxidase activity (Fig. 3A and B). Esterase activity (Fig. 3C) increased at 24 and 72 h and 14 d with respect to the two initial sampling points (0 and 4 h post-challenge) whilst the protease activity dropped after 7 d compared to 0 and 4 h (Fig. 3D). Finally, the antiprotease activity observed at the end of trial increased compared to all the experimental times (Fig. 3E).

3.5. Humoral factors measured in plasma

The lysozyme (Fig. 4A) and peroxidase (Fig. 4B) activities increased in challenged fish after 72 and 48 h of the experiment, respectively, compared to the values observed in the unchallenged group. Similarly,

Table 3
Absolute values (× 10^4 μl) of peripheral blood leucocytes (thrombocytes, lymphocytes, monocytes and neutrophils) of control (unchallenged) Senegalese sole juveniles and challenged specimens with *T. maritimum* for 24 h after 0, 4, 24, 48, 72 h, 7 and 14 days.

Experimental times	Experimental groups	Thrombocytes	Lymphocytes	Monocytes	Neutrophils
		(× 10^4 μl)	(× 10^4 μl)	(× 10^4 μl)	(× 10^4 μl)
0 h	Control	8.8 ± 0.5a	3.5 ± 0.4	0.1 ± 0.0	0.5 ± 0.1a
	Challenged	5.7 ± 0.5	2.4 ± 0.4	0.2 ± 0.1	0.3 ± 0.1AB
4 h	Control	7.5 ± 1.6 ab	3.8 ± 0.9	0.1 ± 0.0	0.5 ± 0.1 ab
	Challenged	8.4 ± 0.2	4.6 ± 0.8	0.2 ± 0.0	0.7 ± 0.1A
24 h	Control	5.4 ± 1.2 ab	4.6 ± 0.9	0.1 ± 0.1	0.3 ± 0.1 ab
	Challenged	5.8 ± 0.7	4.0 ± 0.3	0.1 ± 0.0	0.4 ± 0.1AB
48 h	Control	6.3 ± 0.4 ab	4.0 ± 0.1	0.1 ± 0.0	0.1 ± 0.0b
	Challenged	5.6 ± 0.7	3.5 ± 0.7	0.1 ± 0.0	0.4 ± 0.0*AB
72 h	Control	6.5 ± 0.5 ab	4.9 ± 0.8	0.1 ± 0.0	0.1 ± 0.0b
	Challenged	5.4 ± 0.2	2.8 ± 0.6	0.1 ± 0.0	0.5 ± 0.1*AB
7 days	Control	4.3 ± 0.9b	3.1 ± 0.2	0.1 ± 0.0	0.3 ± 0.1 ab
	Challenged	4.1 ± 0.9	2.7 ± 0.4	0.1 ± 0.0	0.1 ± 0.0B
14 days	Control	6.9 ± 1.2 ab	4.5 ± 0.5	0.1 ± 0.1	0.3 ± 0.0 ab
	Challenged	5.8 ± 1.3	5.7 ± 1.3	0.1 ± 0.0	0.5 ± 0.1AB

Values are expressed as mean ± SEM (n = 6). Asterisks denote significant differences between experimental groups (T-test; $p < 0.05$), whilst small (unchallenged) and capital letters (challenged) denote significant variations between experimental groups regarding time (ANOVA; $p < 0.05$).

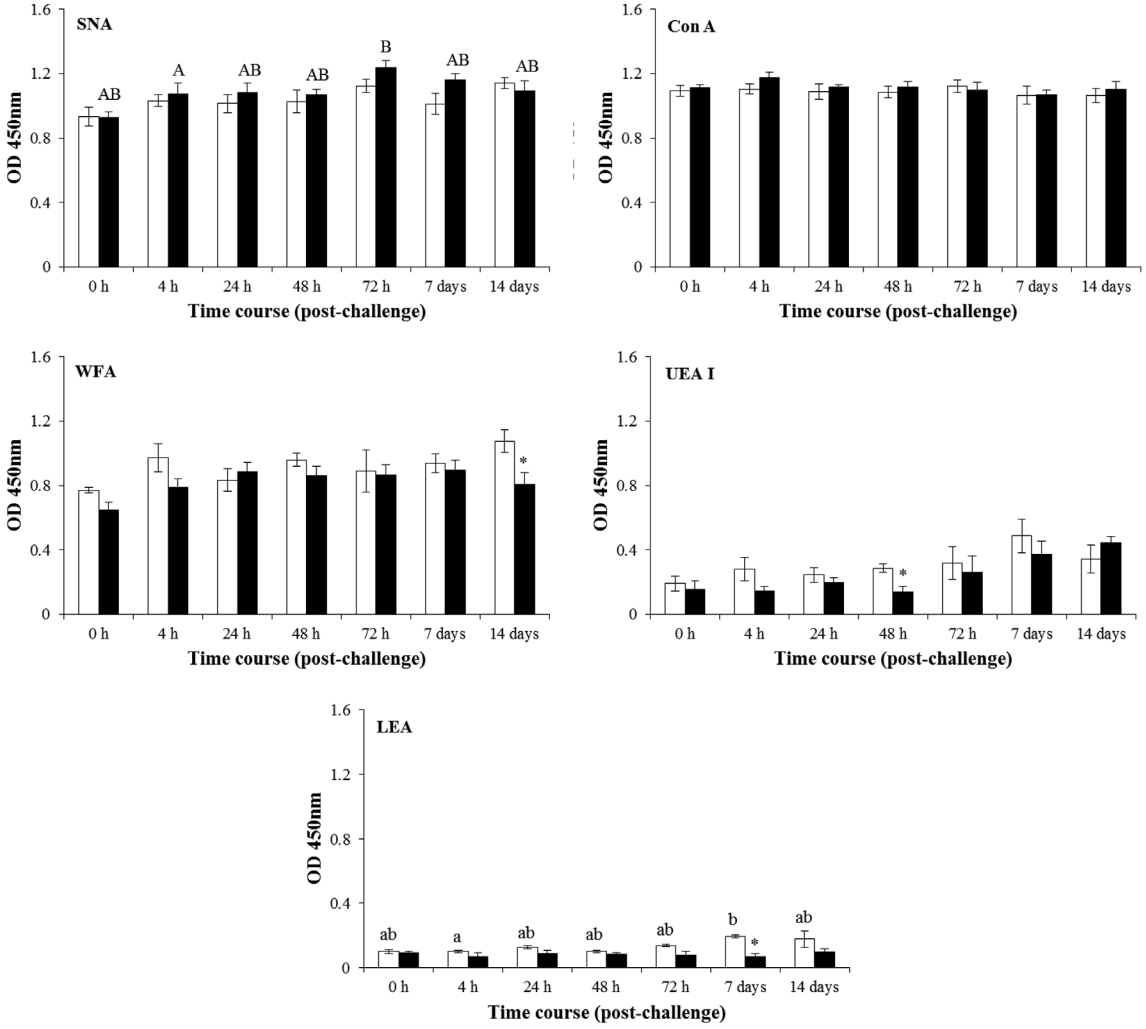

Fig. 1. Lectin binding (OD 450 nm) to *N*-acetylneuraminic acid (SNA), α-D-mannose, α-D-glucose (Con A), *N*-acetyl-D-galactosamine (WFA), α-L fucose (UEA I) and *N*-acetyl-β-D-glucosamine (LEA) carbohydrates present in the skin mucus of control (unchallenged) Senegalese sole juveniles and challenged specimens with *T. maritimum* for 24 h after 0, 4, 24, 48, 72 h, 7 and 14 days. Bars represent the mean ± SEM (n = 6). Asterisks denote significant differences between experimental groups for a given time (T-test; $p < 0.05$), whilst small (unchallenged) and capital letters (challenged) denote significant variations within each experimental group regarding time (ANOVA; $p < 0.05$).

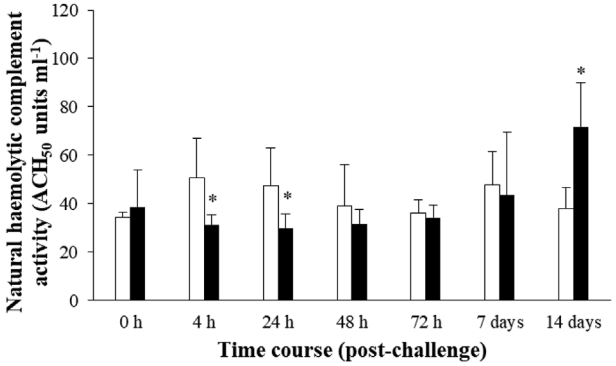

Fig. 2. Natural haemolytic complement (ACH_{50} units ml^{-1}) in skin mucus samples of control (unchallenged) Senegalese sole juveniles and challenged specimens with *T. maritimum* for 24 h after 0, 4, 24, 48, 72 h, 7 and 14 days. Bars represent the mean ± SEM (n = 6). Asterisks denote significant differences between experimental groups for a given time (T-test; $p < 0.05$).

both activities increased in challenged fish after 7 d post-challenge with respect to the control group. Antiprotease activity showed an increase in the challenged fish at the end of the experiment (14 d) compared to the unchallenged fish (Fig. 4C).

Lysozyme activity of the challenged fish increased from 72 h until the end of the trial (14 d) compared to the rest of the sampling points (Fig. 4A). However, peroxidase activity increased in the unchallenged fish at the end of the experiment with respect to the values found at 0, 48 and 72 h. Similarly, peroxidase activity increased in the challenged group after 48 h, 7 d and 14 d compared to the other sampling points (Fig. 4B). The antiprotease activity from the control group decreased after 14 d of the experiment compared to values observed at 0, 4 and 24 h, and this activity was lower at 24 h than the values at 0 h in the challenged fish (Fig. 4C).

3.6. Bactericidal activity in skin mucus and plasma

The bactericidal activity of the skin mucus against *Pdp* and *T. maritimum* is shown in Fig. 5A and B, respectively. In both cases, the bactericidal activity of the skin mucus from the challenged fish increased at the end of the trial (14 d) compared to the unchallenged group. In contrast, bactericidal activity in the challenged fish decreased against both pathogens after 4 and 48 h in the case of *Pdp* and after 0 and 48 h against *T. maritimum* in comparison to the control fish. Over time, the bactericidal activity against *Pdp* in the control group

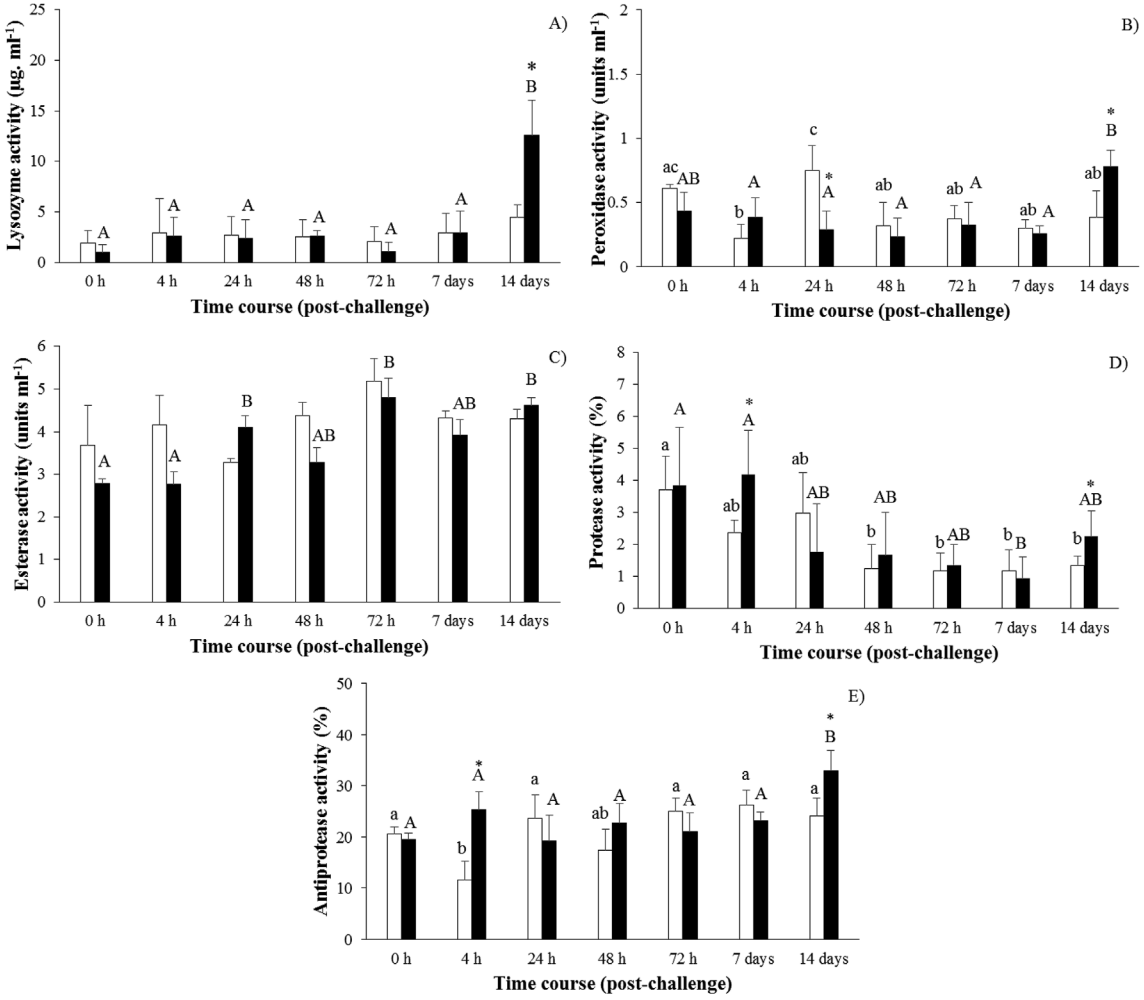

Fig. 3. Lysozyme (µg ml^{-1}) (A), peroxidase (units ml^{-1}) (B), esterase (units ml^{-1}) (C), protease (%) (D) and antiprotease (%) (E) activities in skin mucus samples of control (unchallenged) Senegalese sole juveniles and challenged specimens with *T. maritimum* for 24 h after 0, 4, 24, 48, 72 h, 7 and 14 days. Bars represent the mean ± SEM (n = 6). Asterisks denote significant differences between experimental groups for a given time (T-test; $p < 0.05$), whilst small (unchallenged) and capital letters (challenged) denote significant variations within each experimental group regarding time (ANOVA; $p < 0.05$).

decreased at 72 h, 7 d and 14 d with respect to values found at the first three sampling points. In the challenged fish, the bactericidal activity against *Pdp* also was reduced at 48 h, 72 h and 7 d compared to the values obtained at 0 h, 4 h and 14 d (Fig. 5A). In the case of bactericidal activity against *T. maritimum*, the values dropped at all sampling points, except at 48 h, compared to values found at 0 h in the unchallenged group, whereas the infected group showed the opposite pattern with an increase at the end of the experiment compared to the rest of experimental times (Fig. 5B).

In the case of plasma, the bactericidal activity against *Pdp* and *T. maritimum* is shown in Fig. 6A and B, respectively. While values against *Pdp* in the challenged fish at 0 h, 72 h and 14 d were higher than in the control fish, plasma bactericidal activity against *T. maritimum* rose at 24 h and 14 d compared to the values found in the unchallenged group. Over time, no changes were observed in plasma from the control fish against both bacteria tested. Moreover, plasma from the challenged fish showed an increase of bactericidal activity against *Pdp* at 14 d compared to values found at 24 h, while those values decreased against *T. maritimum* at 48 h, 72 h and 7 d compared to 24 h after the bath challenge.

4. Discussion

Fish are constantly exposed to waterborne pathogens and skin mucus is considered the first line of defence against an extensive range of them [28,29]. Although the elimination of skin mucus results in higher mortalities following a challenge with bacteria [30], the persistent shedding of mucus secretions is essential for flushing out invading bacteria [31]. Fish mucosal barriers contain a wide assortment of innate immune compounds including complement proteins, lysozyme, proteases, esterase, antimicrobial peptides (AMPs), C-reactive proteins and trypsin-like enzymes [12,32,33]. To the best of our knowledge, the present study is the first structured attempt to assess the abundance of terminal carbohydrates, immune-related enzymes and bactericidal activity of the skin mucus against *T. maritimum* in Senegalese sole.

In the present study, the haematological profile and the kinetics of cell migration post-infection were also studied. Recently, Fazio [34] highlighted the use of haematological parameters as an important tool for aquaculture in the diagnosis of disease and nutritional status of fish. Results from the present study showed an increase of haemoglobin concentration and MCH in the challenged Senegalese sole at 24 h post-challenge compared to unchallenged specimens, whilst the RBC remained unaltered. The increased haemoglobin and MCH levels could be related to an altered oxygen demand due to the disease progression in the challenged fish. In contrast, Bandeira Junior et al. [35] observed lower haematocrit, RBC and haemoglobin levels in infected silver catfish (*Rhamdia quelen*) with pathogenic *Citrobacter freundii* after 18 d

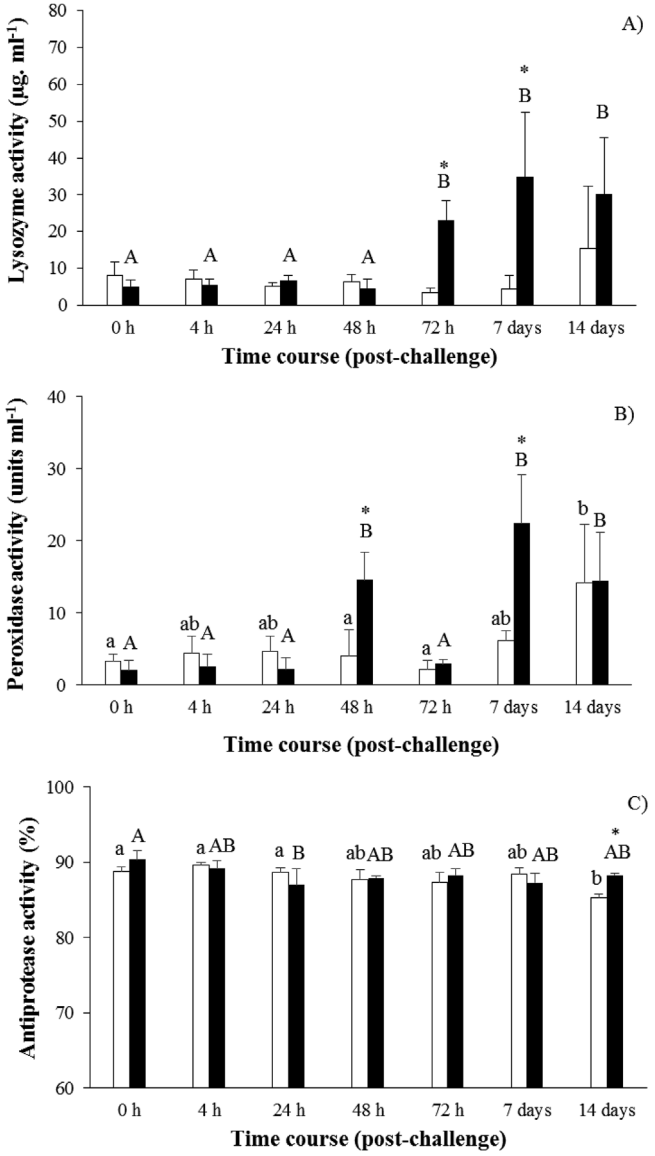

Fig. 4. Lysozyme (µg ml^{-1}) (A), peroxidase (units ml^{-1}) (B) and antiprotease (%) (C) activities in plasma samples of control (unchallenged) Senegalese sole juveniles and challenged specimens with *T. maritimum* for 24 h after 0, 4, 24, 48, 72 h, 7 and 14 days. Bars represent the mean ± SEM (n = 6). Asterisks denote significant differences between experimental groups for a given time (T-test; $p < 0.05$), whilst small (unchallenged) and capital letters (challenged) denote significant variations within each experimental group regarding time (ANOVA; $p < 0.05$).

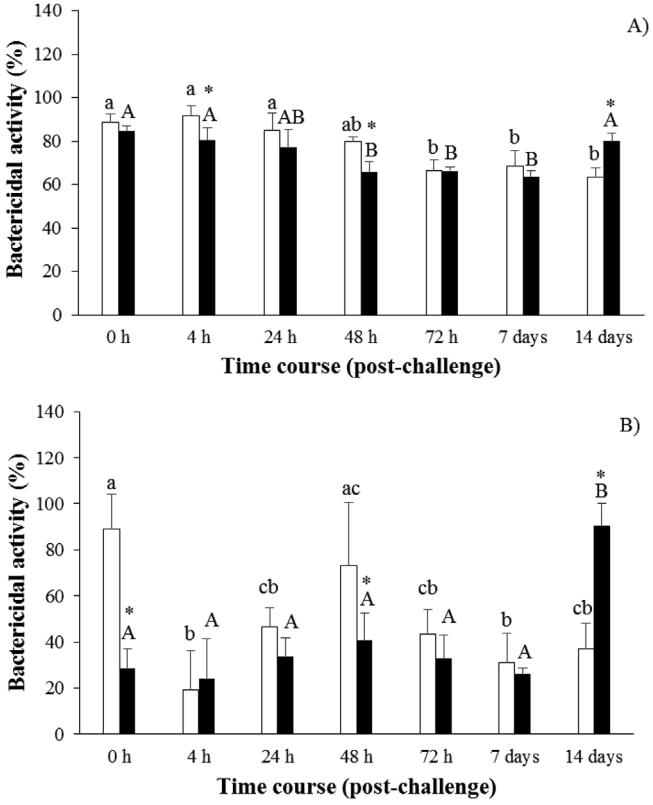

Fig. 5. Bactericidal activity (%) against *Photobacterium damselae* (A) and *Tenacibaculum maritimum* (B) in skin mucus samples of control (unchallenged) Senegalese sole juveniles and challenged specimens with *T. maritimum* for 24 h after 0, 4, 24, 48, 72 h, 7 and 14 days. Bars represent the mean ± SEM (n = 6). Asterisks denote significant differences between experimental groups for a given time (T-test; $p < 0.05$), whilst small (unchallenged) and capital letters (challenged) denote significant variations within each experimental group regarding time (ANOVA; $p < 0.05$).

post-infection. Those contrasting results are most likely linked to different methodological approaches as well as host responses against different pathogens. In this study, a lower concentration of WBC was observed in infected soles at 0 h post-challenge compared to control fish. Similar results were reported in pacu (*Piaractus mesopotamicus*), rainbow trout (*Oncorhynchus mykiss*) and silver catfish (*Rhamdia quelen*) infected by *Aeromonas hydrophila* [36], *Vibrio anguillarum* [37] and *C. freundii* [35], respectively. In contrast to the results found in this study, Nile tilapia (*Oreochromis niloticus*) injected with *Enterococcus* sp showed an increase of WBC after 24 h of injection [38]. However, these authors justified that these results may be related to the stress of handling and not due to the bacterial injection itself. Concomitantly, further studies should be performed to further our knowledge of the haematological changes caused by bacterial infections.

On the other hand, most of the invading pathogens use sugar-binding proteins as lectins to recognize and bind to the host's terminal carbohydrates, which is the first step in the initiation of defensive responses [39]. The carbohydrate residues tested in the present work were selected because they have been described to be related to the infection's progress [39,40]. The strong binding affinity of Con A, SNA and WFA lectins to the skin mucus of Senegalese sole suggests that the most abundant terminal carbohydrates glycosylations are α-D-mannose, α-D-glucose, *N*-acetylneuraminic acid and *N*-acetyl-D-galactosamine. Overall, results from this study showed slight variations, mainly decreases, in the carbohydrates abundance in the skin mucus of fish following the bath challenge. For instance, a decrease in the amount of *N*-acetyl-D-galactosamine (WFA lectin binding) was observed at the end of experiment (14 d), which is related to the correct cell-to-cell communication, important both for normal systemic function and disease processes [41]. However, those results contrast to the ones reported by Schroers et al. [42], who observed a marked increase in the amount of mannose and *N*-acetyl-β-galactosamine in the intestinal mucus of common carp (*Cyprinus carpio*) after 6 d of a perorally administered challenge with *A. hydrophila*.

Considering disease progression, results from the present study showed a higher quantity of *N*-acetylneuraminic acid (detectable by SNA) in the skin mucus of challenged fish at 72 h compared to values found at 4 h. This predominant sialic acid also seems to be involved in immunity and associated with infection resistance. In fact, its role in the progression of infection was corroborated in a previous study where sialic acid could initially protect the intestines of fish from the invasion and dispersion of an intestinal parasite, *Enteromyxum leei* [43]. Nevertheless, a dramatic decrease in the expression of *N*-acetylneuraminic

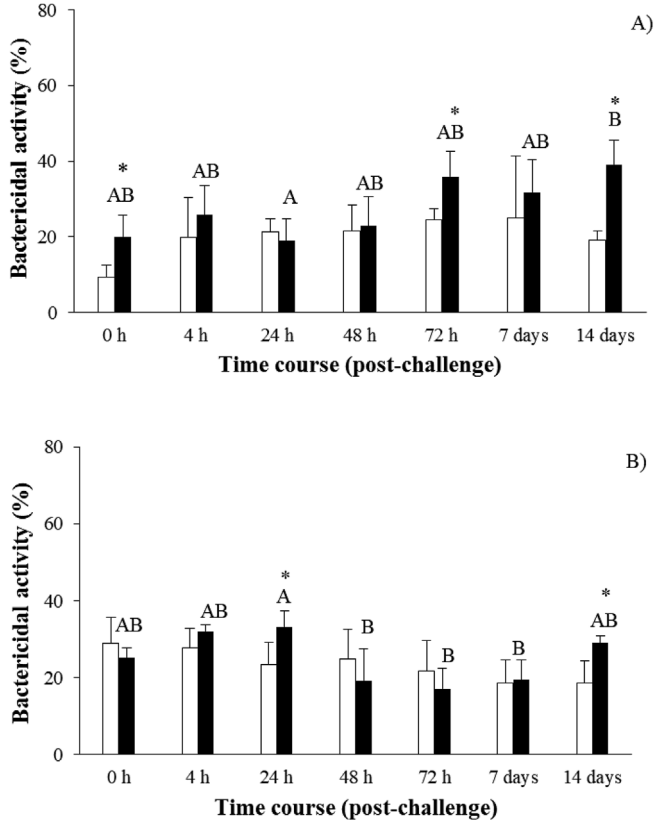

Fig. 6. Bactericidal activity (%) against *Photobacterium damselae* (A) and *Tenacibaculum maritimum* (B) in plasma samples of control (unchallenged) Senegalese sole juveniles and challenged specimens with *T. maritimum* for 24 h after 0, 4, 24, 48, 72 h, 7 and 14 days. Bars represent the mean ± SEM (n = 6). Asterisks denote significant differences between experimental groups for a given time (T-test; $p < 0.05$), whilst small (unchallenged) and capital letters (challenged) denote significant variations within each experimental group regarding time (ANOVA; $p < 0.05$).

acid was reported by those authors after the development of infection. In contrast, another study reported an increase in the amount of *N*-acetylneuraminic acid in the intestines following a bacterial infection in common carp [42]. In the present study, *T. maritimum* did not cause important variations in the skin mucus's carbohydrates profile. Therefore, further in-depth studies are needed to characterise the skin mucus's carbohydrates to understand their precise role in pathogen adhesion and invasion and whether their presence is related to disease resistance.

Regarding systemic immune responses to the bath challenge with *T. maritimum*, the observed increase in circulating neutrophils after 48 h was positively correlated to the increments detected in peroxidase activity in the plasma of the challenged fish at the same time ($R^2 = 0.953$; $P = 0.047$), whilst this activity was also correlated to higher lysozyme activity at 72 h following the bath challenge ($R^2 = 0.966$; $P = 0.002$). Cases of blood neutrophilia have been reported in several fish species following a challenge with different bacterial pathogens [37,44–46]. For instance, similar results were reported by Costas et al. [46], who stated that the blood neutrophilia observed following a *Pdp* challenge was related to plasma peroxidase activity in Senegalese sole. In fact, these phagocytes are considered to be the source of the plasma's lysozyme and peroxidase [47], and increases in both lytic enzymes have been associated with cases of neutrophilia [46,48]. Moreover, Balfry et al. [49] attributed the decline of serum lysozyme activity to the low number of neutrophils observed in the blood of Nile tilapia following a challenge with *Vibrio parahaemolyticus*. In the present study, the blood neutrophilia observed at 72 h could be correlated to plasma lysozyme activity, which was increased. However, although both activities (peroxidase and lysozyme) were also enhanced in the plasma of challenged fish compared to the control group at 7 d following the bath challenge, no correlations were observed with respect to the values of neutrophilia found. In the case of the plasma's antiprotease and bactericidal activities, an increase in the challenged fish compared to the unchallenged fish was observed at the end of the experiment (14 d). Therefore, overall results at a systemic level suggest that the Senegalese sole's immune machinery is activated by *T. maritimum* and is prolonged for at least 14 d after contact with the pathogen. This sustained immune response by the host is probably related to the ability of this particular pathogen to strongly attach to the external body surface of the fish, which could mean that the bacterium did not overcome the fish immune system at the sub-lethal concentration tested.

Indeed, *T. maritimum* has the ability to produce a substantial amount of extracellular polymers or 'slime', allowing them to adhere more firmly to the host's skin [50]. Results from the present study showed a differential response over time for most of the immune-related parameters tested in the skin mucus. Bath challenged fish presented a decrease in those values at earlier times of the experiment (between 0 and 48 h), whereas a generalized increase in all activities was observed at the end (14 d). In the case of protease and antiprotease activities, both were increased in the challenged fish at 4 h and 14 d, suggesting early and late host responses against bacterial colonization. This delayed stimulation of activities in the skin mucus of the challenged fish observed at 14 d could be indicative of a successful disease proliferation. This theory is supported by the peroxidase, lysozyme and haemolytic complement values at the same experimental time. The possible location of *T. maritimum* within the mucus layer could justify the late response observed since this bacterium could remain in the aquatic environment for a long time, utilizing fish mucus as a reservoir in line to what was reported by Avendaño-Herrera [17]. For instance, Magariños et al. [16] revealed that *T. maritimum*, regardless of its origin and degree of virulence, adhered strongly to the skin mucus of three fish species (turbot [*Scophthalmus maximus*], gilthead seabream [*Sparus aurata*] and European seabass [*Dicentrarchus labrax*]) and resisted its bactericidal properties, which suggests that the skin may be a possible portal of entry for this pathogen into the fish body. This hypothesis could be further supported by the suppression observed in some immune parameters at early stages of the challenge whilst the activation of the local immune response (skin mucus) was delayed for 14 d. The ability of *T. maritimum* to resist local and systemic bactericidal host responses is known [18], which could also be related to certain evolving strategies to withstand the innate immunity of fish as already recorded in many other Gram-negative pathogens [51]. Therefore, although the mechanisms of *T. maritimum´s* evasion still remain to be elucidated, the present study could provide some information regarding the *in vivo* response either locally (i.e. skin mucus) or systemically (i.e. plasma) after a bacterial bath challenge.

The lytic activity of the complement system has been well recognized as one of the key killing mechanisms of bacteria in teleosts [10,47,52]. The complement acts both directly via the formation of a membrane attack complex (MAC) and synergistically through the opsonization of pathogens and the attraction of phagocytic cells. The alternative complement pathway, which is antibody independent, is activated by a variety of microorganisms including bacteria and can result in lysis of the bacterial cell [53]. The current study revealed a significant reduction in the haemolytic complement activity in the skin mucus of challenged fish at 4 and 24 h post-challenge, which could be related to *T. maritimum's* evasion strategies and continuous cell proliferation. Interestingly, the opposite pattern was recorded after 14 d, which could be related to the activation of the mucosal system. Likewise, the downregulation of immune-related genes in the skin mucus of channel catfish (*Ictalurus punctatus*) during the early time points following a challenge with *Flavobacterium columnare* could support our results [54]. Although lipopolysaccharides (LPS) are widely known as

small molecular motifs conserved within Gram-negative bacteria, the composition analysis of the LPS from *T. maritimum* revealed an O-chain composed of a disaccharide that contained an unusual linkage ([R]-2-hydroxyglutaric acid residue), which seems to be unique for this bacterium, and suggested that it might have a role in biofilm formation within the host's tissues [55]. Cells within the biofilm could evade complement factor recognition and thus subsequent WBC recruitment and killing [56].

The available data concerning the *in vivo* bactericidal activity of skin mucus after a bacterial challenge is limited to some fish species and still not fully elucidated. Therefore, the present study also investigated the bactericidal activity against two pathogenic bacteria (*Pdp* and *T. maritimum*) of Senegalese sole's skin mucus following an experimental bath challenge with *T. maritimum*. It was observed that the skin mucus of both the unchallenged and challenged fish had a stronger bactericidal activity than the plasma's, reinforcing the fundamental role of fish skin mucus in preventing bacterial colonization, as has previously been reported [33,57,58]. The opsonizing activities of complement together with its synergistic action with lysozyme are considered an important defence mechanism against pathogen invasion. In fact, the delayed increase of the skin mucus's bactericidal activity was consistent with the increment observed in the peroxidase, lysozyme and haemolytic complement activities at the same time (14 d), suggesting that the late response is mainly due to the phagocytes' recruitment of the mucosal surfaces against *T. maritimum*. However, the reduction of *T. maritimum* bactericidal activity in the skin mucus of challenged fish compared to unchallenged ones at both 0 and 48 h could suggest that the bacterium successfully reduced the mucosal antibacterial activity at the beginning of the experiment. The greater bactericidal activity after 14 d suggested that the immune system of the fish strengthened to get around the evasion system of the bacterium. Nevertheless, further studies are needed for this hypothesis to be clarified.

In summary, *T. maritimum* appears not to cause important variations in the glycoprotein composition of Senegalese sole's skin mucus during the disease's progression since it could withstand and overcome the skin mucus's immunity using different evasion strategies, such as passively changing the glycosylation pattern. This pathogen seemed to trigger an activation of the Senegalese sole's innate immune response by improving the recruitment of neutrophils, followed by the stimulation of the systemic and mucosal innate immunity. The generally delayed mucosal immune machinery seems to align with the incipient plasma peroxidase and lysozyme activities, suggesting the involvement of bacterial extracellular toxins or enzymes in their evading strategy and supporting the systemic infection. At the end of trial, the bactericidal activities of the plasma and skin mucus reflect the activation of systemic and local immune responses against invading opportunistic pathogens. Remarkably, the bactericidal activity in the skin mucus is higher than in the plasma, supporting the crucial role of skin mucus in fish immunity.

Acknowledgements

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