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Physiopathological responses of sole (*Solea senegalensis*) subjected to bacterial infection and handling stress after probiotic treatment with autochthonous bacteria

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ABSTRACT

This study aimed to evaluate the protective effects of four autochthonous bacteria isolated from juvenile sole (*Solea senegalensis*) intestine as dietary probiotic supplement against bacterial pathogen infection and handling/transport stressors. Growth performance and immune responses were evaluated after 85 days of feeding trial. Sole (IBW = 16.07 ± 0.11 g) were fed six experimental diets, a control diet (CTRL, without the dietary probiotic supplementation), and five diets supplemented with probiotic bacteria: PB1 (*Shewanella hafniensis*), PB2 (*Enterococcus raffinosus*), PB3 (*Shewanella hafniensis* + *Arthrobacter soli*), PB4 (*Pseudomonas protegens* + *Arthrobacter soli*) and PB5 (*Shewanella hafniensis* + *Arthrobacter soli* + *Enterococcus raffinosus*). All bacteria were selected based on their *in vitro* antimicrobial activity. After the growth trial, fish were submitted to a stress factor (transport) and then each dietary group was divided in two additional groups: non-infected (placebo) and infected with *Photobacterium damselae* subsp. *piscicida*. Immune and antioxidant responses were evaluated at day 10 post-infection. In infection trial A, fish were infected on the same day of transport, whereas in trial B fish were infected after a 7-day recovery from the transport stress. At the end of the feeding trial, fish fed with PB2 and PB4 showed lower final body weight when compared with the other dietary groups. Respiratory burst activity and nitric oxide production were not affected by probiotic supplementation. Fish fed with PB5 presented lower peroxidase activity compared to CTRL. Lysozyme and alternative complement pathway activity (ACH50) showed no significant differences between treatments. The innate immune responses were significantly affected after handling stress and bacterial infection. In trial A, the ACH50 levels of infected fish were significantly lower than the placebo groups. On the other hand, in trial B fish infected with *Pdp* demonstrated higher ACH50 levels when compared to placebos. Peroxidase levels were strongly modulated by bacterial infection and handling stress. In trials A and B, infection had a clear downgrade effect in peroxidase levels. Lipid peroxidation, catalase, glutathione S-transferase and glutathione reductase were altered by both bacterial infection and transport.

Overall, dietary probiotic supplementation did not influence growth performance of sole. The immune and oxidative defenses of sole responded differently to infection depending on the probiotic and the synergy between pathogen infection and transport.

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1. Introduction

The fast growth of the aquaculture sector has been accompanied by several challenges such as fish intolerance to stress factors, both abiotic and biotic. In intensive aquaculture, severe stress may result in massive production losses [1]. Previous studies report the effects of stress events in animal condition [2–5]. Animals response to stress is accompanied by physiologic and behavioral changes that are either compensated by internal mechanisms, or ultimately can lead to death [6]. The allostatic load derived from a stress event causes shifts in metabolism, that may activate alternative metabolic pathways leading to a new state of stasis [6]. Fish physiological response to abiotic stressors [3,7–9] results in negative effects on fish condition such as reduced growth [10,11], diminished immune function [12] and consequent reduced resistance to diseases [13]. Moreover, the metabolic adjustments caused by abiotic stress factors can lead to greater vulnerability to pathogenic infections [14] triggered by opportunistic bacteria/virus. This culminates in suboptimal production, which risks the industry profits. Still, in fish, the mechanisms of accommodation to a long-term stress are not yet fully understood. Nowadays, a joint effort between producers and researchers, targets to understand how fish condition is modulated, so infectious outbreaks can be diminished [15]. This can be achieved through the interpretation of biomarkers of both the immune and antioxidant systems, which might allow the timely application of prophylactic measures [16–20].

Aquaculture sector response to pathogenic epidemics has varied from the indiscriminate use of antibiotics [21], to more environmentally-friendly and sustainable methodologies. The application of antibiotics, generally in large amounts, leads to their ubiquitous dissemination in aquatic systems wielding a strong and lasting selective pressure in the environment [22]. Alternatively, bacteriophages, antivirulence therapy, chromosome II replication inhibition and probiotic administration [23–26] have also been applied as mitigating measures for disease outbreaks. These unconventional methodologies have been gaining supporters in both industrial and scientific fields since they have a narrower/specific spectrum of activity [27]. These strategies to control bacterial infections in aquaculture entail a lower chance of bacterial resistance development than antibiotics. Considering such, a more rational approach in the search for agents which can exert a biocontrol effect over pathogens lead to the isolation of endemic probiotic bacteria recruited from the aquatic environment. These bacteria have been used to improve water quality, as well as control bacterial infections [28]. Currently, probiotics potential benefits encompass improvements in nutritional retention [29], antagonistic properties towards bacterial proliferation [30], nutrient competition towards possible pathogens [31], competition for accommodation space [32], modulation of the host immune system responses [33] and probiotics modulatory influence over antioxidants activity and/or availability. Probiotic strains efficiency depends on their capacity to survive, at adequate concentrations, on the hosts intestine, which may be correlated with their origins [34]. Therefore, the autochthonous source strategy, which comprehends the isolation of bacteria from the gut of mature animals, has been successfully applied in fish [35]. Moreover, multi-strain probiotic products have been suggested as more effective and more consistent than single strain probiotic, as mixed cultures may contain bacteria that complement each other's health effect and thus have synergistic probiotic properties [34].

Senegalese sole (*Solea senegalensis*, Kaup 1858) is a marine species with increasing importance for aquaculture production in Portugal and Spain [36]. However, sole is highly susceptible to microbial pathologies, which lead to heavy economic setbacks [37,38]. *Photobacterium damselae* subsp. *piscicida* (*Pdp*) is the most frequent pathogen in marine fish, including Senegalese sole [39,40]. The *Pdp*, formerly known as *Pasteurella piscicida*, is a gram-negative rod which causes a disease in fish known either as pseudotuberculosis or fish pasteurellosis [41].

Overall, the current study aimed to evaluate whether fish preconditionally fed with probiotic supplemented diets are more resilient to opportunistic pathogens after handling and/or transport stress situations. Specifically, the current study determined the cross effects of dietary probiotic supplementation, *Pdp* infection and handling stress on growth performance, oxidative stress and innate immune responses in juvenile sole (*Solea senegalensis*).

2. Material and methods

The current study was conducted under the supervision of an accredited expert in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGV Portugal, following FELASA category C recommendations), according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE.

2.1. Experimental organisms and rearing conditions

Senegalese sole juveniles of 16.07 ± 0.11 g were obtained from a commercial fish farm (Aquacria S.A., Torreira, Portugal) and transported to CIIMAR (Porto, Portugal) facilities. Fish were acclimated to the experimental facilities and rearing conditions for 2 weeks prior to the beginning of the experiment, while fed a basal diet (Aquasoja, Ovar, Portugal) at 1.5% level of average body weight.

The water temperature was set to 17 ± 1 °C and salinity to 25 ± 1‰. Dissolved oxygen levels were maintained by aeration at 8 ± 1 $mg.L^{-1}$, ammonia and nitrite concentration remained below 1 $mg.L^{-1}$ and water pH at 8 ± 0.5. Photoperiod was set for 12:12 h light:dark periods.

2.2. Experimental diets

All ingredients and proximate composition of the basal formulation of the experimental diets are presented in Table 1.

All chemical analyses were carried according to AOAC [42]. Diets were analyzed for dry matter content (105 °C for 24 h), ash (Nabertherm L9/11/B170; Bremen, Germany; 550 °C for 5 h), crude protein (N × 6.25, Leco N analyser, Model FP-528, Leco Corporation, St. Joseph, USA), crude lipid (petroleum ether extraction, 40–60 °C, Soxtherm, Gerhardt, Germany) and gross energy (adiabatic bomb calorimeter, Werke C2000, IKA, Germany).

The basal diet (SPAROS Lda., Olhão, Portugal) was prepared to meet the *S. senegalensis* nutritional requirements, with 54% crude protein, 18% crude lipid and 22 $Kj.g^{-1}$ gross energy. The control diet (CTRL) was the basal diet without the dietary probiotic supplementation. The levels of dietary probiotic inclusion are depicted in Table 2. Diets PB_1 and PB_2 are mono-species probiotic diets. Diet PB_1 was supplemented with *Shewanella hafniensis* and diet PB_2 with *Enterococcus raffinosus*. The remaining diets were supplemented with multi-species blends of two or three bacteria. Diet PB_3 was supplemented with *S. hafniensis* and *Arthrobacter soli*. Diet PB_4 was supplemented with *Pseudomonas protegens* and *A. soli*, whereas PB_5 diet was supplemented with three bacteria, *S. hafniensis*, *A. soli* and *E. raffinosus*.

The autochthonous bacteria tested in the current study as candidate probiotics for *S. senegalensis* were selected from a pool of 250 bacteria strains, isolated from the gut microbiota of farmed healthy animals, using *in vitro* antagonism tests based on the production of inhibitory compounds against several common fish pathogens. Thereafter, the 16 bacteria strains with the best results (unpublished results) were tested for their stability and viability during feed processing with or without encapsulation (unpublished results). Among those 16 bacteria tested, the most favorable strains were *A. soli*, *S. hafniensis*, *E. raffinosus* and *P. protegens*, due to their *in vitro* antimicrobial activity coupled with good processing stability. Each strain was individually grown in Luria Bertani Broth (LB medium, Invitrogen) for 36 h at room temperature and with constant agitation. After incubation, bacterial cultures were centrifuged

Table 1
Ingredients and proximal composition of the basal diet.

Ingredient (% Dry Weight)	Basal diet
Fishmeal 70 LT[a]	18.5
Fishmeal 60%[b]	25
CPSP 90%[c]	2.5
Squid meal	2.5
Soybean flour	5
Soycomil PC[d]	6.2
Pea flour (Lysamine GP)	10
Wheat flour	7.3
Corn gluten	5
Fish oil	9.3
Vitamins[e] & Minerals Premix 1%[f]	1
Di-calcium phosphate	1.5
Aquatex 8071[g]	5
Binder	1
Proximate analyses (% Dry Weight)	
Dry matter	93.66
Crude protein	56.34
Crude lipid	16.64
Ash	14.29
Gross energy (Kj g^{-1})	21.69

[a] Peruvian fishmeal LT (71% crude protein, 11% crude fat, EXALMAR, Peru).
[b] Fair Average Quality (FAQ) fishmeal (62% crude protein, 12% crude fat, COFACO, Portugal).
[c] Soluble fish protein hydrolysate (87% crude protein, 6.5% crude fat, Sopropêche, France).
[d] Soycomil-P (soy protein concentrate, 65% crude protein, 0.7% crude fat, ADM, The Netherlands).
[e] Vitamins (mg or IU kg−1 diet): vitamin A (retinyl acetate), 20,000 IU; vitamin D3 (DL-cholecalciferol), 2000 IU; vitamin K3 (menadione sodium bisulfite), 25 mg; vitamin B1 (thiamine hydrochloride), 30 mg; vitamin B2 (riboflavin), 30 mg; vitamin B6 (pyridoxine hydrochloride), 20 mg; vitamin B12 (cyanocobalamin), 0.1 mg; vitamin B5 (pantothenic acid), 100 mg; vitamin B3 (nicotinic acid), 200 mg; vitamin B9 (folic acid), 15 mg; vitamin H (biotin), 3 mg; betaine, 500 mg; inositol, 500 mg; choline chloride, 1000 mg; vitamin C (stay C), 1000 mg; vitamin E, 100 mg.
[f] Minerals (% or mg kg−1 diet): Mn (manganese oxide), 9.6 mg; I (potassium iodide), 0.5 mg; Cu (cupric sulphate), 9 mg; Co (cobalt sulphate), 0.65 mg; Zn (zinc oxide), 7.5 mg; Se (sodium selenite), 0.01 mg; Fe (iron sulphate), 6 mg; Cl (sodium chloride), 2.41%; Ca (calcium carbonate), 18.6%; NaCl (sodium), 4%.
[g] Dehulled, grinded yellow pea grits, 24% crude protein, 1% crude fat, SOTEXPRO, France).
[h] Diatomaceous earth: Kielseguhr: LIGRANA GmbH, Germany.

for 20 min at 5000 rpm and 4 °C. The resulting pellet was washed with saline buffer (0.85% NaCl, Panreac) and resuspended in 150 mL saline buffer. *Shewanella hafniensis* was inactivated by heat (1 h at 60 °C) prior to incorporation. The remaining bacterial suspensions were quality controlled and plated for colony forming units (CFUs) evaluation. The probiotic strains were added to the diets by cold extrusion which was determined as the most viable method for these experiments.

2.3. Pathogenic bacteria - *Photobacterium damselae subsp. piscicida (Pdp)*

The bacterial strain PC566.1 used in the present study was kindly provided by Dr Ana do Vale (Institute for Molecular and Cell Biology, University of Porto, Portugal) and isolated from Senegalese sole (Spain) by Professor Alicia E. Toranzo (Departamento de Microbiología y Parasitología, Facultad de Biología, University of Santiago de Compostela, Spain). Bacteria were routinely cultured at 22 °C in tryptic soy broth (TSB) and were stored at −80 °C in TSB-1 supplemented with 15% (v/v) glycerol.

To prepare the inocula for peritoneal injection stocked bacteria were cultured in tryptic soy agar (TSA) (VWR, Portugal), supplemented with NaCl (Sigma, Portugal) to a final concentration of 1.5% (w/v), for 48 h at 22 °C and then inoculated into tryptic soy broth (TSB, Fluka, Sigma Aldrich, Portugal) supplemented with 1.5% NaCl. The bacterial suspension was incubated overnight at 22 °C, with constant agitation (100 rpm). Exponentially growing bacteria were collected by centrifugation at 3500*g* for 30 min and the resulting pellet was resuspended in Hank's Balanced Saline Solution (HBSS, Gibco, UK) and adjusted to 1.84×10^3 colony forming units (CFU) mL^{-1}, based in the bacteria growth equation which was previously determined by CFU and optical density correspondence. Serial dilutions of the suspension were plated onto TSA plates and the number of CFU following incubation at 22 °C confirmed bacterial concentrations.

2.4. Experimental design

2.4.1. Growth trial

Five hundred and twenty Senegalese sole juveniles were randomly distributed in 18 tanks of 50 L each connected to a seawater circulating system. Each dietary treatment was tested in triplicate. Fish were weighed at the beginning and the end of the 85-day experiment. Soles were manually fed (n = 28 $fish.tank^{-1}$) three times a day (10:00, 14:00 and 18:00) until apparent visual satiation. At the end of the feeding trial, 12 $fish.treatment^{-1}$ were anaesthetized using ethyl 3-aminobenzoate methanesulfonate (MS-222; 200 mg L^{-1}; Sigma-Aldrich, Germany), weighted and blood was withdrawn from the caudal vein for plasma collection, using heparinized syringes, and immediately centrifuged at 10,000 G, for 10 min, at 4 °C. Fish were euthanized by cervical dislocation before tissues collection.

The growth performance was evaluated based on the following parameters: Specific Growth Rate (SGR, %. day^{-1}) = $[(\ln W_t - \ln W_0)/(t - t_0)] \times 100$, where W_t, t, W_0 and t_0 are the weight and time (in days) at the end and at the beginning of the experiment, respectively. The Condition Factor (K, g. cm^3) = (Final weight * 100)/(Final $length^3$) and the hepatosomatic Index (HSI, g. kg^{-1}, wet weight basis) = liver weight/whole body weight.

2.4.2. Handling and pathogen stress trials

After 85 days of growth trial, 30 fish per dietary treatment were stressed by simulating a transport for 1 h, in densities close to 37.5 kg m^{-3} and oxygen levels near saturation using pure oxygen aeration. At the end of the transport stress, fish were divided into two groups: one (Trial A) to be immediately infected with a non-lethal concentration of *Pdp* (1.84×10^3 CFU mL^{-1}); and the second group

Table 2
The bacteria inclusion levels in the experimental diets.

Probiotics	Experimental diets (CFU Kg^{-1} diet)					
	CTRL	PB_1	PB_2	PB_3	PB_4	PB_5
Shewanella hafniensis	–	5.13×10^{11}	–	2.53×10^{11}	–	2.53×10^{11}
Enterococcus raffinosus	–	–	6.50×10^{10}	–	–	4.33×10^{10}
Arthrobacter soli	–	–	–	1.22×10^{11}	1.22×10^{11}	1.22×10^{11}
Pseudomonas protegens	–	–	–	–	2.33×10^{11}	–

(Trial B) allowed to acclimate for 7 days, under similar conditions to the growth trial, before *Pdp* infection.

The infection was carried out by intraperitoneal injection of 0.1 mL of either HBSS (placebo groups) or bacterial suspension (infected groups). For 10 days post-infection fish were carefully monitored. Mortalities were confirmed as resulting from the bacterial infection by re-isolation of *Pdp* from liver and kidney from deceased fish. An additional confirmation was later obtained using DNA amplification by PCR 16 S (Macrogen, Japan).

At 10th day post infection, the infection trials were terminated due to the absence of deaths for two consecutive days. Hereupon, 6 fish per treatment were anaesthetized and blood and liver were sampled for the analyses of immune and oxidative stress indicators.

2.4.3. Innate immune parameters

Cellular and humoral parameters were analyzed in head-kidney and plasma, respectively. All analyses were conducted in triplicates and spectrophotometrically analyzed in a Power-WaveTM 178 microplate spectrophotometer (BioTek Synergy HT, Vermont - USA).

2.5. Cellular parameters

The respiratory burst activity and nitric oxide production in leucocytes were evaluated as described by Secombes, Ellis and Hardie [43]. Briefly, samples of head-kidney were collected under aseptic conditions and macerated using a cell strainer (100 μm). The cells were then suspended in Leibovitz L-15 medium (L-15: Gibco, Scotland, UK) supplemented with penicillin (100 $IU.mL^{-1}$; Gibco), streptomycin (100 $mg\ mL^{-1}$; Gibco), heparin (20 $U\ mL^{-1}$; Sigma) and 2% FBS (Fetal Bovine Serum, Gibco). The obtained cell suspension was then placed in a Percoll gradient (Sigma) and centrifuged (400 × g, 40 min at 4 °C). Leucocytes were then collected and washed in Leibovitz L-15 with 0.1% FBS, P/S and heparin. Cells were counted using a hemocytometer, and the suspension concentration adjusted to 2×10^7 $cells.ml^{-1}$.100 μL of the cell suspension was plated in 96-well flat bottom sterile plates (Sarstedt, Germany) and incubated at 18 °C for 24 h.

The respiratory burst activity was determined according to Secombes, Ellis and Hardie [43], based on the reduction of ferricytochrome C method for the detection of extracellular O_2 production.

Nitric oxide production was based on the Griess reaction [44] modified by Tafalla and Novoa [45], that quantifies the nitrite content of the supernatant [46]. The molar concentration of nitrite was determined using a standard curve of sodium nitrite (100 Mm to 1.562 μM).

2.6. Humoral parameters

Lysozyme activity was determined by a turbidimetric assay based on the method described by Ellis [47] with minor modifications [48]. Plasma lysozyme lytic activity was measured using *Micrococcus lysodeikticus* and defining that 1 unit of lysozyme activity produced a decrease in absorbance of 0.001 min^{-1}.

Total peroxidase activity was measured following the procedure described by Quade and Roth [49] and determined by defining that one unit of peroxidase produces an absorbance change of 1 OD.

Alternative complement pathway (ACH50) was measured using rabbit red blood cells (RBC, 2.8×10^8 cells mL^{-1}, Probiológica, Portugal) as described by Sunyer and Tort [50]. The reciprocal of the serum dilution causing 50% lysis of RBC is designated as the ACH50.

2.7. Oxidative stress biomarkers

Oxidative stress was analyzed in liver after homogenization with k-phosphate buffer (pH 7.4, 0,1 M). Protein quantification was spectrophotometrically determined at 600 nm according to Bradford [51] and adapted by Guilhermino, Lopes, Carvalho and Soares [52], using bovine γ-globulins as protein standard.

Part of the homogenate was used to determine lipid peroxidation levels (LPO) by measuring the thiobarbituric acid reactive species (TBARS), according to Ohkawa, Ohishi and Yagi [53] and Bird and Draper [54], with the adaptations of Torres, Testa, Gáspari, Masutt, Panitz, Curi-Pedrosa, Almeida, Mascio and Filho [55].

The remaining homogenate was centrifuged at 10,000 G for 20 min at 4 °C to isolate the post-mitochondrial supernatant (PMS). The PMS was used for determinations of catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activities, as well as the contents of total (TG), reduced (GSH) and oxidized glutathione (GSSG).

CAT activity was determined by measuring the H_2O_2 consumption at 240 nm according to Clairborne [56]. GPx activity was determined by measuring the decrease of NADPH at 340 nm [57]. TG and GSSG were measured by the concomitant reaction of the GSH with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), as it is recycled by the abundant GR, allowing a reading at 412 nm [58,59]. Difference being that in GSSG measure, present GSH is trapped with 2-Vinyl-pyridine (Griffith 1980). Finally, GSH content was calculated subtracting the calculated GSSG to the TG. All analyses were conducted in triplicates on a Power-WaveTM 178 microplate spectrophotometer (BioTek Synergy HT, Vermont - USA).

2.8. Statistical analysis

Statistical analyses were carried out following the methods outlined by Zar [60]. Data concerning the growth trial was analyzed by one-way ANOVA. Data resulting from the infection trial were analyzed by two-way ANOVA (IBM SPSS STATISTICS) using infection and diet (probiotic treatment) as dependent variables. Data were scrutinized for normality (Shapiro-Wilk test) and homogeneity of variance (Leven's test). When significant differences were observed, Tukey's post hoc tests were carried out to identify significantly differences between dietary groups. When data did not meet ANOVA assumptions, the non-parametric Kruskal–Wallis test was performed. Results are expressed as mean ± standard error and with a $P < 0.05$ as level of significance.

3. Results

3.1. Feeding trial – growth performance

At the end of the growth trial (85 days) fish fed PB_2 and PB_4 diets showed significant lower final body weight when compared to fish fed CTRL diet (Table 3). The specific growth rate (SGR) varied between 0.41% day^{-1} (PB_2) and 0.58% day^{-1} (CTRL), however no significant differences were observed between diets. The condition factor (K) ranged from 1.09 to 1.20% and hepatosomatic index (HSI) ranged from 1.16 to 1.26%, both without significant differences between dietary treatments.

3.2. Feeding trial - immune parameters

At the end of the growth trial, cellular immune parameters, respiratory burst activity (ROS) and nitric oxide production (NO) were not affected by the probiotic supplementation ($P < 0.05$, Table 4), ranging from 0.32 (CTRL) to 0.86 (PB_5) O_2^- nmoles. NO varied from 6.88 (PB_3) to 6.95 (PB_5) nitrites μmoles.

In humoral parameters analyses, PB5 diet presented lower peroxidase activity (38.68 ± 4.42 EU. mL^{-1}) compared to CTRL (90.30 ± 22.99 EU. mL^{-1}). Lysozyme and alternative complement pathway activity (ACH50) showed no significant differences among treatments, with lysozyme ranging from 1401.85 (CTRL) to 2510.19 EU. mL^{-1} (PB_1) and ACH50 between 10.08 (PB5) to 16.77 $U\ mL^{-1}$ 88 (PB_3).

Table 3
Growth performance and morphometric indexes of *Senegalese sole* after 85-days of probiotic treatment. Values presented as mean ± standard error (n = 84). Different superscript letters indicate significant differences between treatments for P < 0.05.

	CTRL	PB_1	PB_2	PB_3	PB_4	PB_5
IBW	16.14 ± 0.28	16.05 ± 0.27	16.04 ± 0.27	16.10 ± 0.26	16.11 ± 0.25	16.01 ± 0.26
FBW	26.56 ± 0.63^{a}	24.45 ± 0.53^{ab}	22.91 ± 0.53^{b}	24.96 ± 0.60^{ab}	24.03 ± 0.48^{b}	24.05 ± 0.54^{ab}
SGR	0.58 ± 0.04	0.49 ± 0.07	0.41 ± 0.06	0.52 ± 0.02	0.47 ± 0.03	0.50 ± 0.03
K	1.19 ± 0.04	1,17 ± 0.03	1.09 ± 0.02	1.15 ± 0.03	1.13 ± 0.04	1.20 ± 0.03
HSI	1.24 ± 0.03	1.16 ± 0.14	1.25 ± 0.08	1.17 ± 0.03	1.21 ± 0.03	1.26 ± 0.04

IBW (g) – Initial body weight (g).
FBW (g) – Final body weight.
SGR (%. day^{-1}) – Specific growth rate in body weight percentage.
K – Condition factor presented as fish body.$lenght^{-1}$ (g. cm^3).
HSI – Hepatosomatic index exhibited in liver percentage relatively to fish bodyweight.

3.3. *Infection trial - innate immune parameters*

After the feeding and infection trials, all fish were closely monitored.

In trial B, mortalities in *Pdp* groups were observed at day eight post-infection, for diets CTRL, PB1, PB2 and PB5, with 7, 5, 7 and 3 death fish respectively. No statistical differences were found between treatments.

3.3.1. *Pathogen infection with transport (Trial A)*

In trial A, ACH50 analysis (Fig. 1A) revealed significant differences for infection, but not for diet or diet × infection interaction. In CTRL, PB1 and PB_3 diets, placebo fish revealed higher ACH50 levels when compared to infected fish.

Lysozyme activity (Fig. 1B) revealed a significant increase in infected fish within PB_5 diet. No interactions between factors were detected for this parameter.

Peroxidase activities (Fig. 1C) were influenced by the interaction between diets and infection. Significant higher peroxidase levels were observed in placebo fish within diets PB_1 and PB_3.

3.3.2. *Pathogen infection without transport (Trial B)*

In trial B, ACH50 levels (Fig. 2A) showed significant influence from infection with *Pdp*. Comparisons within diets revealed reduced levels for placebo groups in PB_2 and PB_4.

Lysozyme analysis (Fig. 2B) revealed an influence from the infection factor. In diet PB_5 placebo fish showed significantly higher values than infected fish.

Peroxidase activity levels (Fig. 2C) revealed the influence of the infection factor. Except for CTRL, all *Pdp* infected groups demonstrated lower peroxidase levels when compared to placebo.

3.4. *Oxidative stress*

3.4.1. *Pathogen infection with transport (Trial A)*

LPO levels in Trial A (Fig. 3A) showed a significant increase in peroxidation, after infection for diets PB_2, PB_3 and PB_5. CAT (Fig. 3B) and GR (Fig. 3D) activities were significantly higher in infected fish fed PB_5 diet when compared to the other treatments. GST (Fig. 3C) showed decreased activity in infected fish fed PB_5 diet. In addition, except for PB1 and infected PB5, probiotic diets showed a significant increase in GST levels when compared with control group (CTRL).

3.4.2. *Pathogen infection without transport (Trial B)*

Lipid peroxidation in Trial B (Fig. 4A) showed increased levels in infected fish fed the CTRL diet. CAT activity (Fig. 4B) was significantly higher in infected fish within PB_4 diet. GST (Fig. 4C) was significantly higher in infected fish within diets PB_1 and PB_2.

No significant differences were detected in GR analysis (Fig. 4D) in this trial.

4. Discussion

4.1. *Growth*

Dietary probiotic influence on fish growth performance has been associated with the bacterial modulatory capacity over digestive processes. Several digestive enzymes have been detected in probiotic strains [29,61] and associated with a more efficient nutrient digestibility [62]. The probiotic bacteria included in the present work have been previously correlated with growth improvements for example in tilapia and rohu [63–66]. In the present work however, dietary probiotic supplementation had no significant impact on growth performance. Although lower final weight was detected in fish fed PB_2 and PB_4 diets, the absence of differences in growth rates suggest these differences as minor variations of similar growth performances. Moreover, PB_2 and PB_3 groups had slightly lower initial weights. Regarding

Table 4
Effects on cellular and humoral immune parameters of Senegalese sole after 85-days of feeding the probiotic treatments. Values presented as mean ± standard error (n = 36). Different superscript letters indicate significant differences between treatments for P < 0.05.

	CTRL	PB_1	PB_2	PB_3	PB_4	PB_5
ROS	0.3 ± 0.2	0.3 ± 0.2	0.5 ± 0.3	0.7 ± 0.3	0.4 ± 0.2	0.9 ± 0.5
NO	6.9 ± 0.2	6.9 ± 0.1	6.9 ± 0.2	6.9 ± 0.1	6.9 ± 0.1	7.0 ± 0.2
PER	90.3 ± 9.4^{a}	52.9 ± 9.4^{ab}	47.0 ± 6.2^{ab}	55.1 ± 12.1^{ab}	67.6 ± 13.7^{ab}	38.7 ± 4.4^{b}
ACH50	15.0 ± 0.6	12.3 ± 2.1	16.8 ± 3.2	10.7 ± 2.4	11.9 ± 2.2	10.1 ± 1.6
LYS	1401.9 ± 283.3	2510.2 ± 536.7	1422.2 ± 464.8	2031.5 ± 566.1	1692.1 ± 460.4	1693.5 ± 198.1

ROS - Respiratory burst activity, expressed in O_2^- nmoles.
NO - Nitric oxide, expressed in nitrites μmoles.
PER - Peroxidase activity, expressed in enzymatic units (EU).
ACH50 - Alternative complement pathway activity expressed in ACH50 units.
LYS - Lysozyme activity, expressed in enzymatic units per mL (EU.mL^{-1}).

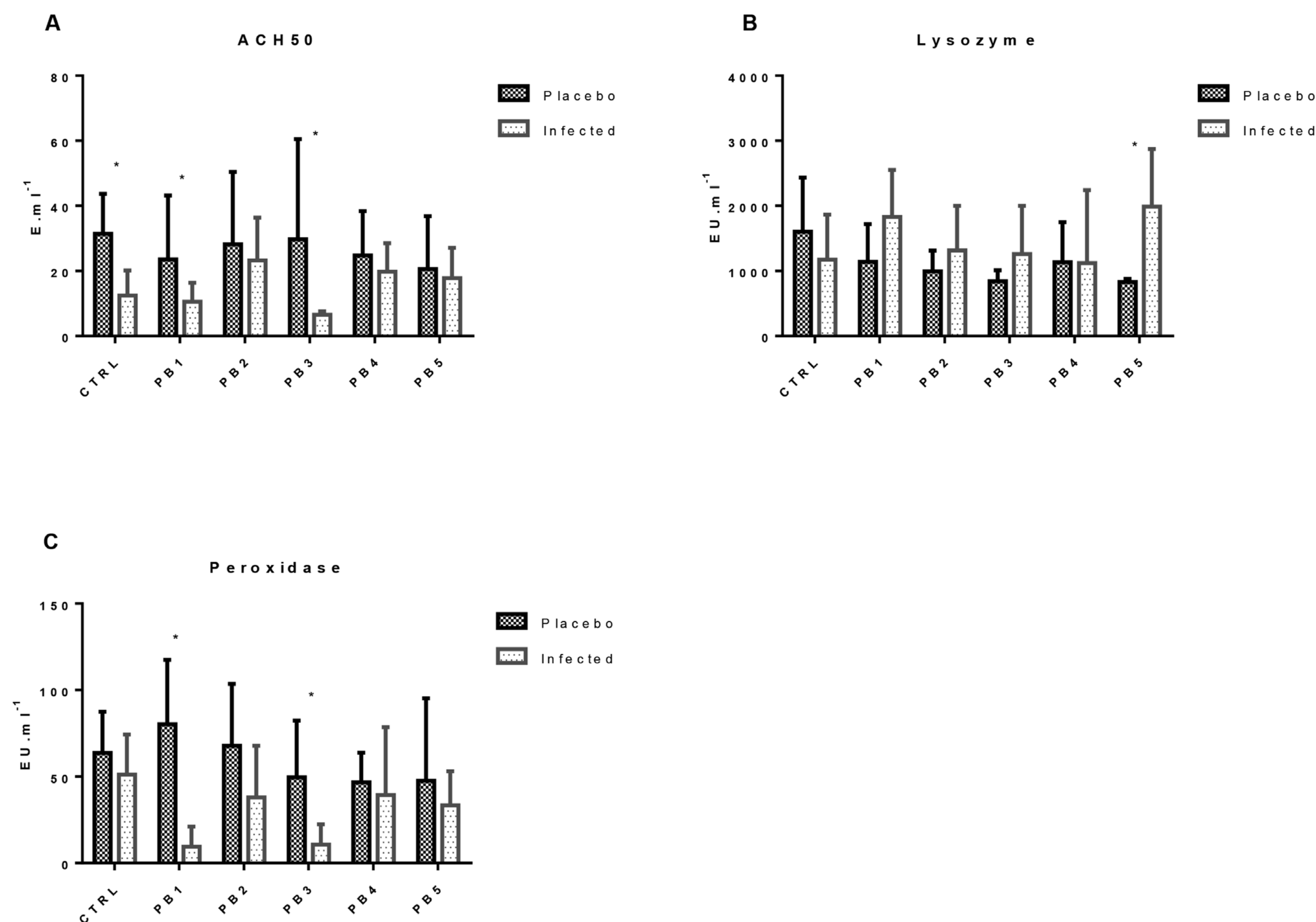

Fig. 1. Innate immune parameters from trial A analyzed in *Solea senegalensis* fed the experimental diets and subjected to an infection with Placebo or *Pdp*. Values represent mean ± standard deviation. * indicates significant differences for infection within diets (ACH50 and Lysozyme – Friedsman test, individual analysis performed with Wilcox test; $P < 0.05$); (Peroxidase - Two-way ANOVA followed by post-hoc Tukey analysis; $P < 0.05$).

condition factor (K) and hepatosomatic index, which are sensitive to a wide variety of stressors [67,68], no significant differences were found between dietary treatments. Discrepancies in growth results regarding probiotic supplementation have been described before and probiotic genera that demonstrated growth improvements in certain studies [69,70], lack congruence in others [30,71,72]. These variations may be related to probiotics colonization success [73] and, perhaps more importantly, to the quorum sensing process [74].

4.2. Innate immune system

As in other vertebrates, fish innate immune system provides the first line of defense against aggressions. However in comparison fish acquired immunity is less developed, which evidences the innate immune system as determinant to battle infections [75]. When facing a pathogen offense, both cellular (e.g. respiratory burst and nitric oxide) and humoral (e.g. complement, lysozyme and peroxidase) defenses interact to initiate the inflammatory response. Reactive oxygen species (ROS) and Nitric Oxide (NO) enhanced activities have been previously described as indicators of a prompter immune condition. ROS was described to increase in tilapia (*Oreochromis niloticus*) fed *Enterococcus* sp [63]. Also reported to have higher activity in Senegalese sole fed *Shewanella putrefaciens* and challenged with *Pdp* [26]. In rainbow trout (*Oncorhynchus mykiss*) fed *Pseudomonas* sp. and challenged with *Flavobacterium psychrophilum*, higher ROS activity levels were also detected. Similarly, NO has been associated with probiotic modulations in rainbow trout fed *Saccharomyces cerevisiae* [76] and *Catla catla* fed *Bacillus amyloliquefaciens*. To our best knowledge and within our bacteria selection, only *Enterococcus* has been analyzed for possible influence over NO production by head kidney macrophages in fish with either null or mitigating effects [77,78]. In the present growth trial, cellular immune parameters revealed no significant differences for NO and ROS activities and match other studies focused on Senegalese sole [78,79].

4.2.1. Innate immune system after infection

The analysis of innate immune responses after the transport stress and infection with *Pdp* revealed a significant modulation by those factors. In trial A, where fish were infected immediately after the transport, ACH50 levels of infected fish were significantly lower than the placebo groups. Accordingly, Costas, Conceição, Aragão, Martos, Ruiz-Jarabo, Mancera and Afonso [80] described a drop in ACH50 levels in Senegalese sole plasma after an acute stress. These evidences suggest that the transport acted as an acute stress, prompting a severe immune response and depleting plasma pro and anti-inflammatory proteins. Since ACH50 seems to be of greater importance for Senegalese sole when fighting *Pdp* infection [81], prophylactic strategies should be considered when handling procedures such as transport are needed in practical farming conditions. On the other hand, in trial B, fish infected with *Pdp* demonstrated higher ACH50 levels when compared to placebos. This response may be related to the recovery time between transport stress an infection, which allowed fish to restore homeostasis. The increase in ACH50 values after infection with *Pdp* has been described for sole before [81], as well as in seabass (*Dicentrarchus labrax*)

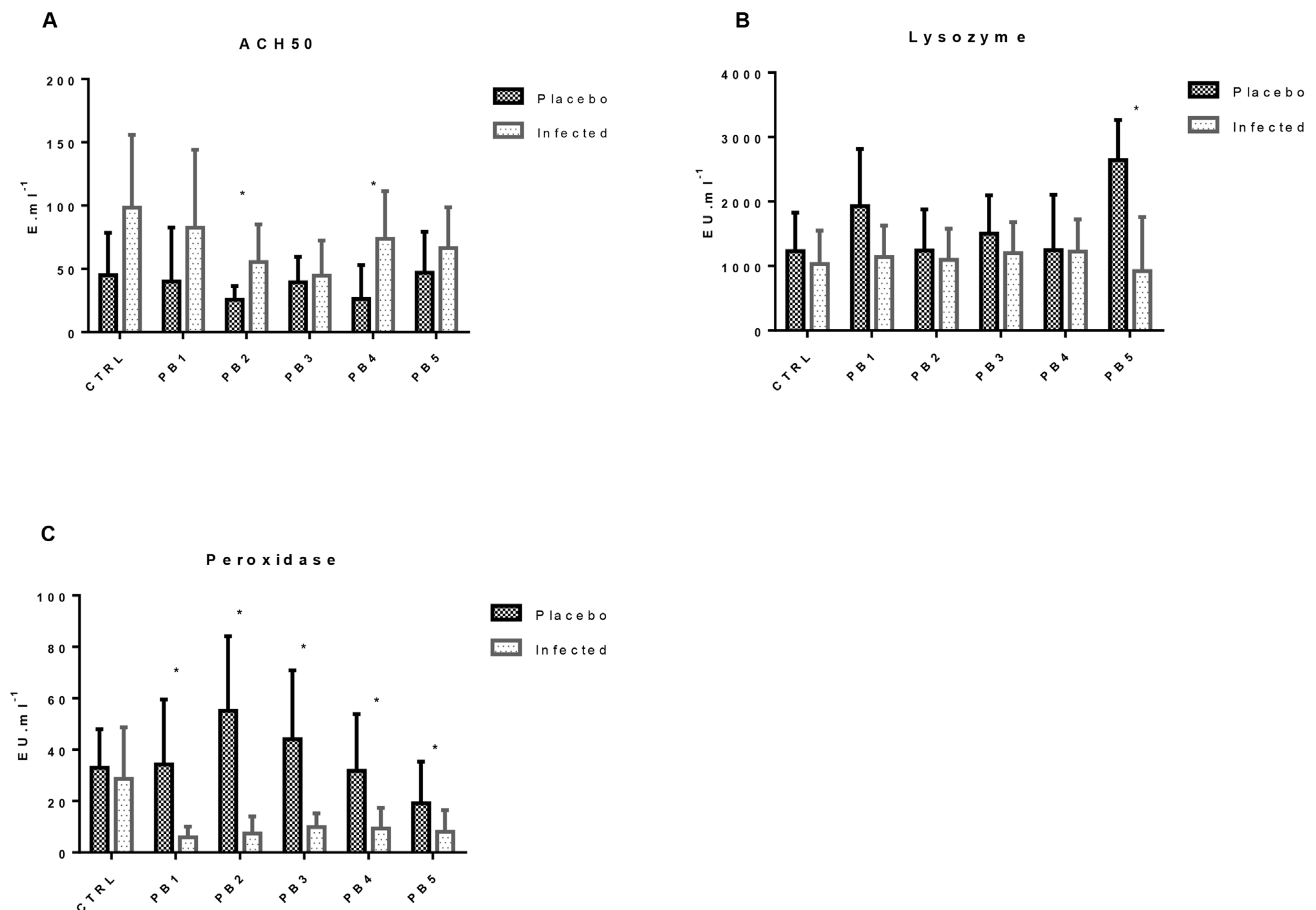

Fig. 2. Innate immune parameters from trial B analyzed in *Solea senegalensis* fed the experimental diets and subjected to an infection with Placebo or *Pdp*. Values presented as mean ± standard deviation. * indicates significant differences for infection. (ACH50 and Lysozyme - Friedsman test, individual analysis performed with Wilcox test; $P < 0.05$); (Peroxidase - Two-way ANOVA followed by post-hoc Tukey analysis; $P < 0.05$).

and gilthead seabream (*Sparus aurata*). The increase in this parameter suggests a stimulated opsonization capacity and therefore a direct contribution to the pathogens phagocytosis processes [82].

When comparing lysozyme activity levels from placebo groups between trials A and B, fish injected immediately after transport revealed lower levels than fish given a recovery time before injection. These results agree with other works where serum lysozyme activity was perceived to decrease when evaluated after handling, crowding and transport stresses [83,84]. Interestingly, when analyzing infection influence in trial A lysozyme levels increased after infection, which is congruent with pathogen exposure responses described in other studies [85,86]. Namely, a study in channel catfish (*Ictalurus punctatus*) reporting increased lysozyme activity in stressed and pathogen exposed fish at the fourth day. On the other hand, in trial B, infected fish showed decreased lysozyme activity, which has also been described for sheat fish (*Silurus glanis)* before [87]. Considering these results, we suggest that lysozyme activity response to stress and infection may differ according to type of stress (biotic or abiotic), but also the timeline after infection (analysis performed at different timings during infection).

In the current work, peroxidase levels revealed a strong influence from both transport and infection. In both trials, infection had a clear downgrade effect in peroxidase levels. Peroxidase levels have been described to increase in Sharpsnout sea bream (*Diplodus puntazzo*) after exposure to *Enteromyxum leei* [88]. In gilthead seabream (*Sparus aurata* L.) exposed to *E. leei*, a depression in peroxidase was also detected [89–91]. Both the duration of the stressor and the stage of infection may modulate peroxidase levels at different time-points. This modulation may be related with the exhaustion of the peroxidase producing active cells and its circulatory levels.

Probiotics potential to stimulate immunity are frequently tested under *in vivo* and *in vitro* conditions [92]. Recent studies focus on disease resistance of probiotic fed organisms, analyzed after pathogenic challenges. In the present study, dietary probiotic influence in sole innate immune parameters revealed no specific modulation pattern. Although it could be argued that probiotics positively influenced immune capacity since CTRL diet revealed a significant lower ACH50 level in trial A, we suggest that the proximity of events (transport stress and infection) may have carried a negative effect in the complement system activity. Differences in peroxidase levels were detected in PB_1 diet, which decreased in both trial A and B. Probiotic negative influence in peroxidase levels after infection is uncommon [92], although there are studies reporting probiotic negative correlation with peroxidase levels. Decreased peroxidase levels detected in fish fed diets PB_1, PB_2 and PB_3, and subjected to *Pdp* infection are believed to be associated with the resulting intestinal microbiota colonization. The immune parameters of both trials analyzed for diets PB_4 and PB_5 showed no evidence of influence from probiotic supplementation. However, lysozyme levels analyzed in trial B for PB_5 placebos were higher when compared to other treatments.

4.3. Oxidative stress

Oxidative stress is described as the balance between the production of reactive oxygen species and their neutralization mechanisms. In the

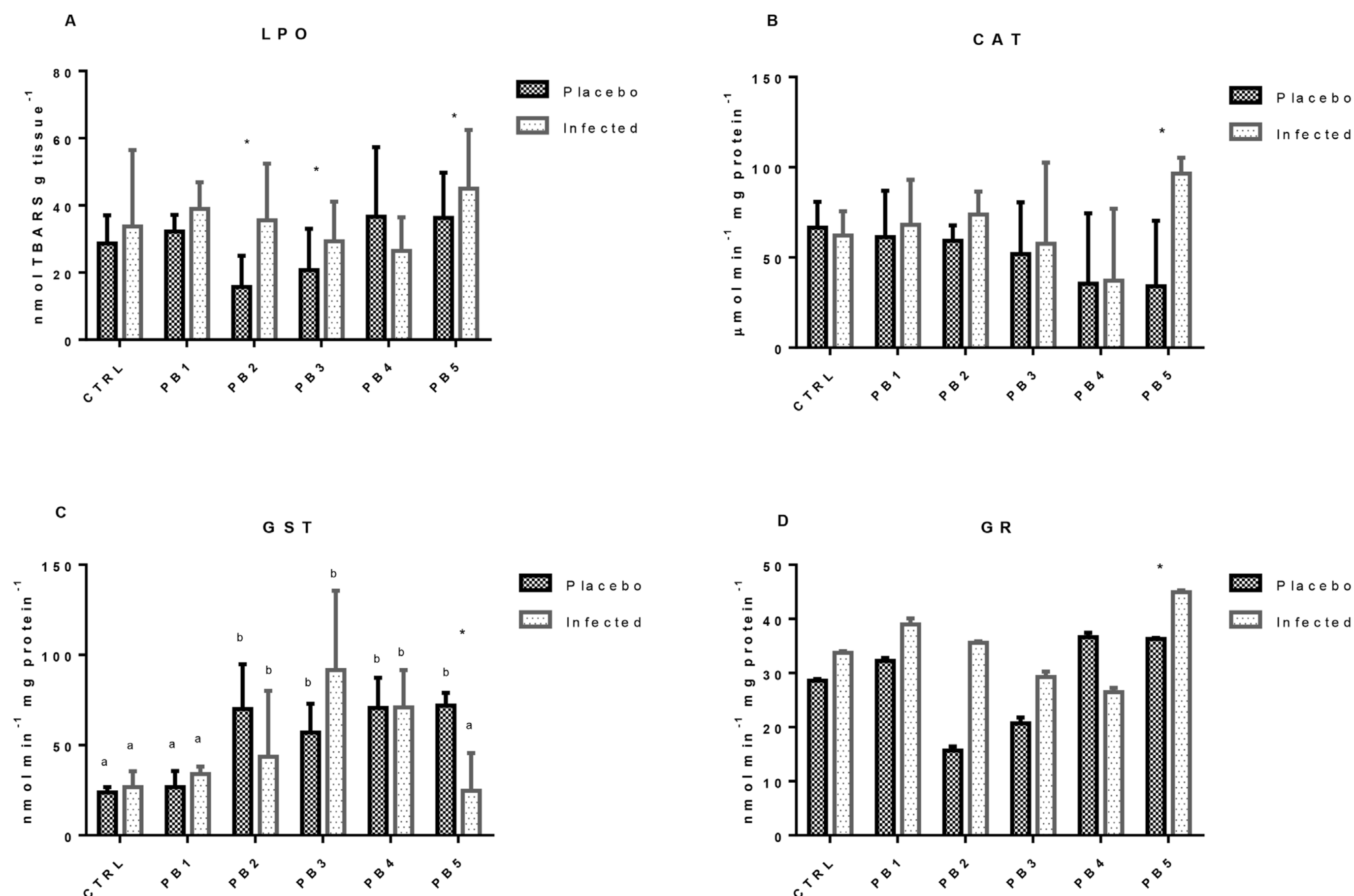

Fig. 3. Oxidative stress parameters in *Solea senegalensis* in trial A, encompassing dietary and infection interaction analysis with Placebo or *Pdp*. Values presented as mean ± standard deviation. Asterisks and different letters represent significant differences within and between diets respectively. All data was cubic root transformed (Two-way ANOVA, followed by a post-hoc Tukey analysis; $P < 0.05$).

neutralization process, several molecules work as anti-oxidants. Dietary probiotic supplementation has been described to improve antioxidant defenses through increased assimilation of dietary antioxidant compounds and the direct intervention in the antioxidant enzymatic activities [93]. Research analyzing the application of Kefir as probiotic supplementation in coruh trout (*Salmo coruhensis*) antioxidant enzyme activities unraveled an interaction between time and dosage of supplementation and oxidative damage results [94].

In trial A, non-infected fish fed PB2 and PB3 diets showed significantly lower LPO levels when compared to non-infected fish fed CTRL diet. This result indicates that diets PB2 and PB3 may protect fish against stressors such as handling or transportation. On the other hand, PB_5 infected fish presented higher LPO levels then all the other treatment groups. This pattern however was not present in trial B, since no significant alterations were detected in probiotic supplemented diets. Such outcome indicates that the antioxidant mechanisms triggered by different stressors are divergent and the dietary probiotic supplementation should be selected accordingly. Moreover, since infected fish (trial B) fed control diet had higher LPO than the other dietary groups probiotic supplementation may aid in mitigating cellular lipid damage when fish are subjected to a pathogenic infection. To our best knowledge, there are limited information of the influence of dietary probiotics treatment on LPO mitigation. Interestingly, probiotic bacteria have been described to produce extracellular superoxide through autoxidation of membrane-associated quinones [95]. Hence, probiotics may not always positively affect the oxidative stress responses, especially when dealing with a combination of stress factors, in this case bacterial infection and transport.

CAT and GST are two antioxidant enzymes reported to respond to different sorts of ROS inducing situations [96–98]. In trial A, CAT activity analyzed within diet PB5, revealed higher activity levels in infected fish. Also, in trial B, CAT activity in diet PB_2 after infection was notoriously higher than any other treatment. CAT activity has been described to increase when *Mycteroperca rosacea* juveniles were exposed to oxidant stressors or exposed to the pathogen bacteria *Aeromonas hydrophila* [99].

Glutathione-based enzymes contribute to the equilibrium trough glutathione regeneration, mainly through GR activity, and detoxification of harmful compounds through GST activity. These enzymes were described to improve with probiotic supplementation in rainbow trout [100,101]. In trial A, GST levels in control and PB_1 diets demonstrated to be lower than most treatments. Additionally, PB_5 diet diminished GST activity after infection. In trial B, GST analyzed in diets PB1 and PB2 revealed to be higher in infected fish. Both increases and decreases have been reported in carp (*Cyprinus carpio* L.) after parasitic infection by *Ptychobothrium* sp. and *Listeria monocytogenes* respectively [102].

GR activitiy in trial A, increased in infected fish when compared to placebo. Probiotic supplementation improvement in GR expression in gut, gills and skin has been described for *Sparus aurata* [103]. Another study reported GR activity increase in *Oncorhynchus mykiss* fed a multistrain probiotic supplement [104]. Although it is still poorly understood how probiotics influence the antioxidant enzymes activities, bacterial modulation of the genes involved in antioxidant pathways appears to be a probable theory.

Overall, probiotic dietary supplementation appears to have no influence over Senegalese sole growth. Also, both immune and oxidative stress parameters revealed different modulatory patterns between trials A and B. Dietary probiotic supplementation role in the innate immune

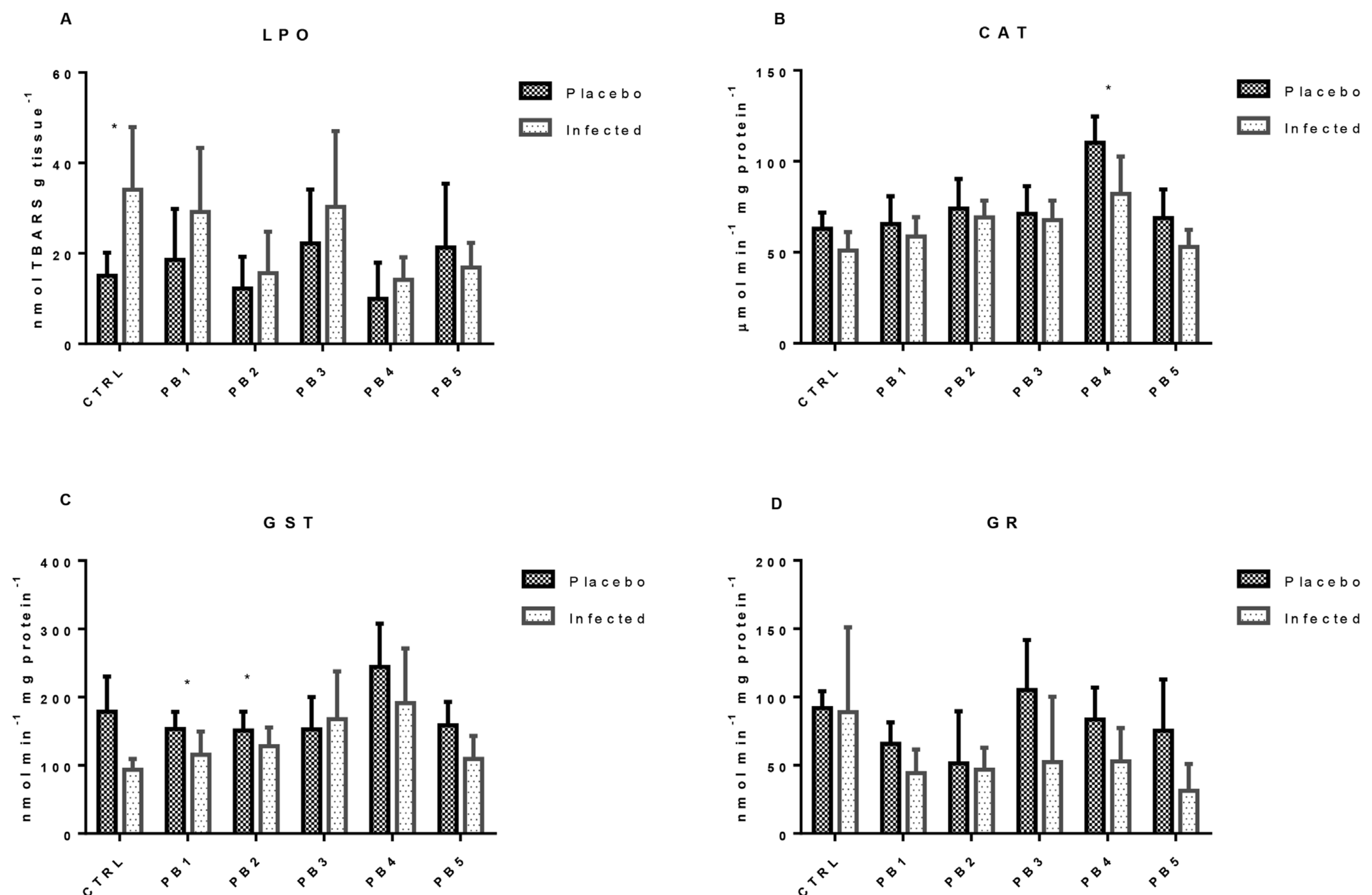

Fig. 4. Oxidative stress parameters in *Solea senegalensis* in trial B, encompassing dietary and infection with Placebo or *Pdp*. Values presented as mean ± standard deviation. Asterisks and different letters indicate significant differences. All data was cubic root transformed (Two-way ANOVA, followed by a post-hoc Tukey analysis; $P < 0.05$).

parameters of Senegalese sole revealed no specific tendencies, showing particular responses in each diet, increasing the complexity regarding bacteria multispecies combinations and modulating factors. Oxidative stress parameters however, proved more conclusive, with abiotic stress shifting the potential benefits of each diet when dealing with an infection.

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