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Effect of Dietary Short-Chain Fatty Acids on the Immune Status and Disease Resistance of European Seabass Juveniles

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Abstract: (1) Background: This study aimed to evaluate the potential of short-chain fatty acids as functional ingredients to improve the immune status and disease resistance of European seabass (*Dicentrarchus labrax*) juveniles. (2) Methods: For that purpose, triplicate groups of fish with an initial body weight of 15.2 ± 0.03 g were fed isoproteic (43% crude protein) and isolipidic (18% crude lipids) diets supplemented with sodium acetate (SA), sodium propionate (SP), and sodium butyrate (SB) at two inclusion levels: 0.25% and 0.50%. An unsupplemented diet was used as a control. After 56 days of feeding with the experimental diets, fish were intraperitoneally (i.p.) injected with 100 µL of *Vibrio anguillarum* (1.2×10^7 Colony Forming Units (CFU)/mL) and mortality was recorded for 3 weeks. At the end of the trial, there were no differences in survival between the treatment groups and the control, but survival was higher in fish fed the diet supplemented with SB 0.50 than SP 0.25 (93.3 vs. 66.7%). Compared to the pre-challenge values, and regardless of diet composition, all hematological parameters (hemoglobin, hematocrit, red blood cells, white blood cells) measured decreased after 4 h of bacterial challenge, except for neutrophils which were increased. Independently of diet composition, lysozyme and nitric oxide decreased at 4 and 24 h post infection. Compared to the control, diets supplemented with SA and SP promoted an up-regulation of both pro- and anti-inflammatory cytokines at 4 h after the challenge, while the diets supplemented with SB promoted an up-regulation of pro- and anti-inflammatory cytokines at 24 h after the challenge. (3) Conclusions: Overall, present results suggest that SA and SP provide a fast response to a bacterial challenge in European sea bass juveniles, while SB provides increased survival.

Keywords: short-chain fatty acids; functional ingredients; immune parameters; fish health; disease resistance

Key Contribution: Dietary SCFA supplementation did not affect the hematological parameters. Survival rates post bacterial challenge showed a tendency to improve with sodium butyrate supplementation. The inflammatory responses are dose dependent, so different doses and SCFA promote different responses.

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Citation: Fontinha, F.; Martins, N.; Bonin, F.; Magalhães, R.; Santos, R.; Peres, H.; Oliva-Teles, A. Effect of Dietary Short-Chain Fatty Acids on the Immune Status and Disease Resistance of European Seabass Juveniles. *Fishes* **2024**, *9*, 363. https://doi.org/10.3390/fishes9090363

Academic Editor: Marina Paolucci

Received: 18 July 2024
Revised: 12 September 2024
Accepted: 14 September 2024
Published: 17 September 2024

1. Introduction

Fish represents about 17% of total animal protein consumption by the human population. Moreover, world fish consumption is expected to continue to increase due to feeding habit modification and the increase in the human population. The amount of fish necessary to meet human population requirements cannot be met only by fisheries due to concerns

about biodiversity and stock conservation. It is therefore necessary to resort to aquaculture and make it more sustainable to fulfill the world fish supply [1].

One of the biggest challenges to aquaculture sustainability is the control of diseases, which cause high losses due to mortality and reduced animal performance and have a huge economic impact [2]. One way to overcome such problems is by using approved antibiotics and chemotherapeutics; however, their use is restricted to the treatment of disease outbreaks, raises consumer concerns regarding aquaculture products, and is not environmentally friendly [3,4]. Thus, intensive research has been devoted to finding alternative strategies to mitigate disease outbreaks in aquaculture, including nutritional strategies that promote animal health and welfare [3,5].

Functional ingredients have been searched as alternatives to the use of antibiotics [6]. Among these, Short-Chain Fatty Acids (SCFAs) are known to have high antimicrobial properties, decreasing the intestinal pH and reducing the pathogenic bacteria [7–9], thus promoting disease resistance [10]. Furthermore, they are easy to include in diets as a feed additive [8]. SCFAs are saturated aliphatic organic acids with 2–6 carbons, with acetate (C2), propionate (C3), and butyrate (C4) being produced as the end products of bacterial fermentation [11], and at the intestine, SCFAs are important in maintaining energy homeostasis, microbiota diversity, gut health, and immunological status [12,13].

By modulating immune cell chemotaxis, reactive oxygen species (ROS), and cytokine release, SCFAs are known to boost the host immune response, improve intestinal structure and function, and disease resistance [14,15]. For instance, rainbow trout (*Oncorhynchus mykiss*) fed diets supplemented with sodium butyrate showed increased red blood cells (RBCs), white blood cells (WBCs), hematocrit (Ht), hemoglobine (Hb), lysozyme, and bactericidal activity [16]; Nile tilapia (*Oreochromis niloticus*) showed an up-regulation of anti- and pro-inflammatory cytokines [17]; and European sea bass (*Dicentrarchus labrax*) showed beneficial effects on major blood constituents [10]. Also in European sea bass, dietary supplementation with sodium propionate increased Hb, Ht, RBC, WBC, and bactericidal activity [10]. In Nile tilapia, dietary sodium propionate supplementation increased lysozyme activity and induced the up-regulation of immune-related genes, namely tumor necrosis factor-α (*tnf-α*) and interleukin-1β (*il-1β*) [18], and in zebrafish (*Danio rerio*), up-regulated inflammatory response genes (*tnf-α, il-1β*) were observed [19]. Dietary sodium acetate supplementation increased the immune function of golden pompano (*Trachinotus ovatus*) by decreasing interleukin-8 (*il-8*), *tnf-α*, and factor nuclear kappa B (*nf-kβ*) and increasing transforming growth factor β (*tgf-β*), and in Siberian sturgeon (*Acipenser baerii*), it improved lysozyme and alternative complement activities [20].

Thus, this study aimed to evaluate European sea bass' immune status and disease resistance at different time points after dietary SCFA supplementation.

2. Materials and Methods

2.1. Experimental Diets

Seven practical diets were formulated to meet the nutritional requirements for European seabass juveniles: isoproteic (43% crude protein) and isolipidic (18% crude lipid) [21], including 0.25% and 0.50% of sodium acetate (Alfa Aesar A13184), sodium propionate (Alfa Aesar A17440), or sodium butyrate (Alfa Aesar A11079). SCFAs were included in the diet mixed with the vitamins, minerals and aminoacids. A diet without SCFA supplementation was used as a control. All dietary ingredients were well mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA) through a 3 mm die. After being dried for 48 h at 50 °C in an oven, the pellets were refrigerated until needed. Ingredient composition and proximate analysis of the diets are presented in Table 1.

Table 1. Ingredient composition (% dry matter) of the experimental diets.

Feedstuffs	CTR	SA (0.25/0.50)	SP (0.25/0.50)	SB (0.25/0.50)
Fishmeal [1]	15.0	15.0	15.0	15.0
CPSP [2]	5.00	5.00	5.00	5.00
Soybean meal [3]	15.0	15.0	15.0	15.0
Corn gluten [4]	17.5	17.6	17.6	17.6
Wheat gluten [5]	5.98	5.98	5.98	5.98
Rapeseed [6]	5.00	5.00	5.00	5.00
Wheat meal [7]	17.6	17.00–17.30	17.00–17.30	17.00–17.30
Fish oil	7.13	7.13	7.13	7.13
Colza oil	7.13	7.13	7.13	7.13
Vitamin [8]	1.00	1.00	1.00	1.00
Mineral [9]	1.00	1.00	1.00	1.00
Choline chloride (50%)	0.50	0.50	0.50	0.50
Binder [10]	1.00	1.00	1.00	1.00
Dicalcium phosphate [11]	0.67	0.68	0.68	0.68
Methionine [12]	0.15	0.15	0.15	0.15
Taurine [13]	0.30	0.30	0.30	0.30
Sodium acetate [14]	0.00	0.25–0.50		
Sodium propionate [15]	0.00		0.25–0.50	
Sodium butyrate [16]	0.00			0.25–0.50
Proximate analysis				
Lipids (%)	17.5	17.4–17.7	18.0–18.2	17.6–17.7
Protein (%)	43.9	43.2–44.0	42.9–43.3	43.1–43.3
Energy (MJ/kg)	22.7	23.1–22.6	22.8–22.9	22.8–22.7
Dry matter (%)	95.8	95.1–95.3	93.9–95.6	94.6–95.6

CP: Crude protein; CL: Crude lipid. [1] Sorgal. S.A.. Ovar. Portugal (CP: 70.3; CL: 12.0). [2] Sorgal. S.A.. Ovar. Portugal (CP: 73.3; CL: 8.3). [3] Sorgal. S.A.. Ovar. Portugal (CP: 49.7; CL: 1.3). [4] Sorgal. S.A.. Ovar. Portugal (CP: 61.3; CL: 1.5). [5] Sorgal. S.A.. Ovar. Portugal (CP: 81.2; CL: 0.6). [6] Sorgal. S.A.. Ovar. Portugal (CP: 32.4; CL: 1.3). [7] Sorgal. S.A.. Ovar. Portugal (CP: 14.5; CL: 1.6). [8] Vitamins (mg kg^{-1} diet): retinol. 18,000 (IU kg^{-1} diet); calciferol. 2000 (IU kg^{-1} diet); alpha tocopherol. 35; menadion sodium bis.. 10; thiamin. 15; riboflavin. 25; Ca pantothenate. 50; nicotinic acid. 200; pyridoxine. 5; folic acid. 10; cyanocobalamin. 0.02; biotin. 1.5; ascorbyl monophosphate. 50; inositol. 400. [9] Choline chloride (50%). Sorgal. S.A.. Ovar. Portugal. [10] Minerals (mg kg^{-1} diet): cobalt sulphate. 1.91; copper sulphate. 19.6; iron sulphate. 200; sodium fluoride. 2.21; potassium iodide. 0.78; magnesium oxide. 830; manganese oxide. 26; sodium selenite. 0.66; zinc oxide. 37.5; dicalcium phosphate. 8.02 (g kg^{-1} diet); potassium chloride. 1.15 (g kg^{-1} diet); sodium chloride. 0.4 (g kg^{-1} diet). [11] Binder. AQUACUBE. Agil. UK. [12] Feed-grade methionine. Sorgal. S.A. Ovar. Portugal. [13] Feed-grade taurine. Sorgal. S.A. Ovar. Portugal. [14–16] Alfa Aesar by Thermo Fisher Scientific. Kandel. Germany.

2.2. Growth Trial

The growth trial was carried out by trained scientists (following FELASA Category C recommendation) according to the European Union Directive (2010/63/EU).

European seabass (*Dicentrarchus labrax*) juveniles were provided by Atlantik Fish, Lda., Castro Marim, Portugal and transported to the experimental facilities at CIIMAR, Porto, Portugal. After transportation, fish were maintained in quarantine for 15 days and then transferred to the experimental system, consisting of a thermoregulated recirculating water system equipped with 21 cylindrical fiberglass tanks of 250 L capacity.

Fish were acclimatized to the experimental conditions for 2 weeks, and then 21 homogenous groups of 25 fish with an initial body weight of circa 15 ± 0.03 g were established. Each experimental diet was randomly assigned to triplicate groups. Fish were fed the experimental diets for 56 days by hand, twice daily (9:30–14:30), 6 days per week, until apparent visual satiety. During the experiment, the water temperature was maintained at 22 ± 1 °C, salinity at 26 ± 1‰, dissolved oxygen above 80% saturation, and photoperiod was maintained at 12 h light and 12 h dark.

At the end of the trial, 3 fish per tank (9 per treatment) were sampled 4 h after the morning meal (Time 0). The blood of each fish was collected from the caudal vein with heparinized syringes and divided into two aliquots. One aliquot was used for the hematological analysis and the other one was centrifuged at 10,000× *g* 10 min at 4 °C and

the plasma collected was frozen on dry ice and stored at −20 °C until further analysis. After blood collection, fish were euthanized by decapitation and the anterior intestine was collected and stored in Trizol reagent (1:10) at −80 °C until gene expression analysis.

2.3. Bacterial Challenge Trial

2.3.1. Bacterial Growth

Vibrio anguillarum DSMZ 21597 was cultivated in brain heart infusion (BHI) for 24 h at 25 °C. The culture was then centrifugated at 4000× *g* for 15 min at room temperature. The bacterial dose was adjusted according to the following protocol: The *vibrio anguillarum* inoculum concentration was estimated based on the growth phase, previously determined in the laboratory. At different time points of the bacterial growth (every 2 h), the optical density (OD600) and the Colony Forming Units (CFUs) of the culture were measured to produce a standard curve showing the relationship between the CFUs and OD. This curve was then used to quickly estimate the number of cells present in the *Vibrio anguillarum* culture, and consequent adjustment to the desired concentration of 1.2×10^7 cfu/mL. To confirm the injection concentration, serial dilution of bacterial suspension was plated and CFU was counted.

2.3.2. Bacterial Challenge

At the end of the growth trial, the remaining fish were reallocated to a challenge room with 28 tanks of 100 L of water capacity. According to the challenge protocol established in the laboratory, the water temperature in the system was maintained at 24 °C, salinity 33‰, and the photoperiod was 12 h light and 12 h dark.

Survival Trial

To assess survival, 15 fish were assigned to duplicate groups in 14 tanks (30 fish per diet) and intraperitoneally injected with 100 uL of *Vibrio anguillarum* 1.2×10^7 CFU/mL. A control group of 30 fish was injected with phosphate-buffered saline solution (PBS) as a control of the experimental handling. Mortality was recorded daily for 3 weeks, and dead fish were weighed and examined for clinical signs. The inoculated bacterium was isolated from the internal organs of moribund fish to confirm mortality.

Time Course Trial

For the time course trial, homogenous groups of 10 fish were established, and each experimental diet was assigned to duplicate groups in 14 tanks (20 fish per diet). Fish were also intraperitoneally injected with 100 uL of *Vibrio anguillarum* 1.2×10^7 CFU/mL and a group of 20 fish were intraperitoneally injected with phosphate-buffered saline solution (PBS) as a control of the experimental handling. At four (time 4 h) and twenty-four (time 24 h) hours post infection, three fish per treatment were sampled for blood and anterior intestine collection as described above.

2.4. Haematological Parameters

Total red blood cell (RBC) and white blood cell (WBC) counts were performed with fresh blood diluted in HBSS using a hemocytometer. Hematocrit was assessed after centrifugation at 10,000× *g* for 10 min at room temperature. Hemoglobin concentration was determined with Drabkin's solution using an analytical kit (SPINREACT kit, ref, 1001230, Girona, Spain).

Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated as follows:

$$\text{MCV } (\mu m^3) = (\text{Ht}/\text{RBC}) \times 10 \quad (1)$$

$$\text{MCH } (\text{pg cell}^{-1}) = (\text{HH}/\text{RBC}) \times 10 \quad (2)$$

$$\text{MCHC } (\text{g } 100 \text{ mL}^{-1}) = (\text{HB}/\text{Ht}) \times 100 \quad (3)$$

Blood smears were performed immediately after blood collection and air-dried. After fixation with formol–ethanol (10% of 37% of formaldehyde in absolute ethanol), the detection of peroxidase activity was performed to identify neutrophils according to Afonso et al. [22]. Wright's stain (Haemacolor; Merck, Darmstadt, Germany) was then applied to the smears and examined (100×) and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes, and neutrophils. For each cell type, the absolute value ($\times 10^4$ μL^{-1}) was calculated.

2.5. Innate Immune Parameters

Plasma lysozyme activity was measured using a turbidimetric assay. For that purpose, 15 µL of plasma and 250 µL of bacterial suspension (*Micrococcus lysodeikticus*) were added in duplicate to a 96-well plate. The reaction was carried out at 25 °C and the absorbance (450 nm) was measured after 0.5 and 4.5 min in a Synergy HT microplate reader. The quantity of lysozyme in the sample was calculated using a standard curve made of serial dilutions of a stock solution of lysozyme (1 mg/10 mL).

For the determination of plasma bactericidal activity, 20 µL of plasma and 20 µL of *Vibrio anguillarum* at a concentration of 1.2×10^7 cfu/mL were added to a U-shaped 96-well plate and incubated for 2.5 h at room temperature. Then, 25 µL of INT (iodonitrotetrazolium) was added to the solution and incubated at room temperature allowing the formation of formazan. Formazan is constituted by living bacteria. After a 10 min centrifugation at 2000× *g*, the precipitate was dissolved in 200 µL of dimethyl sulfoxide. Bactericidal activity is expressed as a percentage of live bacteria.

For the determination of anti-protease activity, 10 µL of plasma was incubated in 1.5 mL microtubes with the same volume of a trypsin solution (trypsin dissolved in $NaHCO_3$) for 10 min at 22 °C. Then, 100 µL of phosphate buffer and 125 µL of azocasein (sigma A2765) were added to the solution and incubated for 1 h at 22 °C. Thereafter, each microtube was filled with 250 µL of trichloroacetic acid and incubated for 30 min at 22 °C. After centrifuging the microtubes for 5 min at room temperature at 10,000× *g*, 100 µL of the supernatant was moved to a 96-well plate with 100 µL of NaOH in each well. The OD was read at 450 nm in a Synergy HT microplate reader, and the percentage inhibition of trypsin activity was compared to the reference sample (phosphate buffer instead of plasma).

To determine protease activity, 10 µL of plasma was incubated in microtubes with 100 µL of phosphate buffer and 125 mL of azocasein for 24 h at 22 °C. Then, 250 µL of trichloroacetic acid was added to stop the reaction. The microtubes were incubated for 30 min at 22 °C and then centrifuged at 10,000× *g* for 5 min at room temperature. After this, 100 µL of the supernatant was transferred to a 96-well plate containing 100 µL of NaOH per well, and the OD was read at 450 nm in a Synergy HT microplate reader. The percentage of protease activity was calculated by comparison to the reference sample (100 uL of sodium bicarbonate and 10 uL of trypsin).

Nitric oxide content was evaluated in plasma using a Nitrate/Nitrite colorimetric kit (Roche Diagnostics GmbH, Mannheim, Germany, ref: 11746081001) in 96-well microplates. To determine Nitric oxide, 5 µL of plasma and 95 µL of distilled water were added to each well plus 50 µL of R2 and 50 µL of R3. The plate was incubated for 30 min at 25 °C with agitation, and then the sample OD was read at 540 nm in a Synergy HT microplate reader. Then 50 µL of R4 and 50 µL of R5 were added and incubated for 15 min and the OD read again at 540 nm.

2.6. Gene Expression

Using a Precellys evolution apparatus (Bertin Instruments, Montigny-le-Bretonneux, France) and the TRIzol reagent (Direct-zolTM RNA Miniprep, Zymo Research, Irvine, CA, USA), each sample's RNA was isolated following the manufacturer's instructions.

The RNA quantity was confirmed by spectrophotometry (µDropTM plate, ThermoScientific, Waltham, VA, USA), and the RNA quality was assessed by electrophoresis on 1% agarose gel. The concentration of RNA was adjusted to 0.5 µg/8 µL H_2O for complemen-

tary DNA synthesis using the NZY First-Strand cDNA Synthesis Kit (NZYTech, MB12502, Lisbon, Portugal).

The expression of the target genes was determined by real-time quantitative PCR analysis in a CFX Connect™ Real-Time System (Bio-Rad, Hercules, CA, USA).

The selected primers used in this study were two pro-inflammatory markers, *tnf-α* (Tumor necrosis factor alpha) and *il-8* (Interleukin 8); two anti-inflammatory markers, *tgf-β* (Transforming growth factor beta) and *il-10* (Interleukin 10); and one apoptotic marker, *casp 3*; one transcriptor factor, *nf-kβ* (Factor nuclear kappa B). The sequences of selected primers are presented in Table 2. To normalize the results of the target genes, two reference genes were used, elongation factor 1α (*ef1α*) and *40s* ribosomal RNA (*40s*). According to Vandesompele et al. [23], the expression levels are expressed as the mean normalized values ± standard error (SE) corresponding to the ratio between copy numbers of the target gene transcripts and the geometric mean of copy numbers of the reference genes.

Table 2. Sequences of primers used for real-time quantitative PCR determination of immune-related genes in European seabass (*Dicentrarchus labrax*).

Gene	Gene Abbreviation	Primer Sequences (5′→3′)	Primer Efficiency	Accession Number	Anel. Temp.
Nuclear factor kappa β	*nf-kβ*	F: GCTGCGAGAAGAGAGGAAGA R: GGTGAACTTTAACCGGACGA	89.57	DLAgn_00239840	60 °C
Tumor necrosis factor-α	*tnf-α*	F: AGCCACAGGATCTGGAGCTA R: GTCCGCTTCTGTAGCTGTCC	105.3	DQ200910	60 °C
Interleukin 8	*il-8*	F: GTCTGAGAAGCCTGGGAGTG R: GCAATGGGAGTTAGCAGGAA	101.3	AM490063	60 °C
Transforming growth factor-β	*tgf-β*	F: GACCTGGGATGGAAGTGGAT R: CAGCTGCTCCACCTTGTGTTG	101.78	AM421619.1	60 °C
Caspase 3	*Casp3*	F: CTGATTTGGATCCAGGCATT R: CGGTCGTAGTGTTCCTCCAT	107.7	DQ345773	60 °C
Interleukin 10	*il-10*	F: ACCCCGTTCGCTTGCCA R: CATCTGGTGACATCACTC	96.8	AM268529	60 °C
Elongation factor 1α	*ef1α*	F: GCTTCGAGGAAATCACCAAG R: CAACCTTCCATCCCTTGAAC	100.92	AJ866727	60 °C
Ribosomal protein S40	*40s*	F: TGATTGTGACAGACCCTCGTG R: CACAGAGCAATGGTGGGGAT	94.5	HE978789.1	60 °C

2.7. Statistics

Data are presented as mean and pooled standard error of the mean (SEM). The probability level for rejection of the null hypotheses was 0.05. Normality and homogeneity of variances were tested by the Shapiro–Wilk and Levene tests, respectively, and normalized when appropriate.

Data were analyzed by three-way ANOVA with time (0, 4, and 24 h), SCFAs (sodium acetate, sodium propionate, and sodium butyrate), and supplementation level (0.25 and 0.50) as main factors. Significative differences between means were calculated by Tukey's multiple range test. When the interaction was significant, data were compared within each factor. Non-orthogonal contrasts were performed to compare the control and the experimental treatments within each sampling time. All statistical analyses were conducted using the SPSS 28.0 software package for Windows (IBM® SPSS® Statistics, Armonk, NY, USA).

3. Results

3.1. Zootechnical Performance

Zootechnical performance was not the aim of this study and data are presented elsewhere. Briefly, fish promptly accepted the diets, and the mortality during the growth trial was low and independent of the experimental diets.

At the end of the growth trial, the final weight of the fish averaged 45 g, and weight gain, feed intake, daily growth index, and feed efficiency were not affected by the experimental diets.

3.2. Hematology and Absolute Values of Peripheral Leucocytes

There were almost no interactions between time, SCFA, and SCFA levels in the parameters analyzed (Tables 3 and 4). Compared to the time of zero hours, at 4 h after the bacterial challenge, all hematological parameters were significantly decreased ($p < 0.001$) (Table 3). At the time of 24 h, hemoglobin, MCHC, and RBC were identical to values observed at 4 h, while hematocrit, MCV, and WBC were higher than at 4 h but were still lower than at the time of 0 h. Dietary SCFA supplementation did not affect the hematological parameters, except for MCV ($p = 0.020$) values which were higher in fish fed the SB diets than in the SA diets.

Table 3. Hematological parameters of European seabass prior to infection (0 h) and 4 and 24 h post infection.

Parameters		Hematocrit (%)	Hemoglobin (g/dL)	MCV (μm^3)	MCH (pg cell^{-1})	MCHC (g 100 mL^{-1})	RBC ($\times 10^6$ μL^{-1})	WBC ($\times 10^4$ μL^{-1})
Control	0 h	31.9	1.83	106	6.80	5.08	2.74	9.97
	4 h	22.2	1.73	107	5.78	5.62	2.15	4.03
	24 h	20.7	1.11	107	5.75	5.39	1.94	5.68
SA 0.25	0 h	32.6	1.82	109	6.06	5.65	3.02	7.31
	4 h	18.8	1.15	79.4 #	4.75	6.15	2.26	4.15
	24 h	22.7	1.24	102	5.69	5.51	2.24 $	7.33
SA 0.50	0 h	28.7	1.76	113	6.86	6.27	2.54	6.72 *
	4 h	22.2	1.38	82.2 #	5.12	6.20	2.73 #	3.65
	24 h	22.3	1.26	105	5.90	5.66	3.65	5.77
SP 0.25	0 h	30.2	1.69	123	6.77	5.63	2.50	8.89
	4 h	21.3	1.13	91.5 #	4.86	4.24	2.37 #	3.70
	24 h	21.3	1.16	97.6	5.29	5.45	2.20	7.13
SP 0.50	0 h	35.8	1.76	137	6.66	5.13	2.68	6.83
	4 h	22.5	1.17	88.3 #	4.60	5.21	2.55	3.37
	24 h	26.0 $	1.35	120	6.18	5.15	2.18	6.13
SB 0.25	0 h	30.5	1.66	108	6.43	4.80	2.55	7.26
	4 h	21.5	1.18	87.6 #	4.81	5.44	2.48 #	3.48
	24 h	24.2	1.38	110	6.22	5.85	2.22 $	5.77
SB 0.50	0 h	29.2	1.51	112	5.74	5.28	2.63	8.54
	4 h	20.2	1.33	106	6.44	6.48	1.94	4.33
	24 h	25.3 $	1.01	114	4.52	3.98 $	2.23 $	5.57
SEM	0 h	0.75	0.05	3.11	0.18	0.17	0.06	0.24
	4 h	0.46	0.10	2.48	0.29	0.32	0.06	0.11
	24 h	0.51	0.04	2.08	0.19	0.15	0.04	0.23
Time		<0.001	<0.001	<0.001	<0.001	0.390	<0.001	<0.001
SCFA		0.233	0.651	0.020	0.989	0.197	0.172	0.754
Level		0.215	0.897	0.073	0.647	0.716	0.700	0.052
SCFA*Level		0.051	0.497	0.595	0.550	0.652	0.242	0.004
SCFA*Time		0.490	0.794	0.233	0.323	0.811	0.347	0.060
Time*Level		0.670	0.484	0.719	0.504	0.389	0.798	0.311
SCFA*Time*level		0.165	0.731	0.206	0.079	0.484	0.001	0.306
Time	0 h	C	B	C	B	-	B	C
	4 h	A	A	A	A	-	A	A
	24 h	B	A	B	A	-	A	B
SCFA	SA	-	-	a	-	-	-	-
	SP	-	-	ab	-	-	-	-
	SB	-	-	b	-	-	-	-

Values are presented as means ($n = 9$ for time 0 and $n = 6$ for 4 and 24 h) and standard error of the mean (SEM). Three-way ANOVA were performed with time, fatty acid, and level as main factors. Capital letters were used for differences between time and small letters for differences between fatty acids. Symbols were used for differences between the control and the SCFA supplemented diets: * at time 0 h, # time 4 h, and $ at time 24 h.

Table 4. Absolute values of peripheral blood leucocytes of European seabass prior infection (0 h) and 4 and 24 h post infection.

Parameters		Thrombocytes ($\times 10^4$ μL^{-1})	Lymphocytes ($\times 10^4$ μL^{-1})	Monocytes ($\times 10^4$ μL^{-1})	Neutrophils ($\times 10^4$ μL^{-1})
Control	0 h	5.87 *	3.95 *	0.14	0.02
	4 h	2.51	1.25	0.07	0.21 #
	24 h	3.27	1.96 $	0.26	0.19
SA 0.25	0 h	4.25	2.89	0.14	0.06
	4 h	2.53	1.31	0.04	0.27
	24 h	4.10	2.79 $	0.24	0.19
SA 0.50	0 h	4.09	2.44	0.15	0.04
	4 h	2.22	1.06	0.08	0.29
	24 h	3.54	2.00	0.15	0.08
SP 0.25	0 h	5.27	3.48	0.12	0.02
	4 h	2.24	1.29	0.04	0.13
	24 h	3.94	2.78 $	0.22	0.14
SP 0.50	0 h	3.99	2.67 *	0.15	0.03
	4 h	2.10	1.11	0.04	0.07 #
	24 h	3.28	2.41	0.31	0.11
SB 0.25	0 h	4.19 *	2.89 *	0.11	0.02
	4 h	2.28	1.01	0.06	0.11
	24 h	3.44	2.06	0.13	0.13
SB 0.50	0 h	4.94 *	3.39 *	0.13	0.07
	4 h	2.71	1.44	0.12	0.07 #
	24 h	3.15	2.11	0.21	0.09
SEM	0 h	0.16	0.11	0.01	0.01
	4 h	0.07	0.05	0.01	0.02
	24 h	0.12	0.11	0.02	0.02
Time		<0.001	<0.001	<0.001	<0.001
SCFA		0.867	0.281	0.896	<0.001
Level		0.117	0.057	0.362	0.108
SCFA*Level		0.002	0.003	0.221	0.808
SCFA*Time		0.049	0.106	0.086	0.001
Time*Level		0.284	0.403	0.627	0.200
SCFA*Time*level		0.279	0.603	0.344	0.540
Time	0 h	C	C	B	A
	4 h	A	A	A	B
	24 h	B	B	C	B
SCFA	SA	-	-	-	b
	SP	-	-	-	a
	SB	-	-	-	a

Values are presented as means n = 9 for time 0 and n = 6 for 4 and 24 h and standard error of the mean (SEM). Three-way ANOVA were performed with time, fatty acid, and level as main factors. Capital letters were used for differences between time and small letters for differences between fatty acids. Symbols means differences between control diets and the diets supplemented with SCFAs: * at time 0 h, # at time 4 h, and $ at time 24 h.

Compared to the control diet, before the bacterial challenge (time 0 h), fish fed the SA0.50 (p = 0.000) diet showed a decreased WBC count. In comparison, at 4 h after the challenge, all dietary groups, except for the SB0.50 group, showed a lower hematocrit count, and the SA and SPgroups showed a lower MCV. Furthermore, no differences between groups were observed in MCH, MCHC, WBC, and RBC. At 24 h after the challenge, fish fed the SP0.50 and SB0.50 diets presented higher hematocrit values, and fish fed the SA0.25, SB0.25, and SB0.50 diets showed higher RBC. In contrast, hemoglobin, MCV, and MCH showed no significant differences between groups.

Compared to the time of 0 h (before the challenge) and 4 h after the i.p,. the thrombocyte, lymphocyte, and monocyte counts decreased ($p < 0.001$) while neutrophils increased ($p < 0.001$) (Table 4). At 24 h, neutrophils remained as high as at 4 h, while monocytes highly increased ($p < 0.001$), surpassing the time of 0 h values. Thrombocytes and lymphocytes also increased but remained lower than at the time of 0 h. Except for neutrophils, which were higher in fish fed the SA than in the SP and SB diets ($p < 0.001$), SCFAs did not affect the other leucocyte count.

The thrombocyte count was lower at 0 h in fish fed the SB diets ($p = 0.026$) than in the control. Further, at 0 h, the lymphocyte count was lower than in the control group in fish fed the SP 0.50 ($p = 0.043$), SB 0.25 ($p = 0.012$), and SB 0.50 ($p = 0.020$) diets. Moreover, at 4 h post challenge, the neutrophil count was lower than in the control in fish fed the SP 0.50 ($p = 0.027$) and SB 0.50 ($p = 0.022$) diets. Furthermore, at 24 h post challenge, the lymphocyte count was higher than that of the control group in fish fed the SA 0.25 ($p = 0.040$) and SP 0.25 ($p = 0.040$) diets.

The monocyte count was not different between groups at any sampling time.

3.3. Innate Immune Parameters

There were almost no interactions regarding time, SCFAs, and SCFA levels in the innate immune parameters analyzed (Table 5). Lysozyme, peroxidase, proteases, anti-proteases, and nitric oxide activity significantly decreased ($p < 0.001$) in all groups during the experiment, while bactericidal activity was not affected by time.

Table 5. Innate immune parameters of European seabass prior infection (0 h) and 4 h and 24 h post infection.

Parameters		Lysozyme (μg mL^{-1})	Peroxidase (Units mL^{-1})	Proteases Activity (%)	Anti-Proteases Activity (%)	Bactericidal Activity (%)	Nitric Oxide
Control	0 h	21.0 *	181.4	15.8 *	87.5 *	50.7 *	711
	4 h	14.9	35.3 #	8.64 #	85.7	43.1 #	429 #
	24 h	8.3 $	143.1 $	10.76	90.3	45.5	492 $
SA 0.25	0 h	16.9	165.8	20.3	87.4	44.7 *	738
	4 h	16.1	42.2	9.82	86.7	52.3 #	532 #
	24 h	4.4 $	120.7	10.30	89.0	44.3	482
SA 0.50	0 h	16.4	191.1	23.7 *	87.2	49.0	719
	4 h	14.1	64.6	9.39	88.0	53.0 #	572 #
	24 h	7.2	139.5	11.33	87.1	44.0	475
SP 0.25	0 h	28.1 *	127.2	18.6	86.9	48.7	731
	4 h	14.6	82.2 #	10.8 #	84.2	47.2	577 #
	24 h	9.7	132.2	10.76	90.3	48.8	492
SP 0.50	0 h	19.0	126.6	16.7	84.2 *	48.5	686
	4 h	16.0	62.0	9.94	83.6	47.7	599 #
	24 h	8.4	47.5 $	10.47	89.8	42.4	352 $
SB 0.25	0 h	24.2	179	18.4	87.9	46.1	732
	4 h	12.1	67.1	11.3 #	78.7	47.0	546
	24 h	8.4	54.2 $	10.38	89.0	48.0	275 $
SB 0.50	0 h	18.9	220	23.3 *	86.2	46.1	747
	4 h	14.1	62.0	10.4 #	85.4	49.2	558 #
	24 h	11.5	35.3 $	10.9	89.7	55.1	255 $
SEM	0 h	1.00	11.6	0.91	0.40	0.74	17.5
	4 h	0.56	4.48	0.25	0.91	1.01	16.2
	24 h	0.44	9.23	0.15	0.19	1.56	21.2
Time		<0.001	<0.001	<0.001	<0.001	0.234	<0.001
SCFA		0.015	0.173	0.408	0.597	0.647	0.057
Level		0.265	0.761	0.491	0.917	0.463	0.454
SCFA*Level		0.355	0.157	0.420	0.168	0.212	0.153
SCFA*Time		0.070	0.002	0.238	0.515	0.011	0.012
Time*Level		0.010	0.196	0.373	0.179	0.909	0.046
SCFA*Time*level		0.297	0.786	0.668	0.171	0.311	0.271

Table 5. *Cont.*

Parameters			Lysozyme (μg mL^{-1})	Peroxidase (Units mL^{-1})	Proteases Activity (%)	Anti-Proteases Activity (%)	Bactericidal Activity (%)	Nitric Oxide
Time		0 h	C	B	B	B	-	C
		4 h	B	A	A	A	-	B
		24 h	A	A	A	C	-	A
SCFA		SA	a	-	-	-	-	-
		SP	ab	-	-	-	-	-
		SB	b	-	-	-	-	-
Time*Level	0.25	0 h	C	-	-	-	-	-
		4 h	B	-	-	-	-	-
		24 h	A	-	-	-	-	-
	0.50	0 h	C	-	-	-	-	C
		4 h	B	-	-	-	-	B
		24 h	A	-	-	-	-	A
SCFA*Time	SA	0 h	-	B	-	-	AB	-
		4 h	-	A	-	-	B	-
		24 h	-	B	-	-	A	-
	SP	0 h	-	-	-	-	-	-
		4 h	-	-	-	-	-	-
		24 h	-	-	-	-	-	-
	SB	0 h	-	B	-	-	A	-
		4 h	-	A	-	-	AB	-
		24 h	-	A	-	-	B	-

Values are presented as means n = 9 for time 0 and n = 6 for 4 and 24 h and standard error of the mean (SEM). Three-way ANOVA was performed with time, fatty acid, and level as main factors. Capital letters were used for differences between time and small letters for differences between fatty acids. Symbols means differences between control diets and the diets supplemented with SCFA: * at time 0 h, # at time 4 h, and $ at time 24 h.

Dietary SCFAs only affected lysozyme activity ($p = 0.015$), with higher activity being observed in fish fed the SB than the SA diet.

Four hours after the bacterial challenge, the anti-proteases activity was decreased compared with time of 0 h ($p < 0.001$), but at 24 h, the activity was increased ($p < 0.001$), regardless of SCFAs or supplementation level.

The peroxidase activity in fish fed the SA diets decreased at 4 h after the challenge but increased to the pre-challenge (0 h) values after 24 h ($p = 0.002$). On the other hand, protease activity in fish fed the SB diets decreased at 4 and 24 h compared to the pre-challenge (0 h) level.

Twenty-four hours after, there was an increase in bactericidal activity in fish fed the SB than at time 0 h and 4 h ($p < 0.001$), while in fish fed the SA diet it was lower at 24 h than at 4 h, and it was not affected by sampling time in fish fed the SP diets.

3.4. Survival

The survival of fish after intraperitoneal injection with *V. anguillarum* is presented in Figure 1. No mortality was observed in the PBS group, while the survival of the experimental groups ranged between 93.3 and 66.7% and was not significantly different between groups, although in absolute values it was higher in groups SB 0.50 (93.3%), SB 0.25 (80%), SA 0.50 (80%), and the control diet (80%).

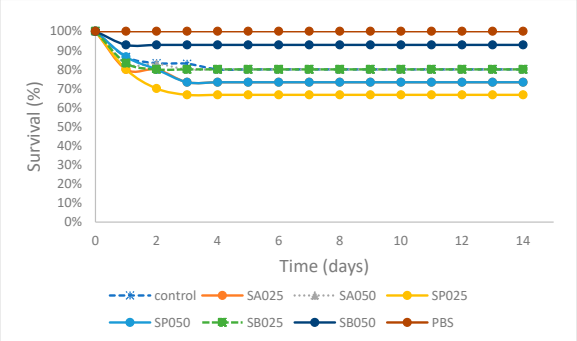
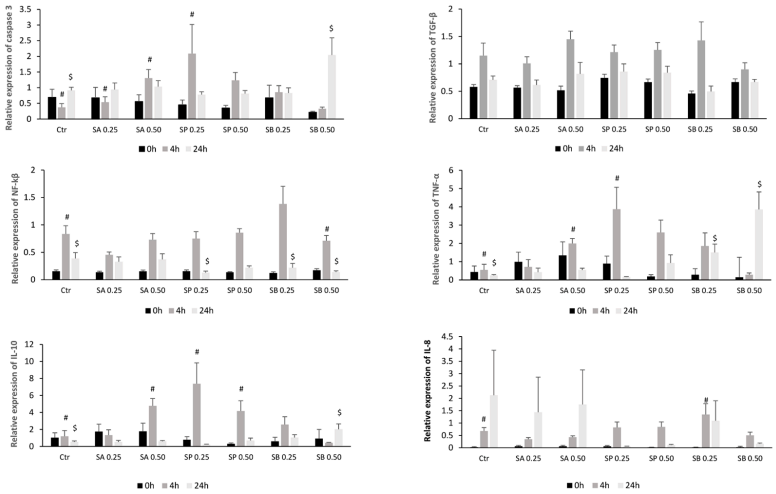


Figure 1. Survival rate (%) of European seabass fed dietary treatments for 56 days and subsequently intraperitoneal injected with 100 µL of *vibrio anguillarum* (1.2×10^7) and PBS.

3.5. Gene Expression

All the immune-related genes measured at the anterior intestine (local of SCFA absorption) responded to the bacterial challenge, showing a higher expression at 4 h after the insult ($p < 0.001$), regardless of dietary treatment, and decreasing their expression at 24 h, except for caspase 3 which remained highly expressed (Figure 2).



Parameters		NF-kβ	TGF-β	IL-10	Caspase 3	TNF-α	IL-8
Time		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Fatty acid		0.193	0.062	0.028	0.953	0135	0.15
Level		0.709	0.491	0.705	0.115	0.955	0.282
SCFA*Level		0.004	0.144	0.050	0.251	0.196	0.009
SCFA*Time		<0.001	0.683	<0.001	0.005	<0.001	0.006
Time*Level		0.394	0.715	0.565	0.215	0.067	0.188
SCFA*Level*Time		<0.001	0.006	0.002	0.009	0.019	0.081
Time	0 h	A	A	A	A	A	A
	4 h	B	B	B	B	B	B
	24 h	A	A	A	B	A	A
SCFAs	SA	-	-	ab	-	-	-
	SP	-	-	b	-	-	-
	SB	-	-	a	-	-	-

Values are presented as means $n = 9$ for time of 0 h and $n = 6$ for 4 and 24 h and standard error of the mean (SEM). Three-way ANOVA tests were performed. If the interaction was significant, two-way ANOVA was performed.

Figure 2. Quantitative expression of immune-related genes in the intestine of European seabass fed experimental diets for 56 days (time of 0 h), challenged with *Vibrio anguillarum* and sampled 4 h and 24 h after injection. Values are presented as means ($n = 9$) for time of 0 h and $n = 6$ for times of 4 and 24 h. Standard error (SE); three-way ANOVA. Symbols means differences between control diets and the diets supplemented with SCFA: # at time 4 h, and $ at time 24 h.

At the time of 0 h, there were no differences in gene expression between the control and the experimental groups. The expression of tgf-β and il-8 in the experimental groups also did not differ from that of the control group at the other times, except for il-8 expression at the time of 4 h in fish fed the SB 0.25 ($p = 0.033$) diet, which was higher than in the control. On the other hand, il-10 expression was increased at 4 h in fish fed the SA 0.50 ($p = 0.020$) and the SP ($p = 0.000$; $p = 0.030$) diets and in the SB 0.50 ($p = 0.001$) diet at 24 h. Also compared to the control, tnf-α expression was increased at 4 h in fish fed the SP ($p = 0.000$; $p = 0.008$) diets and at 24 h in fish fed the SB0.50 ($p = 0.000$) diet, and nf-kβ expression was decreased in fish fed SB0.50 ($p = 0.019$) at 4 h and also decreased 24 h after the challenge fish fed SB ($p = 0.022$; $p = 0.014$) and SP 0.25 ($p = 0.010$) diets.

4. Discussion

SCFAs are important modulators of inflammation, primarily exerting anti-inflammatory effects through multiple mechanisms, including immune cell regulation, cytokine modulation, and maintaining intestinal integrity [24].

The effect of dietary SCFAs was evaluated on the immune response of European seabass juveniles after being challenged by virulent bacteria. Although several studies showed that SCFAs have an immunostimulant effect in fish [25,26], their efficacy in promoting disease resistance is scarcely studied.

Dietary SCFAs were also shown to have beneficial effects on the hematological parameters of several fish species like Indian carp (*Cirrhinus mrigala*) [27], Nile tilapia [28,29], goldfish (*Carassius auratus*) [30], barramundi, (*Lates calcarifer*) [31], and European seabass [8,10]. An improvement in humoral parameters, such as lysozyme and alternative complement activities, in fish fed SCFA-supplemented diets has also been reported in barramundi [31] and in Siberian sturgeon [20].

In the present study, no significant differences were observed in the hematological parameters of unchallenged European sea bass juveniles fed diets supplemented with SCFAs. Regardless of treatment, all hematological parameters decreased 4 h after the bacterial challenge, and by 24 h, they returned to levels close to those observed before the challenge. Accordingly, in Nile tilapia fed sodium butyrate, the blood parameters were also not affected [27]. Kumar et al. [27] observed increased WBC, RBC, and hemoglobin in Indian carp juveniles after a challenge with *Aeromonas hydrophilla* bacteria.

The decrease in the hematological parameters observed in this study may at least partially be related to the fast recruitment of WBCs to the site of infection, reducing their number in circulation, as also observed in other studies [32]. However, despite the total plasmatic WBC count being decreased after the challenge, neutrophil count was maintained high both at 4 and 24 h.

Neutrophils are granulocytes with cytoplasmatic granules filled with defense peptides and enzymes [26] and are critical innate immune cells, comprising the first line of defense against insults [33]. Neuthrophiles are key players in the production of antimicrobial compounds, such as lysozyme, peroxidase, and nitric oxide (NO) [34]. As expected, in the present study, the neutrophil number increased 4 h after the bacterial challenge, but unexpectedly the peroxidase activity and NO level were decreased, indicating that non-oxidative killing mechanisms were used instead of peroxidase and NO production, as also reported in other studies [35].

Moreover, the decreased number of blood leucocytes may also explain the decrease in NO level, lysozyme, proteases, anti-proteases, and peroxidase activity in the plasma at 4 h after the bacterial challenge. However, contrary to what was observed in the other treatments, fish fed diets supplemented with sodium butyrate SB 0.50 presented higher bactericidal activity at 24 h after the intraperitoneal (i.p) injection.

According to Ellis, 2001 [36], bactericidal activity is the ability to kill and eliminate pathogenic bacteria. Abd El-Naby et al. 2019 [37] showed that Nile tilapia fed SB presented a higher bactericidal activity against *A. hydrophila* in a dose-dependent manner. These results

suggest that SB contributes to improved intestinal integrity and development, stimulates the immune system, and avoids intestinal colonization with pathogenic bacteria [38].

The inflammatory process is regulated by the balance between pro- and anti-inflammatory cytokines [39] and the immune response is a steady state between pro- and anti-inflammatory responses [40]. The up-regulation of the pro-inflammatory cytokines may be balanced with the up-regulation of anti-inflammatory cytokine expression [14]. In the present study, the expression of all the immune-related genes analyzed, either pro- or anti-inflammatory genes, was up-regulated at 4 h after the bacterial challenge, returning to the basal values at 24 h, except for caspase.

In the present study, the up-regulation of both pro-inflammatory and anti-inflammatory genes 4 h after the challenge indicates that the immune system was actively responding to the bacterial infection, and the return to basal levels (prior to infection) 24 h after the challenge suggests that the organism already managed the acute phase of the immune response and is moving toward resolution and recovery of the inflammation.

It is known that bacterial infections and pro-inflammatory cytokines induce nf-kβ activation [41] and, accordingly, in this study, nf-kβ expression was up-regulated 4 h after the challenge. In rainbow trout, a diet with 0.2% SB enhanced both pro- and anti-inflammatory cytokines [42], while diets with 1.5, 2.5, and 5 g/kg SB showed an up-regulation of *tnf-α* after a challenge with *Streptococcus iniae*, while an up-regulation of *il-10* and *tgf-β* and a down-regulation of *il-8* was only observed when fed the diet with 2.5 g/kg SB, and an up-regulation of *tnf-α* and *il-8* was only observed when fed the diet with 5 g/kg SB [43].

Furthermore, in crucian carp, diets supplemented with 1% of SA, SP, or SB up-regulated the expression of *tnf-α*, *tgf-β*, and *il-8* [7], while SB also up-regulated the expression of *tgf-β* and *il-8*. In Nile tilapia, a mixture of formic acid, propionic acid, and calcium propionate supplemented in the diet at 1 and 2 g/kg acted as acidifiers and up-regulated tnf-α expression [29].

Overall, these results indicate that the inflammatory responses are dose dependent, which makes it difficult to compare results in different species, using different SCFAs and supplementation doses, and employing different sampling times.

Although there were no statistically significant differences between groups in survival against the bacterial insult, fish fed the SB 0.50 diet showed a tendency to have a higher survival (93.3%) than the other groups (80–66.7%). This lack of significance may be related to the high variability observed in survival, and this potentially increased survival related to the dietary supplementation of SB deserves to be confirmed in another study including higher dietary SB concentrations. Indeed, in a study with zebrafish, the diet that promoted a higher survival rate included 1% SB [7].

5. Conclusions

Overall, fish fed SB diets showed a trend for increased survival after the challenge with *Vibrio anguillarum*, suggesting that this SCFA may be more effective as a functional ingredient than SA and SP for European sea bass juveniles.

Further studies are necessary to better understand the mechanism of action, the correct dose, and the duration of administration of SCFAs on the immune response and to further validate the potential of SCFAs to improve the disease resistance of European sea bass.

Author Contributions: F.F. was responsible for all analytical procedures and wrote the manuscript. N.M. assisted with analytical procedures and manuscript writing. F.B. conducted the experimental trial. R.M. assisted with analytical procedures. R.S. assisted with bacteria preparation. A.O.-T. was responsible for financial support, designed the experiment, and supervised the manuscript writing. H.P. was responsible for financial support, designed the experiment, and supervised the manuscript writing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Fundação para a Ciência e a Tecnologia, Portugal and Fundo Europeu de Desenvolvimento Regional (FEDER). This research was partially supported by

the Strategic Funding UIDB/04423/2020 and UIDP/04423/2020 through national funds provided by FCT. Filipa Fontinha was supported by FCT grant (2020.07212.BD).

Institutional Review Board Statement: All the animals were maintained according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) Guidelines for Reporting Animal Research and approved by the ORBEA Animal Welfare Committee of CIIMAR (ORBEA; reference ORBEA_CIIMAR_27_2019) according to the European Union directive 2010/63/EU and the Portuguese Law DL 113/2013.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data generated during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

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