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Physiological Response of European Sea Bass (*Dicentrarchus labrax*) Juveniles to an Acute Stress Challenge: The Impact of Partial and Total Dietary Fishmeal Replacement by an Insect Meal

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Abstract: This study aimed to explore the effect of FM substitution by defatted *Tenebrio molitor* larvae meal (dTM) on the response of European seabass to an acute stress challenge. An FM-based diet was used as a control and two other isoproteic/isoenergetic diets were formulated to replace 50 and 100% of FM by dTM. Each diet was tested in quadruplicate groups of 15 fish (69 ± 5 g) fed until visual satiety for 16 weeks. After the feeding trial, fish were subjected to 1 min air exposure followed by 1 h of recovery before sampling. The haematological profile, plasma metabolites, and humoral immunity biomarkers, as well as hepatic oxidative stress and antioxidant capacity, were analysed. A clear response to acute stress was observed by a significant increase in haemoglobin, haematocrit, red blood cells, and almost all evaluated plasma metabolites and humoral parameters, regardless of dietary treatment. The obtained results demonstrated that partial substitution of FM by IM did not affect the stress response of seabass. However, total FM replacement increased the hepatic activity of total peroxidase and superoxide dismutase in fish fed TM100.

Keywords: aquaculture; aquafeed; sustainable protein sources; *Tenebrio molitor*

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1. Introduction

In 2020, more than half (i.e., 56%; 88 million tonnes) of the aquatic food available for human consumption came from aquaculture production, and this share is forecasted to increase by 22%, reaching 106 million tonnes by 2030 [1]. The expansion of the aquaculture sector necessarily implies an increase in aquafeed production, and consequently in raw materials availability. The use of fishmeal (FM) and fish oil (FO) from fish by-products (i.e., heads, viscera, skin, bones, and scales) has increased over the last years, but the production of these commodities (particularly FM) is still highly dependent on the processing of whole small pelagic fish species obtained from fisheries [2]. Thus, to guarantee the responsiveness and sustainability of the aquaculture industry over the upcoming years, it is imperative to diminish its dependence on FM and FO obtained from wild fish and promote the strategic use of these ingredients [1]. Over the past few years, the use of vegetable proteins, terrestrial processed animal proteins (PAPs), and more recently, single cell proteins as alternatives to FM has been explored extensively [3]. However, as outlined in the latest research by Glencross et al. [4], each ingredient has its own set of strengths and weaknesses, and there is not a single ingredient that can be considered perfect.

Using insects as a source of nutrients is not a novel concept, as the consumption of edible insects has been a longstanding tradition in Latin America, Africa, and Asia. [5]. However, in Europe, the interest in insect protein has increased since the inclusion of this ingredient in aquafeed was authorized by the European Union (EU) in 2017 [6]. According to Cottrell et al. [7], insect protein stands out as the most promising alternative to fishmeal when compared to other emerging protein sources like micro- and macroalgae, bacteria, and yeast. Indeed, insect meal (IM), predominantly derived from *Hermetia illucens* (HI) and *Tenebrio molitor* (TM) larvae, has demonstrated an enormous potential as an alternative protein source to partially and totally replace FM in diets for both freshwater and marine fish species, including European seabass (*Dicentrarchus labrax*), a key fish species in Mediterranean aquaculture [8]. Despite this, the majority of studies have primarily focused on fish growth, nutrient digestibility, intestinal health, and the final quality of fish intended for human consumption, overlooking the effect of the dietary inclusion of IM on fish response to stress, which in the long term may result in adverse effects on animal health and overall performance [9].

In the past few years, the expansion of aquaculture production has led to the emergence of certain practices (e.g., fish overcrowding, grading, transport, etc.) that induce stress in fish [10,11]. Stress response can be categorized into primary, secondary, or tertiary. The primary response is initiated by the activation of endocrine pathways, triggered by the central nervous system's recognition of the stressor, resulting in the release of catecholamines and cortisol. The secondary response is orchestrated by the hormones responsible for the primary response. This involves cardiovascular and respiratory changes (e.g., increased distribution of oxygen and release of energy substrates into circulation, such as glucose and lactate), as well as hydromineral dysfunction (e.g., adrenaline modifies gills' blood flow and permeability patterns). In addition, this second stage of the stress response is marked by cortisol-mediated immunosuppression. Finally, the tertiary response is an outcome of the incapacity of the animal to achieve homeostasis, resulting in suppressed immunity, metabolic dysfunctions, and impaired growth performance [12]. Moreover, stress may disturb the balance between reactive oxygen species (ROS) produced and the antioxidant scavenging ability of tissues, resulting in increased ROS levels and ultimately leading to oxidative damage. To counteract the harmful effects of ROS, the organism initiates the activation of antioxidant enzymes [13]. Nutrition plays a crucial role as a major modulator of oxidative stress. The consumption of a well-balanced diet containing antioxidant compounds can fortify the animal's redox status, contribute to allostasis, and improve flesh quality. For instance, Zarantoniello et al. [14] recently demonstrated that replacing 3 and 20% FM by full-fat spirulina-enriched black soldier fly (*H. illucens*) prepupae meal resulted in decreased lipid peroxidation of European seabass flesh. On the other hand, oxidative stress can also be triggered by feed ingredients, either through the presence of anti-nutritional factors, amino acid deficiencies, or even an excess of macronutrients [9]. Thus, to better understand the feasibility of using a novel ingredient in aquafeeds, such as IM, it is of paramount importance to assess its effects beyond growth. Specifically, evaluating its impact on immune function and the oxidative stress response becomes imperative.

In this context, the main aim of this study was to investigate, for the first time, the effects of partially and fully replacing fishmeal with defatted (d-) *Tenebrio molitor* (TM) on humoral immunity and hepatic oxidative stress in juvenile European seabass following exposure to an acute stress challenge, reflecting common handling procedures in fish farms.

2. Materials and Methods

2.1. Ingredients and Experimental Diets

Three diets were formulated and extruded by SPAROS Lda. (Olhão, Portugal) according to European seabass nutritional requirements [15]. A control diet (CTRL) was formulated to include 40% of high-quality FM and 14% FO, and two other experimental diets were obtained by replacing 50% (TM50) and 100% (TM100) of FM by dTM. All experimental diets were isoproteic, isolipidic, and isoenergetic (47% protein, 20% lipids, and

24 kJ g^{-1} on a dry matter (DM) basis, respectively) and supplemented with DL-methionine. The TM100 diet was also supplemented with L-lysine, L-threonine, and L-tryptophan. Monocalcium phosphate was added to dTM diets. Feed ingredients and proximate composition of the dTM and experimental diets are presented in Table 1. Experimental diets are the same in this and our previous studies [16–18].

Table 1. Ingredients and proximate composition of defatted *Tenebrio molitor* larvae meal and the experimental diets.

	dTM	CTRL	TM50	TM100
Ingredients (%)				
Fishmeal [1]		40	20	-
Defatted *Tenebrio molitor* larvae meal		-	20.5	40.4
Soy protein concentrate [2]		10.5	10.5	10.5
Soybean meal [3]		13	13	13
Rapeseed meal [4]		5	5	5
Wheat meal [5]		16.2	15.2	14.3
Fish oil [6]		14.0	13.3	12.5
Vitamin and mineral premix [7]		1	1	1
Vitamin C		0.1	0.1	0.1
Vitamin E		0.1	0.1	0.1
Monocalcium phosphate		-	1.0	2.0
L-Lysine		-	-	0.2
L-Threonine		-	-	0.2
L-Tryptophan		-	-	0.1
DL-Methionine		0.1	0.2	0.3
Proximate composition (% dry matter (DM) or kJ/kg DM)				
Dry matter	97.8	93.1	92.6	92.5
Crude protein	71.0	46.9	47.3	47.2
Crude fat	12.1	19.7	19.8	19.0
Gross energy	24.3	23.2	23.5	24.0
Ash	4.8	10.2	8.1	6.3
Phosphorus	0.8	1.2	1.2	1.0

The abbreviations for the experimental diets stand for: CTRL, control diet; TM50 and TM100, diets with 50 and 100% of the fishmeal protein replaced by defatted *Tenebrio molitor* larvae meal. [1] Peruvian fishmeal super prime: 71% crude protein (CP), 11% crude fat (CF), Exalmar, Peru; [2] soy protein concentrate: 65% CP, 0.7% CF, ADM Animal Nutrition, The Netherlands; [3] soybean meal 48: dehulled solvent extracted soybean meal: 48% CP, 2% CF, Cargill, Spain; [4] rapeseed meal: 36% CP, 3% CF, PREMIX Lda., Portugal; [5] wheat meal: 10% CP, 1% CF, Casa Lanchinha, Portugal; [6] sardine oil, Sopropêche, France; [7] vitamin and mineral premix: WISIUM, ADM Portugal S.A., Portugal.

2.2. Ethical Issues

The fish trial and all procedures involving animals were approved by the CIIMAR ethical committee for Managing Animal Welfare (ORBEA), in compliance with Directive 2010/63/EU from the European Union and the Portuguese Decree-Law n° 113/2013 on "The protection of animals used for scientific purposes". Sampling procedures were performed by accredited scientists in laboratory animal science from the Portuguese Veterinary Authority (1005/92, DGAV-Portugal, following FELASA category C recommendations).

2.3. Acute Stress Challenge and Fish Sampling

Before the acute stress challenge, a growth trial was carried out for 16 weeks as described by Basto et al. [16]. Briefly, juveniles of European seabass were obtained from a commercial fish farm (Acuinuga—Acuicultura y Nutrición de Galicia S. L., Spain) and transported to the experimental facilities of CIIMAR (Portugal). After a quarantine period of 3 weeks, 12 homogeneous groups of 15 fish (69 ± 8 g) were randomly allocated to 160 L tanks of a recirculation aquaculture system (RAS) and kept at 22 ± 1 °C, 35 ± 0.5 salinity, 6 L min^{-1}, oxygen level > 90% ± 1 saturation, and an artificial 12 light/12 dark photoperiod. Water quality parameters were monitored daily as described by Basto et al. [16]. Fish were fed by automatic feeders three times a day. Each experimental diet was tested in

quadruplicate for 16 weeks. After the feeding trial, and following a 48 h fasting period, 3 fish per tank (n = 12 fish) were anesthetized (ethylene glycol phenyl ether, 300 μL L^{-1}) and sampled for evaluation of innate immune status. Blood was withdrawn from the caudal vein using heparinized syringes and centrifuged (5000× g for 5 min at 4 °C), and the resulting supernatant plasma was kept at −80 °C until the metabolites and innate immunity-related parameters analysis. Then, fish were euthanized by spinal cord section, and a portion of the liver (≈150 g) was collected, instantly frozen in dry ice, and kept at −80 °C until the analysis of the activity of oxidative stress-related enzymes. To evaluate the outcome of IM dietary incorporation on oxidative stress, antioxidant capacity, and immune response to stress, other 3 fish per tank (12 fish per dietary treatment) were subjected to an acute stress challenge, consisting of 1 min of air exposure. After 1 h of recovery, the blood, plasma, and liver were sampled as previously described. All fish were individually weighted at the beginning and at the end of the experimental trial to calculate growth performance.

2.4. Haematological Parameters

The haematocrit (Ht), haemoglobin (Hb; kit ref. 1001230, Spinreact, Barcelona, Spain), and total red and white blood cell (RBC and WBC, respectively) counts were determined according to Machado et al. [19]. The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were then calculated as follows: MCV (μm^3) = (Ht/RBC) × 10; MCH (pg $cell^{-1}$) = (Hb/RBC) × 10; MCHC (g 100 mL^{-1}) = (Hb/Ht) × 100.

2.5. Plasma Metabolites and Innate Immunity-Related Parameters

Cortisol levels were measured using an ELISA kit (ref. RE52061, IBL International Gmbh, Hamburg, Germany) following the manufacturer's instructions and according to Azeredo, et al. [20]. Lactate and glucose were enzymatically determined using commercial kits (ref. 1,001,330 and 41,011, respectively, Spinreact, Barcelona, Spain) adapted to the microplate format. Lysozyme activity was determined according to Hutchinson and Manning [21]. Total peroxidase activity was assessed following the method described by Quade and Roth [22]. Alternative complement pathway activity (ACH50) was determined based on the lysis of rabbit blood cells according to Sunyer and Tort [23].

2.6. Hepatic Oxidative Stress and Antioxidant Capacity

Liver samples intended for the determination of lipid peroxidation (LPO) and antioxidant enzyme analysis were homogenized with phosphate buffer (0.1 M, pH 7.4) at a ratio of 1:10 (w/v), as described by Resende et al. [24]. Before enzymatic activity assessment, the hepatic soluble protein content was determined using a commercial kit (ref. 23,225, Thermo Fisher Scientific Inc., Pleasanton, CA, USA) for standardizing enzyme activity measurements. The activity of catalase (CAT), glutathione s-transferase (GST), glutathione peroxidase (GPx), and glutathione reductase (GR) was determined according to Pereira et al. [25]. Superoxide dismutase (SOD) activity and total antioxidant capacity (TAC) were determined using commercial kits (ref. 19,160 and MAK187, respectively, Sigma-Aldrich, St. Louis, MO, USA). All measurements were performed in triplicate in a Synergy HTX Multi-Mode Microplate Reader (BioTek Instruments Inc., San Diego, CA, USA).

2.7. Statistical Analysis

Data were tested for normality and homogeneity of variances by Kolmogorov–Smirnov and Levene's tests, respectively, and transformed whenever necessary. Differences were tested by a two-way ANOVA, with stress (stressed and non-stressed) and dietary treatment (CTRL, TM50, and TM100) as the main factors. A significance level of 95% ($p < 0.05$) was considered followed by a post hoc Tukey HSD test to identify significant differences amongst groups using Statistica™ 13.5.0.17 (TIBCO Software Inc., Palo Alto, CA, USA). To discriminate and classify the existing groups, a multivariate canonical discriminant analysis (DA) was performed on

the dataset to evaluate linear combinations of the original variables that would best separate the groups (discriminant functions) using Addinsoft XLSTAT 2022 system software. Each discriminant function explains part of the total variance of the dataset and is loaded by variables contributing the most to that variation. Discriminatory effectiveness was assessed by Wilk's λ test, the distance between group centroids was measured by squared Mahalanobis distance, and Fisher's F statistic was applied to infer significance.

3. Results

3.1. Growth Performance

Before and after the acute stress challenge, no mortality was observed, and European seabass fed diets with partial and total FM replacement by dTM showed similar final body weight after 16 weeks of feeding (Figure 1). Therefore, the experimental diets ensured 100% survival and proper zootechnical performance, and no behavioural alterations were observed in the fish throughout the feeding experiment. Detailed results on the impact of these dietary formulations on fish growth performance are presented in Basto et al. [16].

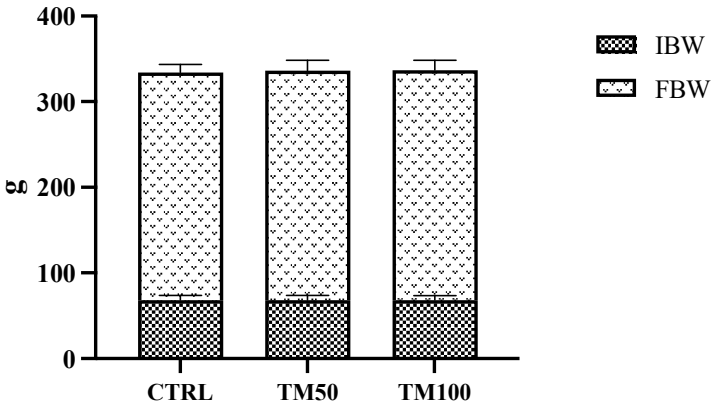

Figure 1. Final body weight (g) of European seabass fed the experimental diets for 16 weeks. The abbreviations are IBW—initial body weight and FBW—final body weight.

3.2. Haematological Profile

Partial and total FM replacement by dTM did not significantly affect haemoglobin, haematocrit, red and white blood cells, mean corpuscular volume, mean corpuscular haemoglobin, or mean corpuscular haemoglobin concentration (Figure 2). On the other hand, haemoglobin, haematocrit, red blood cells, and mean corpuscular haemoglobin concentration significantly increased after the acute stress challenge, while mean corpuscular volume and mean corpuscular haemoglobin decreased, regardless of the dietary treatment (Figure 2).

3.3. Plasma Metabolites and Innate Immunity-Related Parameters

After the acute stress challenge, and irrespective of the dietary treatment, European seabass exhibited a significant increase in plasma cortisol, glucose, and lactate levels, coupled with a significant increase in lysozyme activity and ACH50 (Figure 3). On the other hand, the levels of peroxidase in the plasma of fish fed TM100 were significantly higher than those observed in fish fed CTRL, regardless of stress condition (Figure 3).

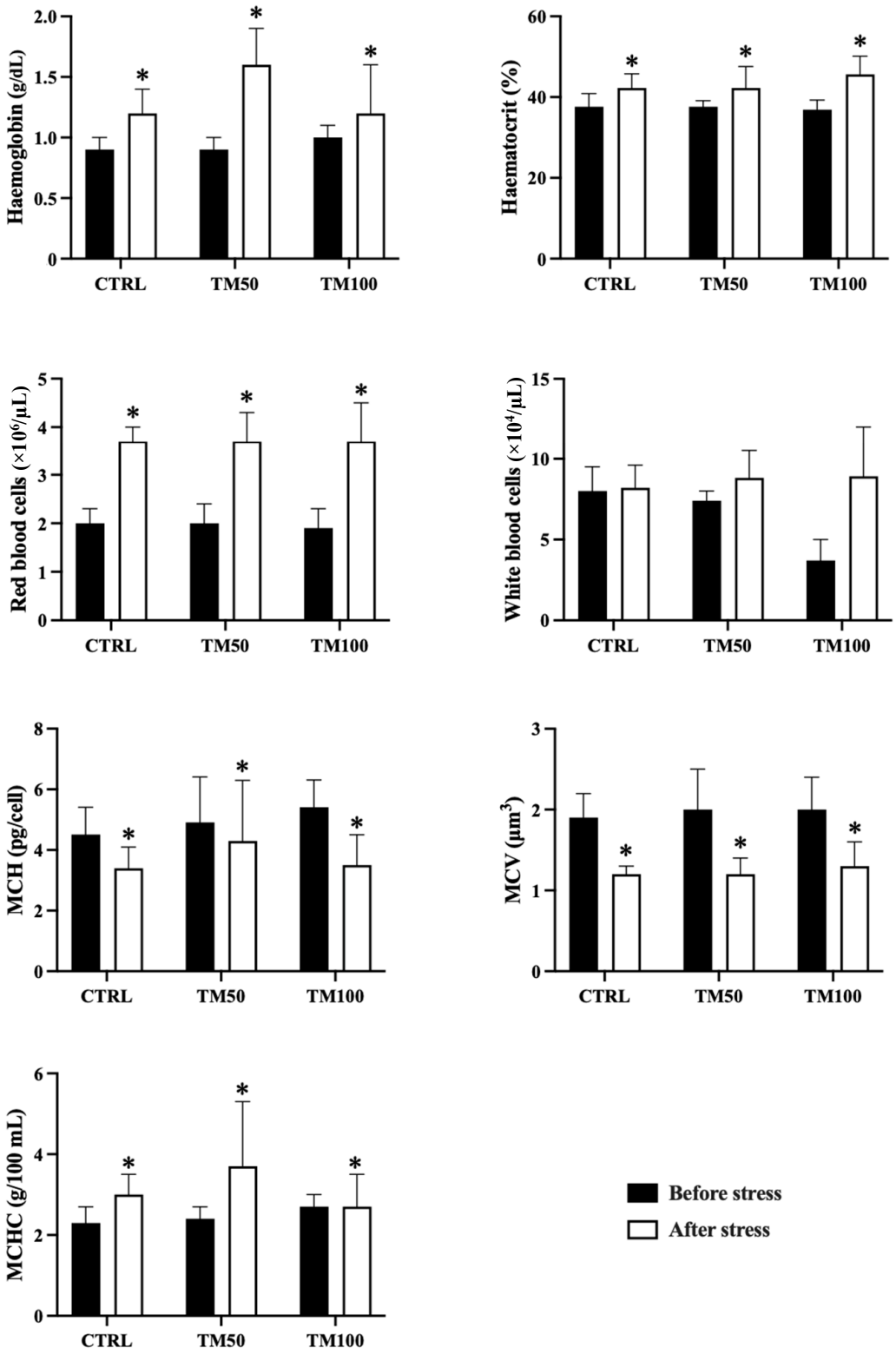

Figure 2. Haematological profile of European seabass fed the experimental diets for 16 weeks and subsequently subjected to an acute stress challenge. Bars represent mean ± standard deviation and the asterisk (*) indicates significant differences ($p < 0.05$) before and after the acute stress challenge, irrespective of dietary treatment.

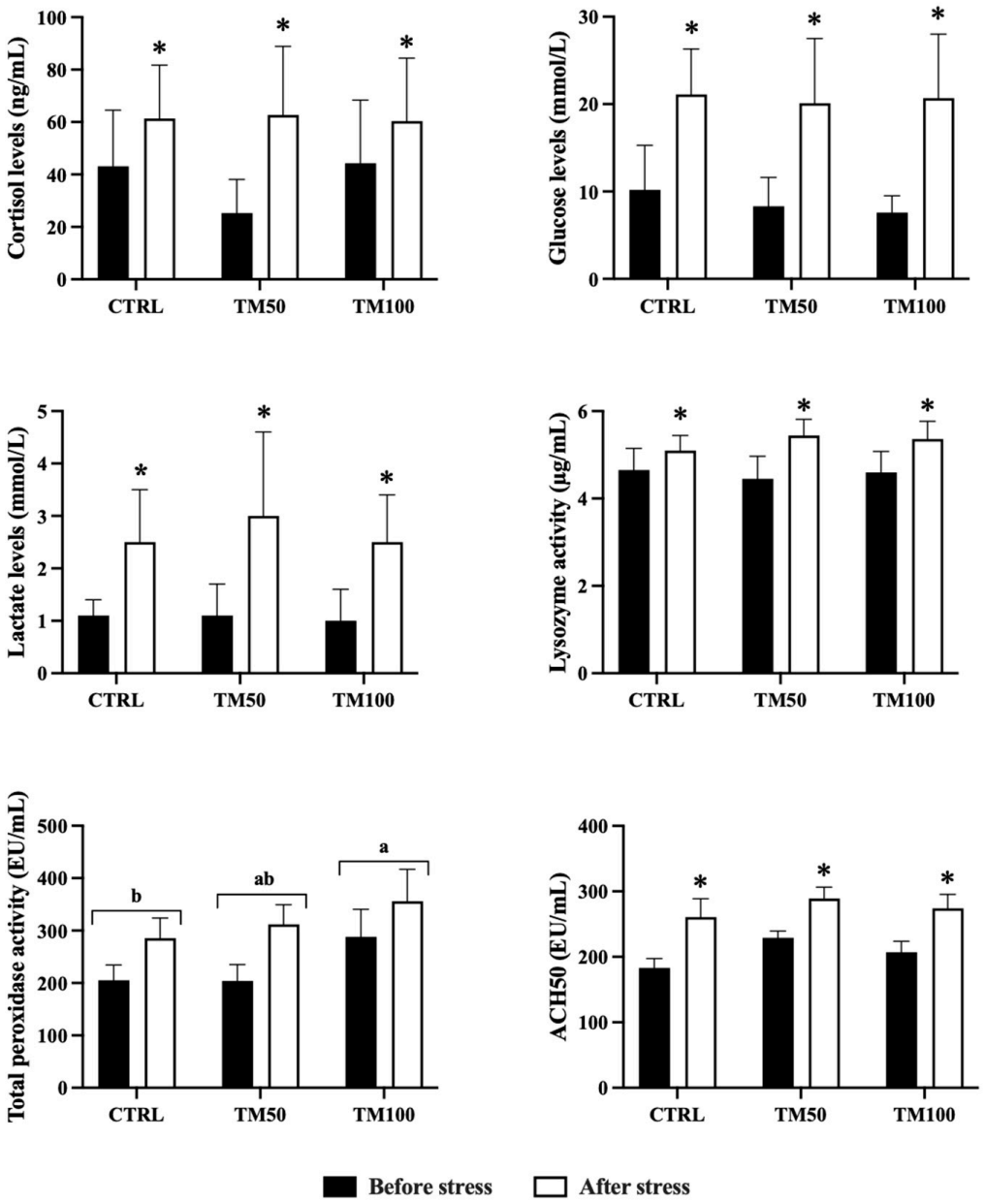

Figure 3. Plasma metabolites and innate immunity-related parameters of European seabass fed the experimental diets for 16 weeks and subsequently subjected to an acute stress challenge. Bars represent mean ± standard deviation. The asterisk (*) indicates significant differences ($p < 0.05$) before and after the acute stress challenge, irrespective of dietary treatment, and different lowercase letters indicate significant differences ($p < 0.05$), irrespective of stress condition.

3.4. Hepatic Oxidative Stress and Antioxidant Capacity

The activity of SOD in the liver of fish fed TM100 was significantly higher than those fed CTRL, irrespective of stress condition (Table 2). The biomarkers CAT, GPx, GST, GR, LPO, and TAC were not affected either by dietary treatment or stress condition (Table 2).

Table 2. Enzymatic activity of oxidative stress-related enzymes, lipid peroxidation, and total antioxidant capacity in the liver of European seabass fed the experimental diets before and after the acute stress challenge.

	Before Stress			After Stress			Two-Way ANOVA *p*-Value		
	CTRL	TM50	TM100	CTRL	TM50	TM100	Diet	Stress	Diet × Stress
SOD	78.5 ± 13.2 ^{b}	80.4 ± 12.8 ^{ab}	90.5 ± 1.8 ^{a}	72.2 ± 13.7 ^{b}	83.9 ± 20.3 ^{ab}	87.1 ± 1.8 ^{a}	0.02	0.46	0.45
CAT	8.1 ± 2.5	6.7 ± 3.4	8.6 ± 2.7	8.7 ± 1.3	9.5 ± 2.7	8.5 ± 2.3	0.81	0.10	0.14
GPx	196.8 ± 11.7	205.8 ± 14.4	200.2 ± 3.2	197.0 ± 5.6	191.3 ± 2.8	190.6 ± 2.7	0.67	0.06	0.06
GST	54.0 ± 9.9	50.7 ± 8.0	56.1 ± 13.6	56.1 ± 6.9	56.3 ± 1.5	61.7 ± 15.5	0.26	0.11	0.84
GR	20.5 ± 5.1	22.8 ± 6.6	23.8 ± 8.4	21.6 ± 2.0	22.3 ± 4.0	22.2 ± 4.6	0.47	0.84	0.71
LPO	10.9 ± 4.1	9.2 ± 3.9	11.0 ± 2.0	9.9 ± 3.9	13.4 ± 7.4	14.8 ± 5.3	0.23	0.06	0.14
TAC	0.21 ± 0.05	0.18 ± 0.05	0.21 ± 0.05	0.23 ± 0.05	0.20 ± 0.05	0.20 ± 0.04	0.15	0.23	0.45

Values represent mean ± standard deviation. Different lowercase letters indicate statistically significant differences ($p < 0.05$) for diet factor. SOD, superoxide dismutase (EU mg protein^{-1}); CAT, catalase (EU mg protein^{-1}); GPx, glutathione peroxidase (nmol mg protein^{-1}); GST, glutathione s-transferase (nmol mg protein^{-1}); GR, glutathione reductase (nmol mg protein^{-1}); LPO, lipid peroxidation (nmol g^{-1} liver); TAC, total antioxidant capacity (nmol Trolox mg^{-1} liver).

3.5. Discriminant Analysis

The overall performance of the analysis indicates good discriminatory ability (Wilks $\lambda = 0.02$, $p < 0.0001$) with the first two discriminant functions accounting for 91.69% of the total dataset variability (Figure 4; F1 83.66% and F2 8.04%). Assessing the linear functions of the variables from the analysed tissues, a clear separation by stress challenge was observed, meaning that fish fed experimental diets before the stress challenge were significantly separated from those after stress, based on the significant Mahalanobis distance of each group's multivariate mean (centroid) ($p < 0.05$). In addition, before stress, the fish fed TM100 were significantly distant from those fed CTRL. The first function (accounting for 83.66% of total dataset variability) was positively loaded mainly by the red blood cell count, haematocrit, haemoglobin, and alternative complement pathway activity, as well as cortisol, glucose, and lactate levels, while being negatively loaded by MCV.

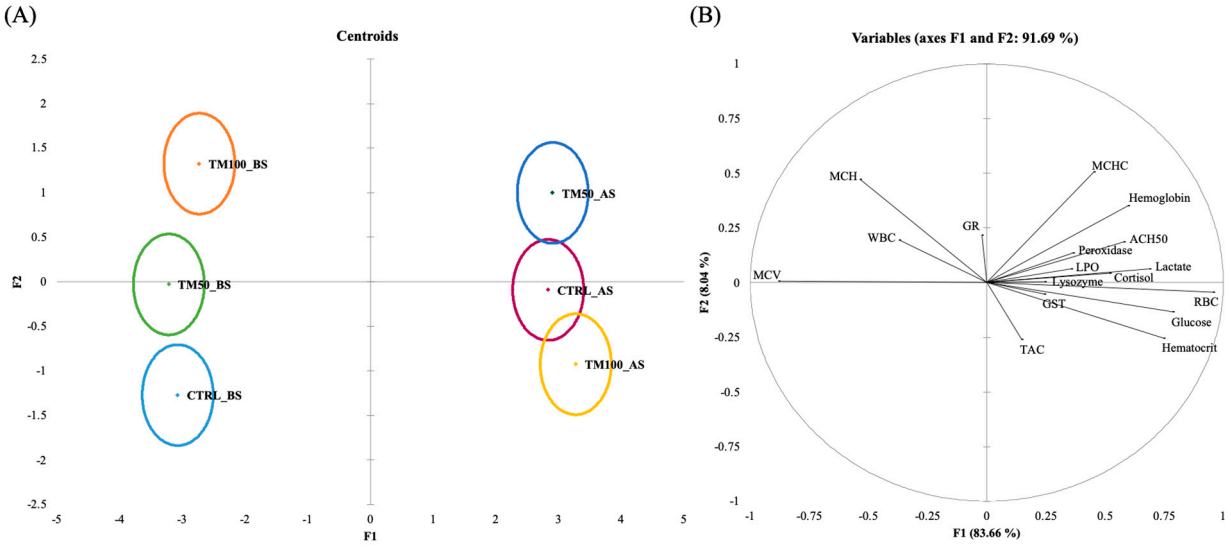

Figure 4. Canonical discriminant analysis of European seabass fed experimental diet before and after the acute stress challenge. (**A**) Canonical discriminant scores of each group. Coloured circles represent groups' centroids. (**B**) Correlation variables/factors (factor loads) for two main discriminant functions (F1 and F2).

4. Discussion

The expansion of the aquaculture sector necessarily implies an increase in aquafeed production, and consequently in raw materials availability. However, the production of conventional marine ingredients and plant protein sources has remained stable, prompting the necessity to explore alternative ingredients [2]. Additionally, the emergence of various environmental and husbandry-related stress factors also poses substantial challenges to the aquaculture industry [26]. Therefore, when a new ingredient is under consideration to be included in aquafeeds, it is increasingly important to understand if it does not compromise the animals' response to stress situations.

Among the most common stressors, handling represents a highly stressful procedure due to its frequent association with air exposure, triggering a notable physiological response [27]. Thus, in the current study, fish were exposed to an acute stress challenge consisting of air exposure, with a total duration of 1 minute, followed by 1 h of recovery before sampling. The significant increase in haemoglobin, haematocrit, red blood cells, mean corpuscular haemoglobin concentration, and cortisol, lactate, and glucose levels after the acute stress challenge validated the applied protocol, as these are typical indicators of acute stress [10,28]. Moreover, a clear separation before and after the stress challenge was observed when a discriminant analysis was applied to the dataset. Additionally, it was clearly observed that when animals were not exposed to a stressful situation, the inclusion of dTM to partially or completely replace FM did not compromise the physiological status of the animals.

After the acute stress challenge, and irrespective of the dietary treatment, European seabass displayed significantly increased values of plasma cortisol. On the other hand, the dietary inclusion of dTM did not affect the circulating levels of this hormone. This suggests that, while the primary stress response of seabass did not exhibit improvement (e.g., decreased circulating cortisol levels) with the inclusion of this protein source, it was also not compromised, even when the FM was completely replaced. To the best of the authors' knowledge, this is the first study evaluating the impact of FM substitution by an IM on the primary stress response of European seabass. In gilthead seabream (*Sparus aurata*), the inclusion of 27.6% of defatted HI to replace 75% of FM did not alter the gill cortisol levels after 17 weeks of a feeding trial [29]. Similarly, in rainbow trout (*Oncorhynchus mykiss*), 50% FM replacement by defatted HI (i.e., 15.7% dietary inclusion) also did not affect the primary stress response parameters, namely, ACTH and cortisol levels, after 18 weeks of feeding with the experimental diets [30].

The secondary stress response is characterized by (1) increased distribution of oxygen and (2) the release of energy substrates into circulation, such as glucose and lactate [10,12,31]. Thus, the observed increased haematocrit, haemoglobin, and red blood cells after air exposure, regardless of dietary treatment, may be related to a splenic contraction to release blood cells into the circulatory system as a strategy to improve the transport of oxygen [32]. In the present study, the partial and total FM replacement by dTM did not significantly affect any of the haematological parameters evaluated. The results obtained herein align with the previous findings of Abdel-Tawwab et al. [33]. Specifically, 50% of FM replacement by defatted HI (i.e., 14.8% dietary inclusion) did not demonstrate any significant impact on the values of different haematological parameters of European seabass juveniles. In Nile tilapia (*Oreochromis niloticus*), total FM replacement by full fat HI, corresponding to 10% dietary inclusion, also did not change the haematological profile of fish [34]. In contrast, in the recent study conducted by Khieokhajonkhet et al. [35], it was demonstrated that red blood cell counts and haemoglobin levels were higher in Nile tilapia fed an experimental diet completely devoid of FM and containing 21.7% giant cricket meal (*Brachytrupes portentosusas*) for 4 weeks. According to those authors, elevated levels of these haematological parameters and their inherent enhanced oxygen transport may lead to a more effective response in challenging circumstances [35]. Nevertheless, to corroborate this hypothesis, further studies would be required involving animals subjected to episodes of stress or infection.

While glucose serves as a crucial energy source in aerobic conditions, providing the fish with the necessary energy substrate to meet heightened energy requirements after stress, the limited oxygen availability in anaerobic conditions hinders the Krebs cycle. In such conditions, pyruvate undergoes conversion to lactate, which is stored in muscle. Once there is a sufficient return of oxygen availability, lactate can be transported to the liver, where it undergoes conversion back to pyruvate for gluconeogenesis. Additionally, lactate can also contribute to meeting energy demands during aerobic metabolism [12]. After the acute stress challenge, and irrespective of the dietary treatment, European seabass exhibited a significant increase in plasma glucose and lactate levels. On the other hand, the dietary inclusion of dTM did not have an impact on any of these stress biomarkers. These results align with findings from other studies that explored the utilization of different types of IM as protein sources in aquafeeds. Similarly, those studies did not observe changes in the plasmatic levels of glucose and/or lactate [36,37]. Altogether, the results herein obtained provide evidence that partial (i.e., 50%) or total FM replacement by dTM, corresponding to dietary inclusion levels of 20% and 40%, respectively, do not compromise the primary or secondary response of European seabass to stress induced by air exposure for 1 min.

Under normal conditions, organisms can neutralize reactive molecules and repair oxidative damage through their natural antioxidant defences. However, exposure to stress can trigger metabolic shifts necessary to meet the increased energy demand, leading to heightened production of ROS as by-products of cellular respiration and other metabolic processes. Oxidative stress occurs when there is an imbalance between the production of ROS and the antioxidant scavenging capacity of tissues through the action of antioxidant enzymes (e.g., SOD, CAT, GPx, GST, and GR), resulting in cellular membrane lipids damage [38,39]. In the liver of fish fed diets completely devoid of FM, the activity of SOD was increased, underscoring the significance of this adaptive response and highlighting its crucial role in antioxidant defence mechanisms. As far as we are aware, this study represents the first attempt to assess the impact of total FM substitution by an IM on the oxidative stress parameters in the liver of European seabass. However, in Atlantic salmon (*Salmo salar*), total FM replacement by dTM (i.e., 21.5% dietary inclusion) did not alter hepatic SOD activity after 12 weeks of a feeding trial [40]. On the other hand, in Siberian sturgeon (*Acipenser baerii*), the inclusion of 37.5% defatted HI to replace 50% FM led to an increase in the hepatic activity of SOD after 16 weeks of a growth trial [41]. Thus, the findings from the existing literature on partial FM substitution by IM in diets for seabass and other fish species are still inconsistent, and further research is required.

Coupled with antioxidant defences, innate immunity is considered the fish's first line of defence, with lysozyme and peroxidase used as conventional biomarkers of the innate immune response in fish. Lysozyme is involved in a defence mechanism, such as bacteriolysis, through action on the peptidoglycan layer of bacterial cellular walls. Peroxidase plays a crucial role as an enzyme that enables the conversion of the superoxide anion to generate hydrochloric acid. Moreover, peroxidase exhibits microbicidal properties by utilizing one of the oxidative radicals to generate hypochlorous acid. This process holds significant importance as it serves as an effective method for eliminating foreign microorganisms [42,43]. While the activity of lysozyme was unaffected by stress conditions or dietary treatments, the peroxidase activity was higher in fish fed TM100. Indeed, several authors state that incorporating both TM or HI in diets for various fish species can enhance the fish immune response, either before or after a bacterial challenge, by increasing lysozyme and peroxidase activities [44–46]. For example, Abdel-Latif et al. [47] observed that the lysozyme activity increased in fish that were fed diets with a 50% FM replacement by partially defatted HI (i.e., 14.8% dietary inclusion) for 8 weeks. Furthermore, fish subjected to this dietary treatment exhibited higher survival rates post-challenge with *Vibrio alginolyticus*. This immunostimulant capacity of IM might be explained by the chitin content of insects, as reviewed by Mohan et al. [48].

In conclusion, the results of this study demonstrate that total FM replacement by dTM increased total peroxidase and SOD activity in fish fed TM100, suggesting an immunostimu-

lant and antioxidant capacity of dTM. But the partial inclusion of dTM did not compromise the physiological stress responses, antioxidant capacity, and innate immunity of European seabass juveniles. However, to better understand the impact of total FM replacement by IM on fish immune response and antioxidant capacity, it would be of high interest to deepen our knowledge of their underlying mechanisms under other challenging rearing conditions (e.g., pathogen or chronic stress exposure).

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Institutional Review Board Statement: All procedures involving animals were approved by the CIIMAR ethical committee for Managing Animal Welfare (ORBEA), in compliance with Directive 2010/63/EU from the European Union and the Portuguese Decree-Law n° 113/2013 on "The protection of animals used for scientific purposes". Sampling procedures were performed by accredited scientists in laboratory animal science from the Portuguese Veterinary Authority (1005/92, DGAV-Portugal, following FELASA category C recommendations).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available upon request.

Conflicts of Interest: Daniel Murta is the founder and R&D advisor of Thunder Foods Lda., which is the promoter of the InFishMix project. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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