



Article

Tryptophan modulatory role in European seabass (*Dicentrarchus labrax*) immune response to acute inflammation under stressful conditions

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Abstract: The present work aimed to study the role of dietary tryptophan supplementation to modulate the European seabass (*Dicentrarchus labrax*) immune condition during stressful rearing conditions (i.e. 15 days exposure to high density) as well as the immune response to acute inflammation after intraperitoneal injection of a bacterial pathogen. Stress alone did not compromise seabass health indicators. In contrast, a clear peripheral and local inflammatory response was observed in response to the inoculated bacteria. Moreover, exposure to a high stocking density seemed to exacerbate the inflammatory response at early sampling points compared to fish stocked at a lower density. In contrast, stressed fish presented some immune suppressing effects on T-cell surface-glycoproteins receptors expression at a late sampling point following inflammation. Regarding the effects of dietary tryptophan, no changes were observed on seabass immune indicators prior to inflammation, while a small number of immunosuppressive effects were observed in response to inflammation, supporting tryptophan role in promotion of immune-tolerance signals during inflammation. Nonetheless, tryptophan dietary supplementation improved the inflammatory response against a bacterial pathogen during stressful conditions, supported by a reduction of plasma cortisol levels, an up-regulation of several immune-related genes at 48 h and an inversion of the previously observed stress-induced T-cell suppression. Finally, the involvement of tryptophan catabolism in macrophages was confirmed by the up-regulation of genes involved in the kynurenine pathway. The present study brings new insights regarding the immune modulatory role of tryptophan during stressful conditions in fish, thus allowing the development of novel prophylactic protocols during vaccination by intraperitoneal injection in the European seabass.

Keywords: tryptophan; innate immunity; aquaculture; functional diets; stress.

1. Introduction

Nowadays it is widely accepted that incorporation of functional ingredients in aquafeeds represents an efficient farming tool that combines the desired immune modulatory properties without neglecting the correct nutritional provisions required for fish normal physiological needs. In this context, it is of utmost importance to unveil the nutraceutical potential of tryptophan in the quest for the definition of new strategies to empower fish in the mitigation of the non-ideal production conditions. Recent works have put into evidence the immune tolerance role of tryptophan dietary supplementation in fish, displaying its potential in the self-restrain mechanisms that compose the immune and stress responses. Machado *et al.* [1] showed that European seabass (*Dicentrarchus labrax*) fed for 14 days a tryptophan supplemented diet and then submitted to an inflammatory in-

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sult, presented a decreased cellular response and dropped plasma nitric oxide and bactericidal activity, compared to fish fed a control diet. In another study, Machado *et al.* [2] showed that dietary tryptophan clearly displayed a role in the attenuation of the immune response inhibiting, once again, the inflammatory mechanisms, and to some degree decreasing seabass disease resistance to *Photobacterium damsela* subsp. *piscicida* (Phdp). In fact, Machado *et al.* [3] in vitro observations pointed to a more direct role of tryptophan in the limitation of self-damage in inflammatory scenarios by the modulation of pathways associated to the assemblage of anti-inflammatory machinery and induction of macrophages polarization towards an anti-inflammatory phenotype. Moreover, a scenario of dietary tryptophan deficiency may equally represent an handicap for the European seabass, since it may lead to a rise in plasma cortisol levels in response to infection which may explain the observed compromised immune cell response and disease resistance to Phdp [2].

Tryptophan has also recognized roles in the neuroendocrine system and therefore works as a player in the neuroendocrine-immune axis. Lepage *et al.* [4] [5] worked on the topic and observed that rainbow trout (*Oncorhynchus mykiss*) fed for 1 week a diet supplemented with tryptophan (up to 4x above the control diet) undergoes different outcomes according to their stress state, with serotonin (5-HT) acting either as a trigger or inhibitor of the hypothalamic-pituitary-interrenal axis. Taking into account that fish showed elevated plasma cortisol levels in response to stress, tryptophan-supplemented feed led to a significant reduction in this stress-induced elevation of plasma cortisol. In contrast, tryptophan-supplemented feed offered to undisturbed fish resulted in a slight elevation of plasma cortisol levels. In accordance, in an attempt to explore the links between immune and neuroendocrine response Azeredo *et al.* [6] observed that an increase in dietary tryptophan (i.e. 4x more tryptophan than the control diet) was beneficial in chronically stressed (i.e. high stocking density) Senegalese sole (*Solea senegalensis*) fed for 38 days. However, undisturbed individuals showed a compromised immune status and lower disease resistance in the latter study.

The previously cited outcomes of dietary tryptophan trust on its role as precursor of compounds responsible for the modulation of stress coping mechanisms, and antioxidant and immune mechanisms [7]. In macrophages, tryptophan catabolism is mediated by indoleamine 2,3-dioxygenase (IDO), which catalyses the first and rate-limiting step of the tryptophan catabolism along the kynurenine pathway. In fact, IDO directly relies on the availability of tryptophan and its transcription is induced by inflammatory stimuli as interferon- γ and cytokines [8; 9]. Afterwards, tryptophan catabolic pathway is responsible for exerting anti-microbial effects, by tryptophan depletion from the extracellular environment, reducing its availability to microorganisms. Moreover, during the kynurenine pathway the formation of specific metabolites as 3-hydroxykynurenine, 3-hydroxyanthranilic acid and quinolinic acid regulate T-cell function, and the same metabolites set up a protector system acting locally to the removal of superoxide radicals modulating oxidative status, thus creating conditions that favour immune suppression and tolerance [8; 10]. Similarly, since both immune and stress responses share common pathways, effector organs and signalling molecules [11], leucocytes are also sensitive to a wide repertoire of other neuroendocrine effectors, such as glucocorticoids. Consequently, important stress signalling molecules, as cortisol, can have profound effects on the fish immune response by influencing the balanced successive secretion of pro- and anti-inflammatory cytokines [12; 13; 14]. Actually, tryptophan works as precursor of relevant functional molecules at the neuroendocrine level. 5-HT is produced from tryptophan by the rate-limiting enzyme tryptophan hydroxylase [15]. This monoamine, which can simultaneously function as a neurotransmitter in the central nervous system and a paracrine or endocrine signal in the gut and blood [16; 17], is able to either induce or inhibit the production of adrenocorticotrophic hormone (ACTH) in the pituitary. ACTH is then responsible for the increase or decrease of interrenal cortisol synthesis [4].

Considering all previous studies, it could be inferred that the consumption needs and physiological fate of tryptophan seems to be modulated according to both physiological status of the individuals (e.g. stress response) and the progress of inflammation. Therefore, and having into account the bi-directional and dependent relationship of both neuro-endocrine and immune responses, dietary tryptophan supplementation may represent a tool in the mitigation of stressful conditions during current farming practices, and consequently avoid unfavourable stress-inducing immune suppressor effects upon an infection episode or an inflammatory inducer. Therefore, the present work intends to study the ability of dietary tryptophan supplementation to modulate the immune response to an acute inflammatory stimulus in European seabass juveniles reared under stressful conditions (i.e. high stocking density).

2. Results

2.1 Response to stress

Exposure to high stocking density related-stress led to a decrease in the haematocrit levels, while increased haemoglobin and both MCH and MCHC ratios were observed (Table 1). In addition, stressed individuals presented lower mc2r mRNA levels than those left undisturbed regardless feed regiment and sampling time (Table S1).

2.2 Inflammatory response

The response to inflammation was perceived looking to the differences found in time regardless any other factor but also in the differences found in time in undisturbed groups (Ø) alone and Ø groups fed exclusively the CTRL diet.

Inflammation alone was responsible for the decrease of peripheral leucocytes numbers, from 48 h to 72 h, but also for the increase of the MCHC from 24 h to 48 h, as well as peripheral monocytes at 4h and total peritoneal leucocytes from 24 h to 48h (Tables 1 and 2). In addition, the number of neutrophils was found increased 4 h after infection while returning to the pre-infection values after 48 h. Plasma cortisol levels reached a peak at 4 and 24 h after infection (Table 3). Gut SOD, CAT and peroxidase activities were progressively reduced by the inflammation development (Table 4). A general up-regulation of most analysed genes in the head-kidney was found in response to inflammation, reaching a peak at 48 h with an overall reestablishment of the initial expression values at the sampling point 72 h (Figure 1 and Table S1). Also, the mRNA expression of *cxc4* (Figure 1- C) was found higher at 0 and 4 h compared to the last sampling points, 48 and 72 h, while *il1β* (Figure 1- D) showed a higher expression at 4 h compared to all the sampling points.

2.3 Stress effect on the inflammatory response

The stress outcome on the inflammatory response was assessed having into account the response over time displayed by stressed fish regardless the feeding regime or fish fed exclusively the CTRL diet. Also, a look was taken at differences displayed between Ø and stressed groups fed CTRL at a specific sampling point.

When fish were reared during 15 days in stressful rearing conditions and the inflammatory mechanisms initiated, a decrease of the haematological indicators, HG, MCH and MCHC (Table 1) as well as gut SOD, CAT and tGSH concentration (Table 4) were observed. In fact, in a pre-inflammation status, the abovementioned haematological parameters were found increased by stress alone (section 2.1), which was not observed in undisturbed individuals upon inflammatory induction. Moreover, leucocytes dynamics upon infection were also found modulated by the previous stress rearing conditions. Circulating total WBC and neutrophils concentrations were found increased at 24 h and 4 h, respectively, in stressed fish when compared to Ø (Tables 2). Curiously, while neutrophils numbers in undisturbed animals rapidly increase in response to inflammation, the number of neutrophils and lymphocytes found in circulation promptly diminished at 4 h in stressed fish, while restoring their initial values in the following sampling times (Table 2).

Regardless diet or in fish fed the control feed (CTRL) regime, stress induced a fast up-regulation (mostly found at early sampling points) of several pro-inflammatory genes (Fig. 1 and Table S1) compared the last sampling points, 48 and 72h. Most importantly, when comparing the response of fish reared under stressful conditions to the undisturbed (Ø) counterparts, when the immune mechanisms were activated, a down-regulation of *tnfa* (Fig. 1- B), *cd8a* (Fig. 1- E), *cd3zeta* (Fig. 1- H), *tcrα* (Fig. 1- I), *afmid* (Fig. 1- K) and *ido2* (Fig. 1- L) at 48 h was observed. In addition, *tgfa* (Fig. 1- A) was found up-regulated by stress at 4 and 24h. On the other hand, high stocking density led to the up-regulation of *tgfb* (Fig. 1- A), *il1β* (Table S1), *cd8β* (Fig. 1- F) and *afmid* (Fig. 1- K) at 4 h, *tgfb* (Fig. 1- A) and *tnfa* (Fig. 1- B) at 24h and *tgfb* (Fig. 1- A) and *cd8β* (Fig. 1- F) at 48 h.

2.4 Tryptophan dietary supplementation

A look into tryptophan dietary supplementation effect in the absence of any other factor was taken. In addition, tryptophan effect during an inflammatory insult in the absence (Ø) and presence of stress was observed.

Regardless stress condition and inflammatory status, tryptophan dietary supplementation was responsible for a reduction in plasma bactericidal activity (Table 3). Moreover, irrespective to stress, 4 h after infection with *Phdp* fish fed TRP presented lower MCV (Table 1) and gut SOD concentration (Table 4) than CTRL-fed fish. Head-kidney gene expression showed that a down-regulation of *il1β* (Table S1) at 4 h and *cd8a* (Fig 1.- E) at 48h following infection in fish fed TRP, while presenting an higher expression of *tnfa* (Fig. 1- B) at 24 h than CTRL-fed fish. Moreover, fish-fed TRP under stressful rearing conditions reduced cortisol 4h after inflammation (Table 3) while displaying a general up-regulation of genes as *tgfb* (Fig. 1- A), *cxc4* (Fig. 1- C), *cd8a* (Fig. 1- E), *cd8β* (Fig. 1- F), *cd3zeta* (Fig. 1- H), *tcrα* (Fig. 1- I), *afmid* (Fig. 1- K) and *ido2* (Fig. 1- L) at 48 h compared to CTRL-fed individuals. Finally, the expression of *tgfb* (Fig. 1- A) and *tnfa* (Fig. 1- B) at 24 h and *cd8β* (Fig. 1- F) at 4 h were found decreased compared to CTRL-fed counterparts.

178 **Table 1.** Haematological parameters of European seabass reared at high density, fed dietary treatments for 15 days (0h), and sampled 4, 24, 48 and 72 hours
179 post bacterial challenge.

Parameters		Dietary treatments									
		CTRL									
		Ø					Stress				
		0h	4h	24h	48h	72h	0h	4h	24h	48h	72h
RBC	($\times 10^6 \mu\text{l}^{-1}$)	1.66 $\pm$ 0.20	1.35 $\pm$ 0.35	1.45 $\pm$ 0.29	1.73 $\pm$ 0.12	1.74 $\pm$ 0.28	1.33 $\pm$ 0.19	1.64 $\pm$ 0.50	1.72 $\pm$ 0.32	1.83 $\pm$ 0.25	1.74 $\pm$ 0.13
HT	(%)	18.17 $\pm$ 2.73	21.50 $\pm$ 2.36	23.50 $\pm$ 3.95	21.17 $\pm$ 1.95	19.33 $\pm$ 4.23	12.80 $\pm$ 1.17	21.50 $\pm$ 3.59	21.33 $\pm$ 5.28	20.17 $\pm$ 3.89	16.83 $\pm$ 1.21
HG	(g dl ⁻¹)	0.20 $\pm$ 0.10	0.09 $\pm$ 0.02	0.24 $\pm$ 0.09	0.30 $\pm$ 0.09	0.11 $\pm$ 0.04	0.69 $\pm$ 0.41	0.09 $\pm$ 0.02	0.08 $\pm$ 0.02	0.34 $\pm$ 0.11	0.16 $\pm$ 0.08
MCH	(pg cell ⁻¹)	1.20 $\pm$ 0.64	0.79 $\pm$ 0.37	1.66 $\pm$ 0.57	1.73 $\pm$ 0.57	0.67 $\pm$ 0.25	5.35 $\pm$ 3.38	0.62 $\pm$ 0.18	0.46 $\pm$ 0.13	1.97 $\pm$ 0.80	0.90 $\pm$ 0.41
MCV	(μm^3)	110.01 $\pm$ 12.03	169.65 $\pm$ 42.08	164.65 $\pm$ 25.36	102.34 $\pm$ 45.65	110.77 $\pm$ 13.03	100.93 $\pm$ 7.72	140.30 $\pm$ 41.05	123.60 $\pm$ 14.99	110.83 $\pm$ 15.77	97.43 $\pm$ 11.64
MCHC	(g 100 ml ⁻¹)	1.11 $\pm$ 0.60	0.45 $\pm$ 0.12	1.05 $\pm$ 0.40	4.04 $\pm$ 5.60	0.64 $\pm$ 0.35	5.22 $\pm$ 3.54	0.44 $\pm$ 0.09	0.39 $\pm$ 0.12	1.76 $\pm$ 0.62	1.00 $\pm$ 0.51

Parameters		Dietary treatments									
		TRP									
		Ø					Stress				
		0h	4h	24h	48h	72h	0h	4h	24h	48h	72h
RBC	($\times 10^6 \mu\text{l}^{-1}$)	1.57 $\pm$ 0.26	1.77 $\pm$ 0.39	2.06 $\pm$ 0.68	1.71 $\pm$ 0.31	1.84 $\pm$ 0.33	1.50 $\pm$ 0.25	1.55 $\pm$ 0.40	1.72 $\pm$ 0.42	1.86 $\pm$ 0.26	1.66 $\pm$ 0.40
HT	(%)	18.83 $\pm$ 5.01	19.00 $\pm$ 1.91	21.33 $\pm$ 4.19	20.83 $\pm$ 2.81	17.40 $\pm$ 2.58	18.33 $\pm$ 1.37	18.83 $\pm$ 5.18	18.67 $\pm$ 3.50	19.40 $\pm$ 1.62	14.67 $\pm$ 1.11
HG	(g dl ⁻¹)	0.31 $\pm$ 0.06	0.11 $\pm$ 0.03	0.23 $\pm$ 0.04	0.29 $\pm$ 0.08	0.21 $\pm$ 0.23	0.66 $\pm$ 0.46	0.10 $\pm$ 0.02	0.12 $\pm$ 0.07	0.29 $\pm$ 0.06	0.24 $\pm$ 0.11
MCH	(pg cell ⁻¹)	1.98 $\pm$ 0.33	0.67 $\pm$ 0.22	1.26 $\pm$ 0.45	1.59 $\pm$ 0.78	1.16 $\pm$ 1.17	4.61 $\pm$ 2.26	0.65 $\pm$ 0.18	0.68 $\pm$ 0.36	1.57 $\pm$ 0.33	1.53 $\pm$ 0.79
MCV	(μm^3)	120.77 $\pm$ 28.73	111.42 $\pm$ 22.61	109.38 $\pm$ 23.21	116.02 $\pm$ 6.09	97.10 $\pm$ 8.77	124.52 $\pm$ 17.00	124.97 $\pm$ 38.85	113.97 $\pm$ 29.44	107.95 $\pm$ 13.53	92.83 $\pm$ 21.66
MCHC	(g 100 ml ⁻¹)	1.72 $\pm$ 0.42	0.61 $\pm$ 0.22	1.17 $\pm$ 0.45	1.86 $\pm$ 0.60	0.67 $\pm$ 0.28	3.53 $\pm$ 2.40	0.56 $\pm$ 0.22	0.65 $\pm$ 0.41	1.55 $\pm$ 0.34	1.66 $\pm$ 0.83

Multifactorial ANOVA

	Diet	Stress	Time	Diet x Stress	Diet x Time	Stress x Time	Diet x Stress X Time	Time				
								0h	4h	24h	48h	72h
RBC	ns	ns	ns	ns	ns	ns	ns					
HT	ns	0.048	0.006	ns	0.035	ns	ns	B	AB	A	AB	B
HG	ns	ns	<0.001	ns	ns	<0.001	ns	A	C	C	B	BC
MCH	ns	0.045	<0.001	ns	ns	<0.001	ns	A	C	B	B	BC
MCV	0.032	ns	<0.001	ns	0.004	ns	ns	ABC	A	AB	B	C
MCHC	ns	ns	<0.001	ns	ns	0.001	ns	A	C	C	B	BC

Diet x Time											Stress x Time									
CTRL					TRP						Ø					Stress				
0h	4h	24h	48h	72h	0h	4h	24h	48h	72h		0h	4h	24h	48h	72h	0h	4h	24h	48h	72h
HT	B	A	A	AB	AB															
HG											#					A*	B	B	B	B
MCH											#					A*	B	B	B	B
MCV	B	aA	A	B	B				b											
MCHC											AB#	AB	B	A	AB	A*	B	B	B	B

Values represent means ± SD (n = 10). Different symbols stand for statistically significant differences attributed to stress. Capital letters stand for statistically significant differences attributed to sampling time. Low case letters stand for significant differences attributed to dietary treatment (Multifactorial ANOVA; Tukey post-hoc test; ns: non-significant; P ≤ 0.05). RBC (Red blood cells-erythrocytes); Ht (Haematocrit); Hg (Haemoglobin); MCH (Mean corpuscular haemoglobin); MCV (Mean corpuscular volume); MCHC (Mean corpuscular haemoglobin concentration).

Table 2. Peripheral and peritoneal leucocyte counts of European seabass reared at high density, fed dietary treatments for 15 days (0h), and sampled 4, 24, 48 and 72 hours post bacterial challenge.

Parameters	Dietary treatments									
	CTRL									
	Ø					Stress				
	0h	4h	24h	48h	72h	0h	4h	24h	48h	72h
WBC	5.02 ± 0.88	4.13 ± 1.00	4.53 ± 0.92	5.03 ± 0.96	4.02 ± 0.42	3.80 ± 1.00	4.50 ± 0.93	4.85 ± 0.69	4.73 ± 0.46	4.83 ± 1.32
Peripheral Neutrophils	0.03 ± 0.03	0.34 ± 0.17	0.40 ± 0.05	0.09 ± 0.05	0.05 ± 0.04	0.04 ± 0.05	0.50 ± 0.20	0.30 ± 0.10	0.05 ± 0.02	0.06 ± 0.05
Peripheral Monocytes	0.09 ± 0.02	0.18 ± 0.11	0.08 ± 0.05	0.25 ± 0.13	0.11 ± 0.06	0.05 ± 0.03	0.21 ± 0.11	0.18 ± 0.10	0.14 ± 0.04	0.21 ± 0.19
Peripheral Lymphocytes	1.46 ± 0.44	0.83 ± 0.24	1.40 ± 0.35	1.31 ± 0.44	1.08 ± 0.26	1.19 ± 0.27	0.53 ± 0.20	1.16 ± 0.47	1.53 ± 0.35	1.54 ± 0.44
Peripheral Thrombocytes	3.51 ± 0.67	3.08 ± 0.85	2.70 ± 0.60	3.39 ± 0.64	2.79 ± 0.47	2.76 ± 0.78	3.30 ± 0.71	3.36 ± 0.31	2.85 ± 0.35	3.04 ± 0.97
Peritoneal Leucocytes	-	5.77 ± 4.51	4.40 ± 2.15	7.88 ± 3.97	6.68 ± 2.16	-	6.58 ± 4.22	4.87 ± 1.23	7.00 ± 2.33	6.25 ± 2.11

Parameters	Dietary treatments									
	TRP									
	Ø					Stress				
	0h	4h	24h	48h	72h	0h	4h	24h	48h	72h
WBC	4.37 ± 1.31	3.93 ± 0.82	4.02 ± 0.86	4.57 ± 0.63	3.95 ± 0.42	5.08 ± 0.89	4.42 ± 1.46	5.53 ± 1.03	3.33 ± 0.61	4.73 ± 0.83
Peripheral Neutrophils	0.03 ± 0.03	0.22 ± 0.14	0.40 ± 0.19	0.09 ± 0.03	0.07 ± 0.10	0.12 ± 0.14	0.49 ± 0.30	0.45 ± 0.20	0.05 ± 0.03	0.03 ± 0.02
Peripheral Monocytes	0.12 ± 0.06	0.21 ± 0.12	0.07 ± 0.03	0.13 ± 0.03	0.14 ± 0.07	0.14 ± 0.14	0.16 ± 0.10	0.18 ± 0.08	0.14 ± 0.05	0.15 ± 0.05
Peripheral Lymphocytes	1.39 ± 0.49	0.92 ± 0.65	1.34 ± 0.27	1.42 ± 0.36	1.21 ± 0.28	1.35 ± 0.36	0.57 ± 0.28	1.48 ± 0.47	0.99 ± 0.19	1.53 ± 0.44
Peripheral Thrombocytes	2.85 ± 0.84	3.11 ± 0.66	2.28 ± 0.52	2.31 ± 0.55	2.53 ± 0.37	3.49 ± 0.64	3.18 ± 0.94	3.50 ± 0.88	2.17 ± 0.52	3.08 ± 0.60
Peritoneal Leucocytes	-	5.60 ± 4.36	5.25 ± 2.69	7.38 ± 6.71	7.53 ± 3.27	-	6.82 ± 3.71	5.20 ± 1.24	7.15 ± 3.16	8.37 ± 3.71

Multifactorial ANOVA

	Diet	Stress	Time	Diet x Stress	Diet x Time	Stress x Time	Diet x Stress X Time	Stress x Time														
								Time					Stress x Time									
													Ø					Stress				
								0h	4h	24h	48h	72h	0h	4h	24h	48h	72h	0h	4h	24h	48h	72h
WBC	ns	ns	ns	ns	ns	0.02	ns						AB	AB	AB#	A	B	AB	AB	A*	B	AB
Peripheral Neutrophils	ns	ns	<0.001	ns	ns	0.011	ns	B	A	A	B	B	B	A#	A	B	B	A	B*	B	A	A
Peripheral Monocytes	ns	ns	0.025	ns	ns	ns	ns	B	A	AB	AB	AB										
Peripheral Lymphocytes	ns	ns	<0.001	ns	ns	0.048	ns	A	B	A	A	A					A	B	A	A	A	
Peripheral Thrombocytes	ns	ns	ns	ns	ns	ns	ns															
Peritoneal Leucocytes	ns	ns	0.045	ns	ns	ns	ns	-	AB	B	A	AB										

Values represent means ± SD (n = 10). Different symbols stand for statistically significant differences attributed to stress. Capital letters stand for statistically significant differences attributed to sampling time. (Multifactorial ANOVA; Tukey post-hoc test; ns: non-significant; P ≤ 0.05).

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Table 3. Plasma humoral parameters of European seabass reared at high density, fed dietary treatments for 15 days (0h), and sampled 4, 24, 48 and 72 hours post bacterial challenge.

Parameters		Dietary treatments									
		CTRL									
		Ø					Stress				
		0h	4h	24h	48h	72h	0h	4h	24h	48h	72h
Peroxidase	(unit ml ⁻¹)	25.70 ± 0.00	39.71 ± 18.83	26.42 ± 23.86	44.30 ± 31.57	107.03 ± 50.21	89.27 ± 65.83	46.51 ± 5.59	70.31 ± 6.54	62.25 ± 31.51	76.80 ± 37.97
Bactericidal activity	(%)	20.48 ± 7.49	24.84 ± 8.98	21.20 ± 4.96	24.04 ± 7.42	16.12 ± 5.05	15.71 ± 8.84	27.53 ± 17.83	28.66 ± 9.61	23.48 ± 10.28	26.28 ± 18.85
Cortisol	(ng ml ⁻¹)	83.82 ± 12.13 B	157.52 ± 38.29 A	170.42 ± 30.29 A	119.35 ± 39.47 AB	68.36 ± 28.77 B	103.76 ± 15.91 AB	174.00 ± 28.08 aA	116.51 ± 19.67 AB	119.35 ± 36.04 AB	80.93 ± 16.47 B

Parameters		Dietary treatments									
		TRP									
		Ø					Stress				
		0h	4h	24h	48h	72h	0h	4h	24h	48h	72h
Peroxidase	(unit ml ⁻¹)	74.24 ± 46.42	42.68 ± 12.26	62.33 ± 30.93	69.64 ± 77.44	63.90 ± 48.50	28.10 ± 1.61	60.03 ± 29.00	82.57 ± 66.71	115.68 ± 69.28	64.74 ± 37.44
Bactericidal activity	(%)	25.26 ± 14.05	21.81 ± 9.09	22.00 ± 11.10	19.24 ± 6.39	13.67 ± 10.28	15.49 ± 7.01	19.46 ± 4.97	11.40 ± 13.65	22.61 ± 11.10	13.59 ± 4.82
Cortisol	(ng ml ⁻¹)	127.452 ± 42.81 AB	152.30 ± 26.32 AB	174.00 ± 30.76 A	80.93 ± 18.05 B	62.19 ± 27.96 B	74.87 ± 33.28	87.77 ± 17.47 b	116.51 ± 21.99	98.20 ± 24.74	48.72 ± 20.18

Multifactorial ANOVA

	Diet	Stress	Time	Diet x Stress	Diet x Time	Stress x Time	Diet x Stress x Time	Diet x Stress				Stress x Time									
								CTRL		TRP		Ø					Stress				
								Ø		Stress		Ø		Stress		Ø		Stress		Ø	
								Ø	Stress	Ø	Stress	0h	4h	24h	48h	72h	0h	4h	24h	48h	72h
Peroxidase	ns	ns	ns	ns	ns	ns	ns					B	B	B	AB	A					
Bactericidal activity	0.033	ns	ns	ns	ns	ns	ns														
Cortisol	ns	<0.001	ns	<0.001	ns	<0.001	0.003	b	a	a*	b#				*	*	A	AB	AB	AB#	B#

Values represent means ± SD (n = 10). Different symbols stand for statistically significant differences attributed to stress. Capital letters stand for statistically significant differences attributed to sampling time. Low case letters stand for significant differences attributed to dietary treatment (Multifactorial ANOVA; Tukey post-hoc test; ns: non-significant; P ≤ 0.05).

Table 4. Gut immune and oxidative stress parameters of European seabass reared at high density, fed dietary treatments for 15 days (0h), and sampled 4, 24, 48 and 72 hours post bacterial challenge.

Parameters		Dietary treatments									
		CTRL									
		Ø					Stress				
		0h	4h	24h	48h	72h	0h	4h	24h	48h	72h
SOD	(U mg protein ⁻¹)	42.50 ± 16.84	55.84 ± 29.61	15.38 ± 4.40	23.51 ± 9.56	17.80 ± 3.54	53.95 ± 35.15	57.60 ± 31.57	58.37 ± 28.88	24.37 ± 8.21	13.03 ± 6.39
CAT	(U mg protein ⁻¹)	57.68 ± 32.03	87.07 ± 35.31	29.76 ± 0.00	47.41 ± 16.13	40.62 ± 1.65	83.74 ± 17.14	107.67 ± 23.65	59.68 ± 6.75	37.31 ± 7.74	45.53 ± 10.19
Total GSH	(µM)	2321.68 ± 749.79 A	3497.65 ± 1490.63 A*	845.78 ± 296.25 B	226.61 ± 77.26 B	283.05 ± 81.95 B	2834.11 ± 790.40 A	1290.81 ± 648.55 B#	895.02 ± 347.87 B	357.92 ± 49.20 B	318.32 ± 86.37 B
Bactericidal Activity	(%)	58.76 ± 6.42	64.83 ± 7.06	66.50 ± 2.90	67.64 ± 1.70	66.59 ± 1.79	59.55 ± 5.18	62.37 ± 2.70	60.01 ± 6.70	68.35 ± 4.65	70.07 ± 3.13
Peroxidase	(unit ml ⁻¹)	28.13 ± 8.60	77.31 ± 78.10	65.66 ± 55.01	89.85 ± 47.17	23.83 ± 4.05	44.34 ± 15.57	56.56 ± 26.38	58.36 ± 34.05	66.72 ± 47.22	21.49 ± 7.53

Parameters		Dietary treatments									
		TRP									
		Ø					Stress				
		0h	4h	24h	48h	72h	0h	4h	24h	48h	72h
SOD	(U mg protein ⁻¹)	59.19 ± 23.38	21.52 ± 15.67	17.24 ± 9.07	36.20 ± 15.68	11.82 ± 8.48	44.55 ± 31.92	29.15 ± 10.78	30.65 ± 25.24	14.67 ± 7.66	11.85 ± 5.40
CAT	(U mg protein ⁻¹)	73.92 ± 18.86	68.06 ± 11.05	44.33 ± 8.15	60.96 ± 12.10	41.25 ± 10.45	66.95 ± 10.48	90.37 ± 32.22	63.27 ± 2.30	38.06 ± 4.43	31.68 ± 1.47
Total GSH	(µM)	2778.96 ± 305.04 A	2839.77 ± 846.36 A	753.84 ± 723.88 B	440.67 ± 108.38 B	354.49 ± 154.28 B	2013.46 ± 460.27 AB	2608.92 ± 1299.99 A	681.46 ± 221.56 BC	546.07 ± 46.62 BC	312.23 ± 170.54 C
Bactericidal Activity	(%)	60.43 ± 5.61 B	65.01 ± 2.65 AB	60.43 ± 3.52 AB	67.85 ± 1.72 AB	72.50 ± 2.45 A	66.91 ± 4.18 AB	58.59 ± 4.33 B	63.92 ± 1.39 AB	69.37 ± 3.09 A	66.91 ± 4.83 AB
Peroxidase	(unit ml ⁻¹)	54.70 ± 14.38	50.71 ± 31.48	44.33 ± 27.21	52.12 ± 27.78	31.24 ± 12.96	77.31 ± 45.37	69.84 ± 30.96	33.71 ± 16.23	64.88 ± 39.43	26.55 ± 7.58

Multifactorial ANOVA

				Diet x	Diet x	Stress x Time	Diet x	Time					Diet x Time										Stress x Time										
	Diet	Stress	Time	Stress	Time		Stress x Time	Time						CTRL					TRP					Ø					Stress				
									0h	4h	24h	48h	72h	0h	4h	24h	48h	72h	0h	4h	24h	48h	72h	0h	4h	24h	48h	72h	0h	4h	24h	48h	72h
SOD	0,037	ns	<0.001	ns	0,044	0,032	ns	A	B	BC	BC	C	A	AB*	ABC	B	C	#		A	AB	B	AB	B	A	B	B	BC	B				
CAT	ns	ns	<0.011	ns	ns	0,018	ns	AB	A	BC	C	C								AB	A	B	AB	B	A	A	BC	C	C				
Total GSH	ns	ns	<0.001	ns	ns	0,009	0,005	A	A	B	B	B								A	A*	B	B	B	A	A#	B	B	B				

Bactericidal Activity	ns	ns	<0.001	ns	ns	ns	0,021	B	B	B	A	A
Peroxidase	ns	ns	0,03	ns	ns	ns	ns	AB	A	AB	A	B

Values represent means ± SD (n = 10). Different symbols stand for statistically significant differences attributed to stress. Capital letters stand for statistically significant differences attributed to sampling time. Low case letters stand for significant differences attributed to dietary treatment (Multifactorial ANOVA; Tukey post-hoc test; ns: non-significant; $P \leq 0.05$).

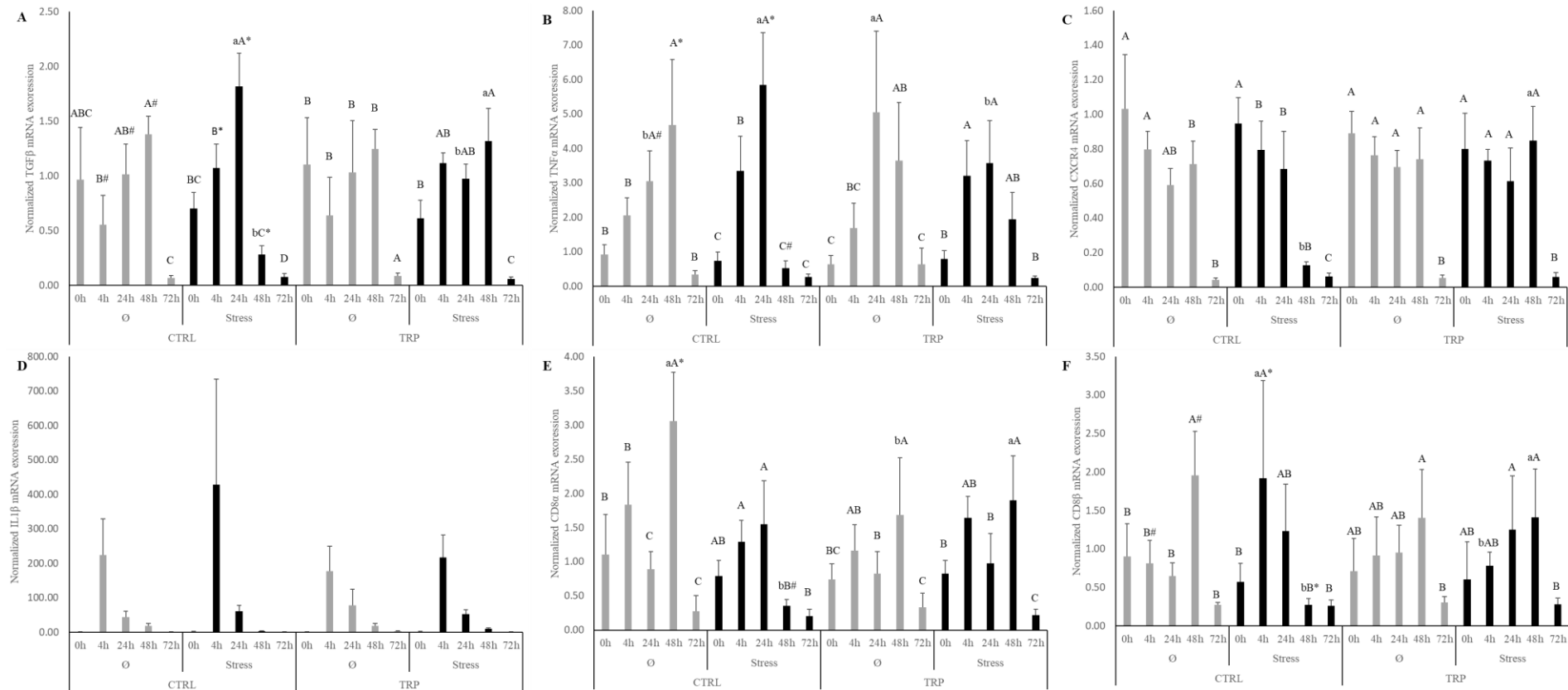


Figure 1. Quantitative expression of (A) Transforming growth factor-beta, (B) Tumor necrosis factor alpha, (C) Chemokine CXCR4, (D) Interleukin 1 beta, (E) Cluster of differentiation 8 alpha chain, (F) Cluster of differentiation 8 beta chain in head-kidney of European seabass reared at high density, fed dietary treatments for 15 days (0h), and sampled 4, 24, 48 and 72 hours post bacterial challenge. Values represent means $\pm$ SD (n = 10). Different symbols stand for statistically significant differences attributed to stress. Capital letters stand for statistically significant differences attributed to sampling time. Low case letters stand for significant differences attributed to dietary treatment (Multifactorial ANOVA; Tukey post-hoc test; ns: non-significant; $P \leq 0.05$).

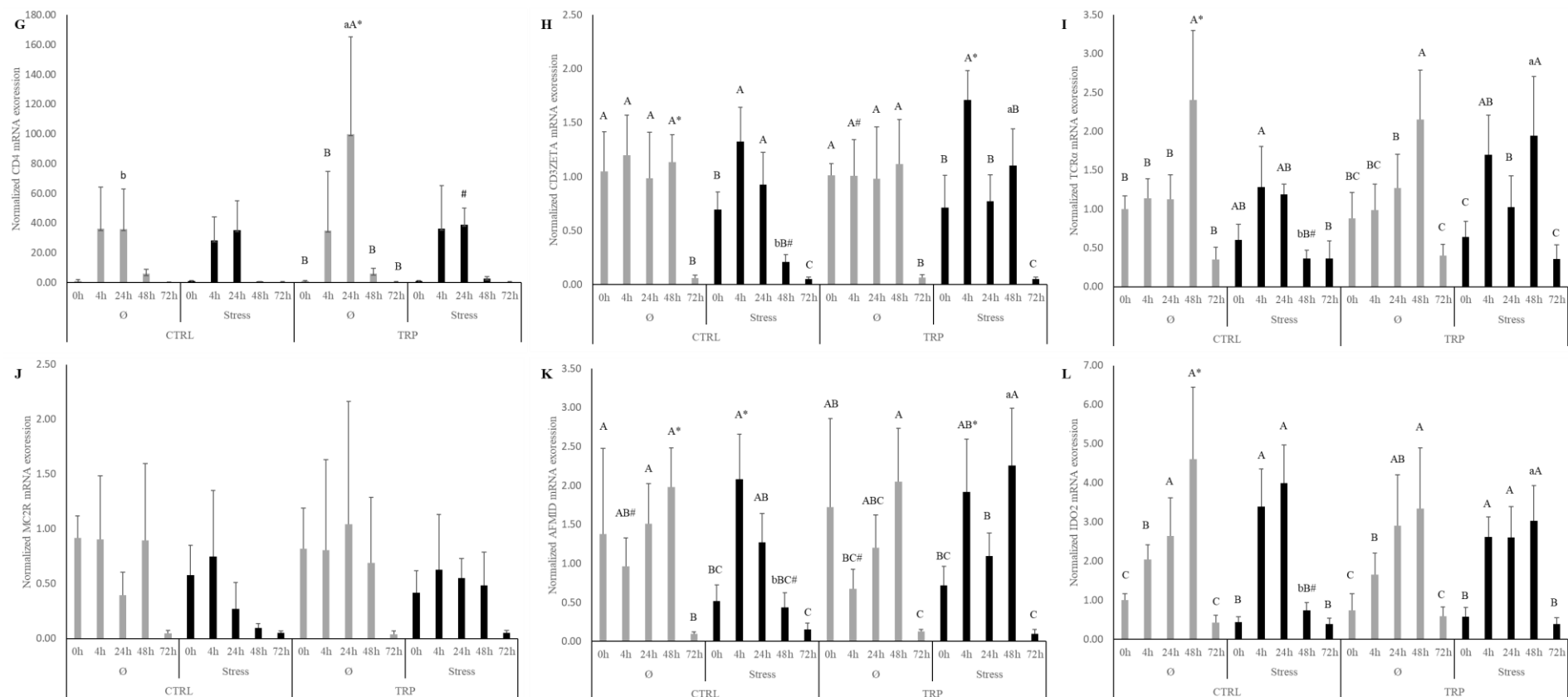


Figure 1 (continuation). Quantitative expression of (G) T-cell surface glycoprotein cd4, (H) T-cell surface glycoprotein cd3 zeta chain, (I) T cell receptor alpha chain, (J) Melanocortin 2 receptor, (K) Arylformamidase-like and (L) Indoleamine -dioxxygenase 2 in head-kidney of European seabass reared at high density, fed dietary treatments for 15 days (0h), and sampled 4, 24, 48 and 72 hours post bacterial challenge. Values represent means $\pm$ SD (n = 10). Different symbols stand for statistically significant differences attributed to stress. Capital letters stand for statistically significant differences attributed to sampling time. Low case letters stand for significant differences attributed to dietary treatment (Multifactorial ANOVA; Tukey post-hoc test; ns: non-significant; $P \leq 0.05$).



3. Discussion

Aiming to understand the outcome of dietary tryptophan supplementation in an interactive scenario of stressful rearing conditions and an activation of inflammatory mechanisms, European seabass juveniles reared at high stocking density and fed a diet supplemented with tryptophan for 15 days were infected with *Phdp*.

Primarily, stress condition alone, irrespective of dietary treatment and infection, did not significantly compromise fish health status, with the exception of a decrease of haematocrit and *mc2r* mRNA expression and an increase of haemoglobin concentration and corpuscular indexes. The melanocortin 2 receptor (*mc2r*) codes an ACTH receptor that regulates synthesis and release of glucocorticoids in response to ACTH release and therefore is essential for fish stress response. However, the observed down-regulation of the gene in response to stress could be the result of a feedback response to an adaptation to the prolonged stress [18]. In fact, cortisol levels tended to be higher in stressed European seabass compared to undisturbed groups at the sampling time 0h, i.e. after a 15 days exposure to high-density conditions. Therefore, as suggested by Agulleiro *et al.* [18], chronic dietary cortisol may directly result in a decreased MC2R expression as an upstream control mechanism of cortisol release.

Differently, immune stimulation itself induced a clear inflammatory response as could be expected, transversally characterized by a promptly enhanced neutrophil and monocyte response (4 h), followed by an increase of the concentration of total leucocytes found in the peritoneal cavity (from 24 to 48 h). In fact, plasma cortisol levels, as well as the expression of the pro-inflammatory cytokine *il1 β* , were found augmented by inflammation right after infection (i.e. 4 and 24 h). Later, the inflammatory process was characterized by the up-regulation of numerous inflammatory genes at 48 h, as the regulatory cytokine *tgfb*, the acute-phase cytokine *tnfa*, both T-cell surface-glycoproteins *cd8 α* and β , and T-cell receptors *cd3zeta* and *tcra*. The observed modulation of the peripheral and local response to the infection with *Phdp* is in line with several previous reports [2; 19; 20; 21; 22], that characterize the acute response to inflammation as a fast neutrophilia and monocytosis within the first 24 h of infection together with the up-regulation of pro-inflammatory genes at the systemic level. Interestingly, intestinal oxidative stress appeared diminished as gathered by decreased antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and total glutathione (tGSH) activity. This lower activity of antioxidant enzymes at gut level might be the reflection of a shift in resources allocation to the inflammatory focus [23], the peritoneal cavity, supported by a sturdy response at the peripheral (blood and head-kidney genes) and local (peritoneal leucocyte) levels.

Nonetheless, it is worth mention that in the present study stressful rearing conditions seemed to exacerbate the inflammatory response. Despite the lower haemoglobin, haemoglobin corpuscular indexes, and activity of the antioxidant enzymes SOD, CAT and tGSH in the gut compared to non-stressed individuals, stressed fish showed a higher activity of several inflammatory indicators. Contrarily to what would be expected in chronically stressed animals (15 days exposure to high stocking density conditions), no clear innate immune suppression due to the rise of cortisol [7] levels seems to have been taken place. In fact, stressed seabass clearly displayed a profile resembling the one normally found in acute-stressed fish [24]. A significant higher concentration of peripheral total leucocytes and neutrophils was found in stressed fish at the early stage of the inflammation (4 and 24 h). In addition, key inflammatory genes such as *il1 β* and *tgfb* (at 4 h), *tnfa* (at 24 h) and *cd8 α* and β (at 48 h) were found highly expressed in stressed European seabass compared to non-stressed groups. This points to a probable enhancement of the inflammatory response by stress, both at the leucocyte and transcriptional levels. Nonetheless, stressed fish presented a significant lower expression of *tnfa*, *cd8 α* , *cd3zeta* and *tcra* than undisturbed fish at a late sampling point (48 h). This may in contrast suggest a certain degree of immune suppressing effect of stress, mostly on T-cell surface-glycoproteins receptors. In fact, some authors have observed not only a down regulation of lymphocytes surface

molecules essential for their activation, but also a restrain in their tissue distribution while the same was not observed in phagocytic cells (neutrophils and monocytes/macrophages) [25; 26]. The disparity of the observed results could be the outcome of several factors such as the type and severity of the stressor used [24; 27], despite the prolonged time of exposure applied in the present study (15 days).

The main goal of the present work was to assess whether dietary tryptophan supplementation during stressful conditions could be of advantage in the event of an infection.

Exclusively from tryptophan dietary supplementation, apart from a decreased plasma bactericidal activity, no further changes were observed. Such inability of dietary tryptophan per se, to induce significant changes in fish immune mechanisms, was previously demonstrated in Persian sturgeon (*Acipenser persicus*) by Hoseini *et al.* [28] and in seabass [1; 2] after a 15 days feeding period. Indeed, most of the attributed immune-related effects of dietary tryptophan were described as dependent on immune stimulation and associated with the induction of the IDO-related kynurenine pathway [1; 2; 8; 9; 28]. In agreement, a small number of immunosuppressive effects were observed in fish fed a higher tryptophan dose in response to inflammation. A decrease of MCV, gut SOD activity and down-regulation of the pro-inflammatory cytokine *il1 β* and T-cell glycoprotein *cd8 α* in the head-kidney were observed. Nonetheless, *tnfa* expression was found up regulated at 24 h in response to infection. In a previous study, dietary tryptophan offered 26 % above the requirement level led to a weaker peripheral and local leucocyte response, to a down-regulation of key immune-related genes and to a concomitant decrease in disease resistance to a bacterial infection (*Phdp*) [2]. Engelsma *et al.* [29] also described a similar effect in common carp (*Cyprinus carpio*). Despite shy, the outcome of the inflammatory response observed in the present study is in agreement with the premise that tryptophan may prime immune suppression by promoting immune-tolerance signals during inflammation [2; 3; 29; 30]. Upon inflammatory induction, tryptophan catabolism in macrophages through the kynurenine pathway contributes to a reduction of its availability for microorganisms consumption, deviates superoxide radicals for the modulation of cell oxidative status and regulates T-cell function, thus creating conditions that favour immune suppression and tolerance [8; 10]. In addition, tryptophan supplementation may contribute to immune tolerance by promoting macrophage differentiation towards the M2/healing phenotype in an inflammatory scenario [3]. Nonetheless, no modulation of either kynurenine-related genes, as the *afmid* and *ido2* were observed in response to tryptophan supplementation in an inflammatory scenario.

In contrast, considering dietary tryptophan supplementation during stressful rearing conditions and its outcome in the inflammatory mechanisms, the present study showed an important reduction of plasma cortisol levels at 4 h of infection compared to CTRL-fed fish. In fact, despite non-significant, tryptophan seemed to induce a reduction in basal cortisol levels in response to stress even prior to the inflammatory stimulus (0 h). This is in agreement with previous reports that point to the ability of tryptophan to counteract stress-induced cortisol increase [4] and concomitantly avoid cortisol immune suppressor effect [2; 7]. This hypothesis is further supported by the up-regulation of several immune-related genes at 48 h following infection. At that sampling point an increased mRNA expression of *tgfb β* , *cxc4*, *cd8 α* and $-\beta$, *cd3zeta* and *tcra* was observed, highlighting a less restrained response. TGF β is a multifunctional cytokine produced by leucocytes and with key roles in several cellular process, while CXCR4 is a membrane protein present in leucocytes that binds to the CXC chemokines family. Moreover, contrarily with the previous observed immune suppressing effect at 48 h of stress, tryptophan spare seems to revert stress-induced T-cell suppression by increasing gene expression of the T-cell surface-glycoproteins and receptors (*cd8 α* and $-\beta$, *cd3zeta*, *tcra*). However, it is described that only about 10 % of total L-tryptophan is consumed through the neuro-endocrine routes [31]. Indeed, in the present experimental conditions an up-regulation of *ido2* and *afmid* was visible at 48 h, pointing to the involvement of the tryptophan catabolic pathway in macrophages mediated by IDO. IDO2 is the first and rate-limiting enzyme of tryptophan catabolism in macrophages, followed by the enzyme arylformamidase (AFMID) responsible

for kynurenine formation. Through this route, tryptophan consumption sets up a protection system mostly characterized by its immune suppressive effect and role during the immune response [3; 8; 10]. Nonetheless, a down regulation of *tnfa*, *cd8β* and *tgfb* at earlier sampling points was perceived.

To conclude, and despite of the main goal of the present study, the multiplicity of factors as stress, inflammation and tryptophan per se, as well as their interaction cannot be ignored. To begin with, stressful rearing conditions alone did not significantly compromise seabass health indicators apart from the expectation of a decreased MC2R expression possibly as control mechanism of the prolonged cortisol release. In contrast, immune stimulation itself induced, as expected, a clear inflammatory response at the peripheral (blood and head-kidney genes) and local (peritoneal leucocyte) levels. However, no clear immune suppression was perceived when chronically stressed fish (15 days under high stocking density conditions) were submitted to an acute inflammation. Contrarily, stress seemed to exacerbate the inflammatory response at early sampling points (up to 24 h). Nonetheless, stressed fish presented some immune suppressing effects mostly on T-cell surface-glycoproteins receptors at a late sampling point (48 h).

Finally, while tryptophan alone failed to modulate seabass immune indicators, a small number of immunosuppressive effects were observed in response to inflammation. Such results are in agreement with the premise that tryptophan may prime immune suppression by promoting immune-tolerance signals during inflammation. Nonetheless, when stress is a present factor, dietary tryptophan supplementation seems to improve the imposed inflammatory response by mitigating stress effects since a clear reduction of plasma cortisol levels is promptly observed in TRP-fed fish. This ability of tryptophan to counteract stress-induced cortisol increase, is further supported by the up-regulation of several immune-related genes at 48 h of infection, and by a reversal of the previously observed stress-induced T-cell suppression. Finally, the involvement of the tryptophan catabolic pathway in macrophages mediated by IDO was confirmed by the up-regulation of genes involved in the kynurenine pathway. Data from the present study could be useful for the development of novel prophylactic protocols during vaccination by intraperitoneal injection in the European seabass, since fish are crowded prior *i.p.* injection and an improved immune response during inflammation could enhance vaccination efficiency.

4. Materials and Methods

4.1 Experimental diets

Two plant protein-based diets with inclusion of fish soluble protein concentrate (5 %) for better palatability were formulated and manufactured by Sparos Lda. (Olhão, Portugal). The CTRL diet was formulated to include an indispensable amino acid (AA) profile meeting the ideal pattern established for European seabass [18]. According to previous works [1; 2; 19], an identical diet was formulated to contain L-tryptophan at 0.3 % of feed at the expenses of wheat meal (TRP). All diets were manufactured as described by Machado *et al.* [32]. Dietary AA content was analysed by ultra-high performance liquid chromatography (UPLC) after acid hydrolysis, as described in Aragão *et al.* [33]. Tryptophan was determined by UPLC, after alkaline hydrolysis (6 M NaOH and methanesulfonic acid at 110 °C over 22 h in nitrogen-flushed glass vials). Formulation and proximate analysis is presented in Table 5 whereas the total AA profile of the experimental diets is presented in Table 6.

Table 5. Ingredients and chemical composition of the experimental diets.

Ingredients (%)	CTRL	TRP
Fish soluble protein concentrate ^a	5.00	5.00
Fish gelatin ^b	2.00	2.00
Soy protein concentrate ^c	25.00	25.00
Pea protein concentrate ^d	6.00	6.00
Wheat gluten ^e	10.00	10.00
Corn gluten meal ^f	15.00	15.00
Wheat meal ^g	15.80	15.50
Fish oil ^h	15.10	15.10
Vit & Min Premix ⁱ	1.00	1.00
Soy lecithin ^j	1.00	1.00
Antioxidant ^k	0.20	0.20
Sodium propionate ^l	0.10	0.10
Monocalcium phosphate ^m	3.00	3.00
L-Lysine ⁿ	0.60	0.60
L-Tryptophan^o	0.00	0.30
DL-Methionine ^p	0.20	0.20
Total	100.00	100.00
Chemical composition (as fed basis)		
Crude protein (%)	45.70	46.00
Crude fat (%)	18.00	18.00
Fiber (%)	1.70	1.70
Starch (%)	13.40	13.20
Ash (%)	6.80	6.80
Energy (MJ/kg)	21.90	21.90

^a CPSP 90: 82.6% crude protein (CP), 9.6% crude fat (CF), Sopropêche, France. ^b Fish gelatin: 88% CP, 0.1% CF, LAPI Gelatine SPA, Italy. ^c Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands; ^d NUTRALYS F85F: 78% CP, 1% CF, ROQUETTE Frères, France. ^e VITAL: 83.7% CP, 1.6% CF, ROQUETTE Frères, France. ^f Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal. ^g Wheat meal: 10.2% CP; 1.2% CF, Casa Lanchinha, Portugal. ^h SAVINOR UTS, Portugal. ⁱ PREMIX Lda, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate, 7.5 mg; sodium chloride, 400 mg; excipient wheat middlings. ^j Lecico P700IPM, LECICO GmbH, Germany. ^k Paramex PX, Kemin Europe NV, Belgium. ^l PREMIX Lda., Portugal. ^m MCP: 22% phosphorus, 16% calcium, Fositalia, Italy. ⁿ Lysine HCl 99%, Ajinomoto Eurolysine SAS, France. ^o L-Tryptophan 98%, Ajinomoto Eurolysine SAS, France. ^p DL-Methionine for Aquaculture: 99% Methionine, Evonik Nutrition & Care GmbH, Germany

Table 6. Amino acid composition of experimental diets.

Amino acids (% DW)	CTRL	TRP
Arginine	4.0	4.1
Histidine	1.2	1.2
Lysine	3.0	3.0
Threonine	1.8	1.9
Isoleucine	2.1	2.1
Leucine	4.2	4.3
Valine	2.3	2.3
Methionine	1.1	1.2
Phenylalanine	3.0	3.2
Cysteine	0.4	0.4
Tyrosine	2.6	2.8
Aspartic acid	3.4	3.2
Glutamic acid	9.7	9.1
Alanine	2.4	2.5
Glycine	2.7	2.7
Proline	3.7	3.6
Serine	2.6	2.6
Tryptophan	0.2	0.4

4.2 *Phdp* inoculum preparation

For the inflammatory bacterial challenge, *Phdp* strain PP3, isolated from yellowtail (*Seriola quinqueradiata*; Japan) by Dr Andrew C. Barnes (Marine Laboratory, Aberdeen, UK), was used. Bacteria were routinely cultured at 22 °C in tryptic soy broth (TSB) or tryptic soy agar (TSA) (both from Difco Laboratories) supplemented with NaCl to a final concentration of 2 % (w/v) (TSB-2 and TSA-2, respectively) and stored at -80 °C in TSB-2 supplemented with 15 % (v/v) glycerol. To prepare the inoculum for injection into the fish peritoneal cavities, 100 µL of stocked bacteria were cultured overnight at 22 °C on TSA-2. Exponentially growing bacteria were collected and re-suspended in sterile TSB-2 and adjusted to a final concentration of 5×10^7 colony forming units (cfu) ml⁻¹, as confirmed by plating the resulting cultures onto TSA-2 plates and counting of cfu, and each fish inoculated intraperitoneally (*i.p.*) with 100 µl (5×10^6 cfu per fish) of the bacterial suspension.

4.3 Experimental design

European seabass juveniles (12.02 ± 2.77 g) were randomly distributed (n= 22-22 per tank) in two independent recirculating seawater systems composed by a total of 16 tanks (Figure 2), at a density of 5 kg m⁻³ and maintained for a 2 weeks acclimatization period (temperature $20 \pm 0.5^\circ\text{C}$; salinity 32 ppt; photoperiod 10:14 hours dark: light). Afterwards, by lowering the water level in one of the systems, fish were submitted to a higher density (10 kg m⁻³; Stress group) to simulate a stressful rearing condition while the other system served as control for the stocking density factor (5 kg m⁻³; Ø group). In a complete randomized design (Figure 2 serves only as example of diet distribution), the two dietary treatments (CTRL and TRP) were randomly assigned in quadruplicate tanks in each recirculating seawater system and fish were fed these diets for 15 days twice a day with a daily

average ration of 2 % of body weight. Samplings were then carried out (n= 2-3 fish per tank, n= 10 per treatment) for the collection of data regarding the haematological profile, peritoneal cells collection, plasma cortisol and immune parameters. In addition, gut samples were collected for innate immune and oxidative stress analysis. Finally, head-kidney was sampled for gene expression analysis. Afterwards, the remaining fish were intraperitoneally (*i.p.*) injected with 100 μ L of Phdp (5×10^7 cfu mL⁻¹). At that point, temperature was increased up to 24 ± 0.5 °C to mimic the natural infection conditions and, similarly to the previous point, fish were sampled (n= 2-3 fish per tank, n= 10 per treatment) after 4, 24, 48 and 72 hours. For all the sampling points fish were euthanized by anaesthetic overdose with 2-phenoxyethanol (1 mL L⁻¹, Merck KGaA).

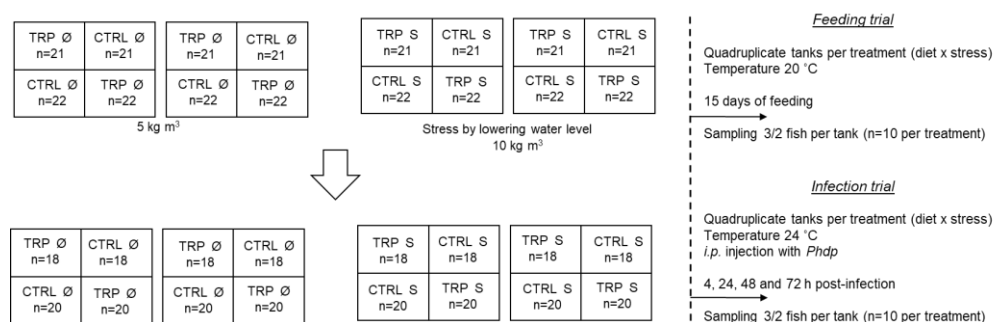


Figure 2. Trial design.

4.4 Haematological parameters

Blood was collected from the caudal vein using heparinized syringes, one part being used for haematological analysis and the remaining centrifuged at $10,000 \times g$ 10 min at 4 °C and the plasma collected, frozen in dry ice and stored at -80 °C for evaluating innate humoral immune response parameters.

The haematological profile was conducted according to Machado *et al.* [1] and comprised total peripheral leucocytes (WBC) and erythrocytes (RBC) counts, as well as haematocrit (HT) and haemoglobin (HG; SPINREACT kit, ref. 1001230, Spain) assessments. Afterwards, the mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were also calculated. Immediately after blood collection, blood smears were performed from homogenized blood and air-dried. After fixation with formol-ethanol (10 of 37 % formaldehyde in absolute ethanol), detection of peroxidase was carried out as described by Afonso *et al.* [19] in order to allow identification of neutrophils. Blood smears were then stained with Wright's stain (Haemacolor; Merck). Slides were examined ($\times 1,000$ magnification), and at least 200 leucocytes were counted and classified as peripheral thrombocytes, lymphocytes, monocytes and neutrophils. Absolute value ($\times 10^4$ mL⁻¹) of each cell type calculated according to the total blood WBC count.

4.5 Peritoneal leucocytes counting

Peritoneal cells were collected according to the procedure described by Costas *et al.* [34] in individuals *i.p.* injected with the bacteria. Briefly, following fish anaesthesia and blood collection from the caudal vein, 5 mL of cold Hank's balanced salt solution (HBSS) supplemented with 30 units heparin mL⁻¹ was injected into the peritoneal cavity. The peritoneal area was then slightly massaged in order to disperse peritoneal cells in the injected HBSS. The *i.p.* injected HBSS containing suspended cells were collected and total peritoneal leucocytes counts were performed with a haemocytometer.

4.6 Plasma innate immune response parameters

Total peroxidase activity in plasma was measured following the procedure described by Quade and Roth [35]. The bactericidal activity assay was performed using Phdp strain PP3. Bacteria were cultured in tryptic soy broth (TSB) (Difco Laboratories) supplemented

with NaCl to a final concentration of 2 % (w/v) (TSB-2) and exponentially growing bacteria were resuspended in sterile HBSS and adjusted to 1×10^6 cfu mL⁻¹. Plating serial dilutions of the suspensions onto TSA-2 plates and counting the number of cfu following incubation at 22 °C confirmed bacterial concentration of the inoculum. Plasma bactericidal activity was then determined following the method described by Graham *et al.* [36] with modifications [1].

Cortisol was assessed by an ELISA Kit (IBL International GmbH, Hamburg, Germany) already validated for European seabass [37], and following manufacturer's instructions. All analyses were conducted in triplicates and absorbance was read in a Synergy HT (Biotek) microplate reader.

4.7 Gut innate immune and oxidative stress parameters

Seabass **gut samples** were weighed and homogenized individually as described Peixoto *et al.* [38].

Total protein concentration in gut was measured using a Pierce BCA Protein Assay Kit as described by Costas *et al.* [39]. Reduced/oxidized glutathione ratio was measured using a Microplate Assay for GSH/GSSG Kit, as described by Hamre *et al.* [40]. Catalase (CAT) activity was determined by measuring the decrease of hydrogen peroxide (H₂O₂) concentration as described by Rodrigues *et al.* [41]. Superoxide dismutase (SOD) activity was determined following Lima *et al.* [42]. Gut bactericidal activity were determined following the method described by Graham *et al.* [36] with modifications [38] using *Tenacibaculum maritimum* strain ACC13.1. Peroxidase activity was analysed according to Quade and Roth [35].

All analyses were conducted in triplicates and absorbance was read in a Synergy HT (Biotek) microplate reader.

4.8 Head-kidney gene expression

Total RNA isolation and DNase treatment (NZY Total RNA isolation kit, MB13402, NZYTech, Portugal) and first-strand cDNA synthesis (NZY First-strand cDNA synthesis kit, MB125, NZYTech, Portugal) were performed according to manufacture guidelines. Primers design and efficiency values and quantitative PCR assays were performed as described by Machado *et al.* [20]. Efficiency of each primer was calculated according to a series dilution of a cDNA pool. DNA amplification was carried out using in a CFX384 Touch Real-Time PCR Detection System (Biorad) with specific primers for genes that have been selected for their involvement in immune responses (Table 3). Accession number, efficiency values, annealing temperature, product length and primers sequences are presented in Table 3. Melting curve analysis was also performed to verify that no primer dimers were amplified. The standard cycling conditions were 95 °C initial denaturation for 10 min, followed by 40 cycles of 94 °C denaturation for 30 s, primer annealing temperature (Table 7) for 30 s and 72 °C extension for 30 s. All reactions were carried out as technical duplicates. The expression of the target genes was normalized using the expression of European seabass 40s ribosomal protein (40s) and elongation factor 1 alpha (ef1α).



Table 7. Forward and reverse primers for real-time PCR.

Acronym	Gene	Gene Bank ID	Eff ¹	AT ²	Product length ³	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
<i>40s</i>	40s Ribosomal protein	HE978789.1	104.44	60	79	TGATTGTGACAGACCCTCGTG	CACAGAGCAATGGTGGGGAT
<i>ef1α</i>	Elongation factor 1 β	AJ866727.1	96.45	57	144	AACTTCAACGCCCAGGTCAT	CTTCTTGCCAGAACGACGGT
<i>tgfb</i>	Transforming growth factor β	AM421619.1	109.67	55	143	ACCTACATCTGGAACGCTGA	TGTTGCCTGCCCACATAGTAG
<i>tnfa</i>	Tumor necrosis factor α	DQ070246.1	108.81	55	112	AGCCACAGGATCTGGAGCTA	GTCCGCTTCTGTAGCTGTCC
<i>cxcr4</i>	Chemokine CXC receptor 4	FN687464.1	90.94	57	171	ACCAGACCTTGTGTTTGCCA	ATGAAGCCCACCAGGATGTG
<i>il1β</i>	Interleukin 1 β	AJ269472.1	87.60	57	105	AGCGACATGGTGCGATTCT	CTCCTCTGCTGTGCTGATGT
<i>cd8α</i>	Cluster of differentiation 8 α chain	DQ090838.1	117.69	60	292	GCGATTCTGGTGTCTTGTCTAAAG	GAGACGCACAGCTGTAAATGC
<i>cd8β</i>	Cluster of differentiation 8 β chain	DLAgn_00090370	104.44	55	223	CGGAACCCAAAAGGCCAAAG	TAGGCTGTAGATGCAGTGCT
<i>cd4</i>	T-cell surface glycoprotein cd4	DLAgn_00058650	98.04	60	131	CTCGGTGGCATCCTTCTGAC	AGCCAGACAACAACCTGTCC
<i>cd3zeta</i>	T-cell surface glycoprotein cd3 zeta chain	DLAgn_00052540	93.08	60	99	GGCTCTCCGAACCTTCTCCAG	CTGCAACGTCGTTTCCGATG
<i>tcra</i>	T- cell receptor α chain	DLAgn_00260540	116.00	60	72	ACACTGGCTGAGAAACATCCT	GGCTGGGTCACTCTGTCTTC
<i>mc2r</i>	Melanocortin 2 receptor	DLAgn_00065140	100.98	60	118	GAGGGCAAGGGGAGCATTTA	GACGGGCAGATGGCAGTTAT
<i>afmid</i>	Arylformamidase-like	DLAgn_00177950	128.26	55	112	CGTTTCCACCTGTTTGACCT	CCTAGCCTGCTGAAGGACTG
<i>ido2</i>	Indoleamine -dioxygenase 2	DLAgn_00014730	108.20	55	74	TGAAGGTGTGAGCAATGAGC	CAAAGCACTGAATGGCTGAA

¹The efficiency of PCR reactions calculated from serial dilutions of tissue RT reactions in the validation procedure.

²Annealing temperature (°C).

³Amplicon (bp)



4.9 Statistical analysis

All results are expressed as mean $\pm$ standard deviation (mean $\pm$ SD). Data were analyzed for normality (Shapiro-Wilk's W test) and homogeneity of variance (Levene's test) and, when necessary, transformed before being treated statistically. Data was analysed by multifactorial ANOVA, with diet, stress and time/inflammation as factors, and followed by Tukey post-hoc test to identify differences between the experimental treatments. All statistical analyses were performed using the computer package STATISTICA 12 for WINDOWS. The level of significance used was $p \leq 0.05$ for all statistical tests.

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