



Full length article

Dietary tryptophan supplementation induces a transient immune enhancement of gilthead seabream (*Sparus aurata*) juveniles fed fishmeal-free diets



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ABSTRACT

European aquaculture is an industry with a high sustainability profile contributing to the supply of safe seafood. However, several diseases can affect farmed fish and it is imperative to find alternatives for chemotherapeutic treatments when disease outbreaks occur. Maintenance of health through nutrition is well-established in modern animal farming, and amino acids (AA) are promising candidates as functional additives to improve fish health. Therefore, the goal of this research is to provide a better understanding of the influence of tryptophan supplementation on nutritional condition and immune mechanisms in fish. Triplicate groups of fish (13.3 ± 0.3 g) previously fed with a fishmeal-based diet were either fed a control diet with an extreme formulation (0% fishmeal) but meeting the AA requirements (CTRL), or the SUP diet, formulated as the CTRL with an increase in tryptophan (TRP) content. After 2 and 13 weeks of feeding, head-kidney (HK), liver (L) and white skeletal muscle (WSM) were collected for gene expression, whereas plasma was suited for humoral immune parameters. A holistic approach using transcriptomic, humoral and zootechnical parameters was undertaken. The expression of 29–31 genes for WSM, L or HK confirms an effect due to the treatment across time. A two-way ANOVA analysis revealed that 15–24 genes varied significantly depending on the tissue, and the multivariate analysis by means of PLS-DA explained (R^2) and predicted (Q^2) with four components up to 93% and 78% of total variance, respectively. Component 1 ($R^2 = 50.06\%$) represented the time effects, whereas components 2 (24.36%) and 3 (13.89%) grouped fish on the basis of dietary treatment, at early sampling. The HK results in particular suggest that fish fed SUP diet displayed an immunostimulated state at 2 weeks. No major differences were observed in plasma humoral parameters, despite an increase in antiprotease and peroxidase activities after 13 weeks regardless of dietary treatment. These results suggest that tryptophan supplementation may improve the seabream immune status after 2 weeks. Hence, the use of functional feeds is especially relevant during a short-term feeding period before a predictable stressful event or disease outbreak, considering that these putative advantageous effects seem to disappear after a 13 weeks feeding period.

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1. Introduction

A sustainable and profitable aquaculture relies on the production of healthy fish, which requires balanced feeds manufactured with high-quality ingredients. Research in last years has focused on the reduction of fish meal and fish oil use in aquafeeds, in order to mitigate the strong reliance of the aquaculture sector on these limited resources. In practice, plant-based ingredients are currently the most studied and immediately available alternatives to reduce the dependence of carnivorous species on marine fisheries resources [1–5], though many other types of ingredients are also increasingly considered (e.g. processed animal proteins, insect meals, algae and yeast biomasses). Thus, good practices in diet formulation are imperative, given the significant impact that nutrition can have on fish performance and health. Moreover, it is important to find feed ingredients and/or additives to compensate for the replacement of traditional marine feed ingredients with more sustainable unconventional feedstuffs [6], as this replacement sometimes brings fish health and welfare issues [7]. Such a fish health and welfare dimension in fish feeds formulation can also help to reduce even further the use of medicated feeds [8,9]. Although there is a wide range of studies involving the successful replacement of marine ingredients with alternative (usually plant-based) ingredients in gilthead seabream, most studies are focused on growth performance, with some drawback effects on welfare and immunity [10]. Still, important advances are expected in next years in the use of functional or fortified feeds. This term describes feeds that go beyond satisfying the minimum nutritional requirements, improving growth performance and overall health status and stress resistance of farmed animals [11]. This new concept in modern animal nutrition can be achieved through the inclusion of a wide range of possible ingredients, additives and purified nutrients (e.g., amino acids, prebiotics, probiotics, glucans, nucleotides, methyl donors, essential fatty acids).

Amino acids (AA) are fundamental nutrients in aquafeeds formulation, their importance going well beyond their role as the building blocks of proteins. AA are also functional compounds, since AA are precursors for the synthesis of other important biologically active molecules such as nitric oxide and polyamines being essential for reproduction, immunity and organism maintenance [12]. AA requirements may increase as consequence of stressful conditions (e.g. handling) and metabolic changes associated with inflammation and infection [7,13,14]. In fact, the dependence of the immune system on the availability of AA is related to their role as signalling molecules which are essential for cellular function [15]. For instance, AA have essential roles in higher vertebrates as they regulate i) activation of T- and B-lymphocytes, natural-killer cells and macrophages; ii) cellular redox stage, gene expression and lymphocyte proliferation; and iii) production of antibodies, cytokines and cytokine substances as nitric oxide (NO) and superoxide [16].

The importance of AA supplementation in aquafeeds is generally acknowledged, and AA such as lysine and methionine are routinely added to commercial diets to compensate their relatively low presence in e.g. most vegetable ingredients. Contrarily, supplementing other essential AA (such as tryptophan, threonine and histidine) when alternative ingredients are used is not as established as with lysine and methionine. As such, it is likely that the supply of these AA is sometimes compromised when low fish meal diets are used, leading to potential negative effects in terms of fish health.

Tryptophan (Trp), in particular, is an essential amino acid with recognized roles in both neuroendocrine and immune systems [17]. Trp is a precursor of bioactive molecules and neurotransmitters such as serotonin, kynurenine and melatonin. Products of tryptophan catabolism can enhance host immunity by inhibiting the production of superoxide, scavenging free radicals and minimizing the production of pro-inflammatory cytokines [17,18]. Nonetheless, the modulatory effects of Trp dietary supplementation on fish immune responses seems to depend on species, level of supplementation, time of administration and

prior stress condition [17].

The present study aimed to explore the effects of tryptophan supplementation on the health status and growth performance of gilthead seabream juveniles in the context of fish-meal free diets. Since there is a current need to gather deeper knowledge on the time-dependent modulatory effects of functional diets, the effects of experimental diets were assessed after short and long-term feeding periods.

2. Material and methods

2.1. Experimental diets

Two different diets were formulated and manufactured by SPAROS Lda. (Olhão, Portugal) to fulfil the known nutritional requirements of gilthead seabream juveniles: a control diet (CTRL) which contained 0% fishmeal, while meeting the estimated AA requirements for this species (five IAA were added to obtain balanced diet) [19,20], along with a supplemented diet (SUP), with the same basal formulation, but with an additional tryptophan supplement. The level of supplementation was chosen based on available data [7,15,17]. Main ingredients were ground (below 250 µm) in a Hosakawa micropulverizer hammer mill, model #1 (Hosokawa Micron Ltd., United Kingdom). Powdered ingredients were then mixed according to the target formulation in a TGC double-helix mixer, model 500L (TGC Extrusion, France), to attain a basal mixture (no oils were added at this stage). All diets were manufactured by extrusion (pellet size 2.0 mm) by means of a CLEXTRAL BC45 pilot-scale twin-screw extruder (Clextral, France) with a screw diameter of 55.5 mm and temperature ranging 105–110 °C. Upon extrusion, all batches of extruded feeds were dried in a convection oven (OP 750-EF, LTE Scientifics, United Kingdom) for 2 h at 60 °C. After this process, pellets were left to cool at room temperature, and subsequently the essential AA were mixed with the fish oil fraction according to the formulation and added under vacuum coating conditions in a Pegasus vacuum mixer (PG-10VCLAB, DINNISEN, The Netherlands) respective mixture. Throughout the duration of the trial, experimental feeds were stored at room temperature. Formulation and proximal composition of experimental diets are presented in Table 1. Proximate composition analysis was performed by the following methods: dry matter, by drying at 105 °C for 24 h; ash, by combustion at 550 °C for 12 h; crude protein (N × 6.25), by a flash combustion technique followed by gas chromatographic separation and thermal conductivity detection (LECO FP428); fat, after petroleum ether extraction, by the Soxhlet method; total phosphorus, according to the ISO/DIS 6491 method, using the vanado-molybdate reagent; gross energy, in an adiabatic bomb calorimeter (IKA).

Total AA content of diets was determined by hydrolysis in 6 M HCl at 116 °C for 2 h in nitrogen-flushed glass vials. Samples were then pre-column derivatised with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, USA). Analyses were done by ultra-high performance liquid chromatography in a Waters reverse-phase AA analysis system, using norvaline as an internal standard. During acid hydrolysis asparagine is converted to aspartate and glutamine to glutamate, so the reported values for these AA represent the sum of the respective amine and acid. The resultant peaks were analysed with EMPOWER software (Waters, USA). Tryptophan was independently determined by HPLC, after alkaline hydrolysis (Silliker Portugal, S.A.) The AA profiles of the experimental diets are presented in Table S1.

2.2. Fish and rearing facilities

The trial was conducted by trained scientists (following FELASA category C recommendations) and according to the European Economic Community animal experimentation guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE at the Ramalhete research station of CCMAR (University of

Table 1
Ingredients and proximate composition of the experimental diets.

Ingredients (%)	CTRL	SUP
Poultry meal 65 ^a	10.00	10.00
Soy protein concentrate ^b	14.00	14.00
Wheat gluten ^c	10.44	8.88
Corn gluten ^d	11.00	11.00
Guar korma 60 ^e	9.00	9.00
Soybean meal 48 ^f	15.20	15.20
Rapeseed meal ^g	3.00	3.00
Wheat meal ^h	5.50	5.40
Fish oil ⁱ	9.20	9.30
Soybean oil ^j	3.00	3.00
Rapeseed oil ^k	3.00	3.00
Vit & Min Premix ^l	1.00	1.00
Binder ^m	0.20	0.20
Antioxidant powder ⁿ	0.20	0.20
Sodium propionate ^o	0.10	0.10
Monocalcium phosphate ^p	3.00	3.00
L-Histidine ^q	0.30	1.00
L-Lysine ^r	1.20	1.20
L-Threonine ^s	0.25	0.95
L-Tryptophan ^t	0.11	0.27
DL-Methionine ^u	0.30	0.30
Proximate analyses (% Dry matter)		
Dry matter (DM, % as fed)	93.94	93.49
Crude protein (% DM)	49.88	50.56
Crude lipid (% DM)	20.70	18.40
Ash (% DM)	6.60	6.48
Gross Energy (MJ kg ⁻¹ DM)	21.88	21.97
AA composition (g AA 100 g⁻¹ CP)		
Tryptophan	0.60	0.93

- ^a Poultry meal: 65%CP, 14.4% CF, SAVINOR UTS, Portugal.
^b Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands.
^c VITAL: 83.7% CP, 1.6% CF, ROQUETTE Frères, France.
^d Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal.
^e Guar meal: KORFEED 60: 60.2% CP, 6.9% CF, India.
^f Soybean meal 48: Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, CARGILL, Spain.
^g Rapeseed meal: Defatted rapeseed meal: 37.7% CP, 2.3% CF, Premix Lda, Portugal.
^h Wheat meal: 10.2% CP; 1.2% CF, Casa Lanchinha, Portugal.
ⁱ Fish oil: SAVINOR UTS, Portugal.
^j Soybean oil: Henry Lamotte Oils GmbH, Germany.
^k Rapeseed oil: Henry Lamotte Oils GmbH, Germany.
^l Vitamin and mineral premix: 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 pantothenate, 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.
^m Binder: Kieselguhr (natural zeolite), LIGRANA GmbH, Germany.
ⁿ Antioxidant: Paramega PX, Kemin Europe NV, Belgium.
^o Sodium propionate: Disproquímica, Portugal.
^p Monocalcium phosphate: 22% P, 18% Ca, Fosfitalia, Italy.
^q L-Histidine: L-Histidine 98%, Ajinomoto Eurolysine SAS, France.
^r L-Lysine: L-Lysine HCl 99%, Ajinomoto Eurolysine SAS, France.
^s L-Threonine: ThreAMINO 98.5%, Evonik Nutrition & Care GmbH, Germany.
^t L-Tryptophan: TrypAMINO 98%, Evonik Nutrition & Care GmbH, Germany.
^u DL-Methionine: DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany.

Table 2

Body weight (BW, g fish⁻¹), relative growth rate (RGR), daily growth index (DGI), feed conversion ratio (FCR), feed efficiency (FE), voluntary feed intake (VFI) and protein efficiency ratio (PER) of gilthead seabream juveniles fed the experimental diets for 15 and 92 days. Values are expressed as mean ± SE (n = 3).

	15 Days		92 Days	
	CTRL	SUP	CTRL	SUP
BW	20.07 ± 0.97	20.65 ± 0.73	68.45 ± 4.46	64.30 ± 3.31
RGR (%)	2.78 ± 0.34	2.94 ± 0.35	1.80 ± 0.07	1.72 ± 0.07
DGI	2.32 ± 0.29	2.46 ± 0.30	1.87 ± 0.10	1.77 ± 0.09
FCR	1.52 ± 0.02	1.66 ± 0.17	1.41 ± 0.05	1.55 ± 0.12
FE	0.64 ± 0.04	0.67 ± 0.12	0.71 ± 0.03	0.65 ± 0.05
VFI	4.17 ± 0.21	4.21 ± 0.25	1.96 ± 0.02	2.10 ± 0.09
PER	1.38 ± 0.10	1.44 ± 0.24	1.55 ± 0.51	1.39 ± 0.10
Intake (g.Kg ABW⁻¹.days⁻¹)				
Dry matter	39.23 ± 2.14	39.44 ± 2.41	18.94 ± 0.15	20.20 ± 0.89
Protein	19.57 ± 1.06	19.94 ± 1.22	9.45 ± 0.75	10.21 ± 0.45
Lipids	8.64 ± 0.47	7.76 ± 0.47	4.17 ± 0.29	3.97 ± 0.18

Algarve, Portugal), from August to November 2016. Fish with an initial body weight of 13 g were randomly distributed in 1000 L tanks and fed a (fishmeal based) commercial diet for 3 weeks to ensure acclimation to the experimental conditions. Triplicate groups of 150 gilthead seabream per treatment were hand-fed *ad libitum* twice a day (except Sundays, when fish were fed once a day) each experimental diet (i.e. CTRL and SUP) for 92 days. Through the trial, fish were subjected to a natural temperature regime, which was logged every day (Suppl. Fig. 1). Seawater was supplied at 2 L/min (mean temperature 23 °C ± 2.6; mean salinity 34 ± 0.7 ppt) in a flow-through system (mean dissolved oxygen above 5 mg L⁻¹). All physical and chemical water parameters were evaluated daily during the experiment.

2.2.1. Experimental procedures

The growth trial was designed to have two sampling points (short and long-term feeding periods). The first stage of the feeding trial lasted 15 days, which was used to assess the effects of a short-term dietary supplementation with tryptophan on gilthead seabream growth- and health-related biomarkers. After a 24 h fasting period, 19 fish per tank were randomly selected and sacrificed with a tricaine methanesulfonate lethal dose (200 µg/L). All fish were sampled for mucus and blood collection. Briefly, skin mucus was collected by gentle scraping the fish dorso-lateral surface using a cell scraper with enough care to avoid contamination with blood, urogenital and intestinal excretions according to [21], snap frozen in liquid nitrogen and stored at -80 °C. Tissue samples (i.e. head kidney, liver and dorsal skeletal muscle) were also obtained from 16 of the sampled fish, snap frozen in liquid nitrogen, and stored at -80 °C until further analyses. The remaining three fish were stored at -20 °C for subsequent analysis to determine body proximal composition. At 36 and 70 days, intermediate biometric samplings of all fish were undertaken to evaluate growth performance, along with cleaning of all rearing tanks. At the end of the trial (92 days), and after a 24 h fasting period, 19 fish per tank were sampled as described for the 15 days sampling point.

2.3. Haematological procedures

Blood was collected from the caudal vein using heparinized syringes. The haematological profile consisted of total white (WBC) and red (RBC) blood cells counts, haematocrit (Ht) and haemoglobin (Hb; SPINREACT kit, ref. 1001230, Spain) as described by Machado et al. [22]. The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated as follows:

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

Table 3

Haematocrit, haemoglobin, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cells (RBC), white blood cells (WBC) and absolute values of peripheral blood leucocytes (thrombocytes, lymphocytes, monocytes and neutrophils) in gilthead seabream fed dietary treatments during 15 and 92 days. Values are expressed as means \pm SE (n = 9). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments.

Parameters	Dietary treatments				Two-way ANOVA		
	15 Days		92 Days		Time	Diet	Time x diet
	CTRL	SUP	CTRL	SUP			
Haematocrit (%)	31.00 \pm 1.40	34.77 \pm 0.68	36.36 \pm 1.27	37.08 \pm 1.36	< 0.005	0.722	0.418
Haemoglobin (g dl ⁻¹)	2.08 \pm 0.18	2.34 \pm 0.07	2.19 \pm 0.16	2.49 \pm 0.11	0.690	0.947	0.749
MCH (pg cell ⁻¹)	7.61 \pm 0.60	8.46 \pm 0.50	7.87 \pm 0.63	9.17 \pm 0.57	0.439	0.581	0.721
MCV (μm^3)	114.22 \pm 2.90	116.54 \pm 1.35	131.25 \pm 1.06	136.60 \pm 9.12	0.038	0.910	0.859
MCHC (g 100 ml ⁻¹)	6.63 \pm 0.33	6.79 \pm 0.34	5.99 \pm 0.32	6.97 \pm 0.27	0.364	0.713	0.331
RBC (x10 ⁶ μl)	2.75 \pm 0.14	3.05 \pm 0.11	2.84 \pm 0.17	2.75 \pm 0.15	0.462	0.284	0.205
WBC (x10 ⁴ μl)	6.66 \pm 0.90	5.80 \pm 0.19	5.15 \pm 0.47	4.99 \pm 0.37	0.437	0.212	0.286
Absolute peripheral blood leucocytes							
Thrombocytes (x10 ⁴ μl)	4.11 \pm 0.50	3.90 \pm 0.15	3.61 \pm 0.20	3.65 \pm 0.30	0.730	0.474	0.217
Lymphocytes (x10 ⁴ μl)	1.85 \pm 0.32	1.38 \pm 0.12	0.32 \pm 0.05	0.61 \pm 0.04	< 0.001	0.261	0.622
Monocytes (x10 ⁴ μl)	0.25 \pm 0.05	0.12 \pm 0.01	0.10 \pm 0.02	0.06 \pm 0.01	0.010	0.048	0.269
Neutrophils (x10 ⁴ μl)	0.32 \pm 0.06	0.37 \pm 0.07	0.83 \pm 0.09	0.66 \pm 0.08	< 0.001	0.637	0.168

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

$$\text{MCHC (g.100 mL}^{-1}\text{)} = (\text{Hb/Ht}) \times 100$$

Blood smears were performed right after blood collection, air dried and fixed with formol-ethanol (10% of 37% formaldehyde in absolute ethanol). Afterwards they were stained with Wright's stain (Hemacolor; Merck). Detection of peroxidase activity was carried out as described by Afonso et al. [23] to facilitate the identification of neutrophils. The slides were examined (1000 \times), and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes and neutrophils. The absolute amount ($\times 10^4 \mu\text{L}^{-1}$) of each cell type was calculated. The remaining blood was centrifuged at 8000 \times g during 8 min at room temperature and plasma was stored at -80°C until assayed.

2.4. Plasma cortisol and humoral immune parameters

Plasma cortisol levels were measured by means of a commercial enzyme-linked immunosorbent assay (ELISA) kit (RE52611 for human serum and saliva; IBL, Hamburg, Germany), as previously described by López-Olmeda et al. [24]. Plasma bactericidal activity was measured according to Graham et al. [25] adapted by Machado et al. [22].

Table 4

Plasma and mucus humoral parameters of gilthead seabream fed dietary treatments during 15 and 92 days.

Parameters	Dietary treatments				Two-way ANOVA		
	15 Days		92 Days		Time	Diet	Time x diet
	CTRL	SUP	CTRL	SUP			
Plasma							
Cortisol (ng/ml)	29.03 \pm 4.23	27.72 \pm 2.48	32.94 \pm 4.35	28.46 \pm 4.36	0.552	0.356	0.751
Bactericidal activity (%)	48.55 \pm 0.71	49.86 \pm 1.45	7.76 \pm 2.38	20.73 \pm 5.73	< 0.001	0.016	0.073
Protease activity (%)	5.37 \pm 0.16	5.18 \pm 0.13	5.31 \pm 0.16	5.43 \pm 0.20	0.167	0.957	0.352
Antiprotease activity (%)	93.26 \pm 0.29	93.48 \pm 0.37	97.76 \pm 0.10	97.48 \pm 0.07	< 0.001	0.962	0.403
Peroxidase activity (units/ml)	41.61 \pm 8.42	26.72 \pm 4.09	47.46 \pm 9.61	42.02 \pm 8.39	0.074	0.456	0.760
ACH50 (units/ml)	23.41 \pm 3.11	31.80 \pm 4.36	30.59 \pm 7.02	33.60 \pm 3.60	0.323	0.204	0.546
Nitric Oxide (μM)	123.33 \pm 16.60	89.04 \pm 14.96	49.55 \pm 4.63	51.38 \pm 9.89	< 0.001	0.454	0.173
Mucus							
Bactericidal activity (%)	33.38 \pm 3.02	33.51 \pm 7.51	35.65 \pm 1.33	35.09 \pm 1.18	0.439	0.931	0.889
Protease activity (%)	7.40 \pm 0.79	6.74 \pm 1.04	7.50 \pm 0.54	7.91 \pm 0.47	0.360	0.857	0.445
Peroxidase activity (units/ml)	26.05 \pm 8.20	23.40 \pm 7.03	11.60 \pm 2.50	16.71 \pm 2.67	0.022	0.782	0.386
ACH50 (units/ml)	2.43 \pm 0.56	2.85 \pm 0.43	3.55 \pm 0.14	2.77 \pm 0.21	0.130	0.599	0.081

Values are expressed as means \pm SE (n = 9). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments.

Photobacterium damsela subsp. *piscida* (*Phdp*), strain PP3 was used. The protease activity was determined using the azocasein hydrolysis assay according to the method of Guardiola et al. [21] with minor modifications. Briefly, 10 μL of plasma was incubated with 100 μL of sodium bicarbonate buffer (5 mg mL⁻¹ NaHCO₃, pH 8.3) and 125 μL of azocasein (20 mg mL⁻¹ in NaHCO₃, 5 mg mL⁻¹, pH 8.3) for 24 h at 22 $^\circ\text{C}$ in polystyrene microtubes with continuous shaking. The reaction was stopped by adding 10% trichloroacetic acid (TCA) (100 mg mL⁻¹) and the mixture centrifuged (6000 \times g, 5 min). Afterwards, 100 μL of the supernatant was transferred in duplicates to a 96-well plate that previously contained 100 μL of NaOH (40 mg mL⁻¹) per well. The OD was read at 450 nm in a Synergy HT microplate reader. Phosphate buffer was added to some wells instead of plasma and served as blank (0% of protease activity), whereas the reference sample was replaced by trypsin solution (5 mg mL⁻¹ in NaHCO₃, 5 mg mL⁻¹, pH 8.3) instead of plasma (100% activity). The percentage of inhibition of trypsin activity compared to the reference sample was calculated. The anti-protease activity was determined as described by Ellis [26] adapted by Machado et al. [22]. Total peroxidase activity in plasma was measured according to the procedures described by Quade and Roth [27]. Alternative complement pathway (ACP) activity was estimated as described by Oriol Sunyer and Tort [28] using horse red blood cells (HorRBC;

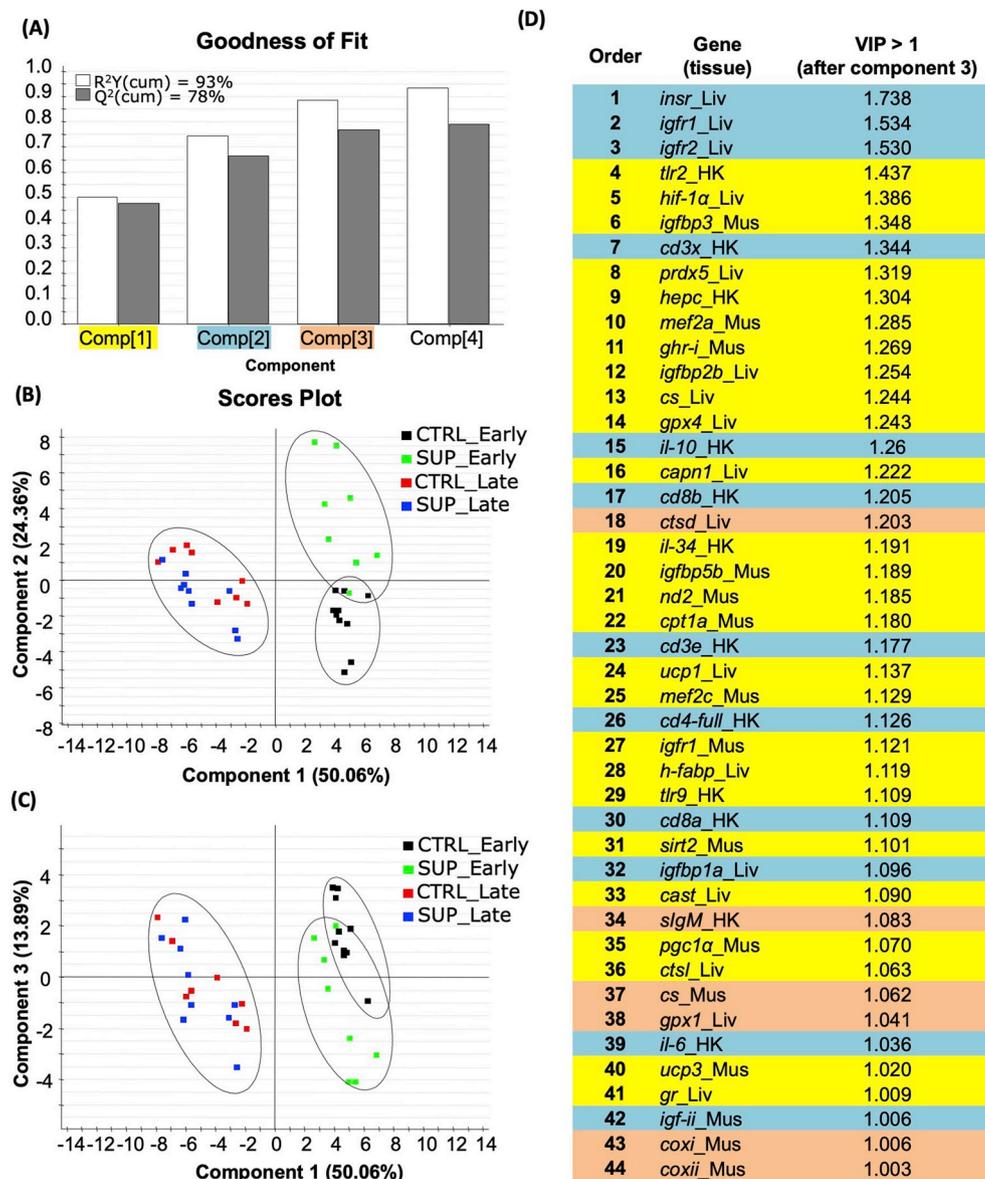


Fig. 1. Discriminant analysis (PLS-DA) of liver, muscle and head kidney molecular signatures of fish fed the experimental diets. Relative expression data of the 89 genes included in the array can be found on Tables 6–8. (A) Cumulative coefficients of goodness of fit (R^2 , white bars) and prediction (Q^2 , grey bars) by each component; the three first components explained 88.31% of total variance. (B and C) PLS-DA score plots of all biomarkers analysed in the three target tissues along the three main components. (D) Ordered list of markers by variable importance (VIP) in projection of PLS-DA model for group differentiation. Markers with VIP values > 1 after the first, second and third components are highlighted in yellow, blue and orange, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Probiologica Lda, Portugal) for ACP determination. Total plasma nitrite and nitrate content was measured using a Nitrate/Nitrite colorimetric kit (Roche Diagnostics GmbH, Mannheim, Germany) by adapting it to a 96-well plate and by following manufacturer's instructions. Since both these compounds are derivatives of endogenously produced NO, they are indicative of NO amount in plasma. Briefly, 10 μ L of plasma were diluted in 90 μ L of distilled water in duplicate and then 50 μ L of reduced nicotinamide adenine dinucleotide phosphate (NADPH) were added, followed by the addition of 4 μ L of nitrate reductase. A blank was produced by adding distilled water instead of plasma. Absorbance at 540 nm was read after 30 min incubation at 25 $^{\circ}$ C. Afterwards, 50 μ L of sulfanilamide and an equal volume of N-(1-naphthyl)-ethylenediamine dihydrochloride were added to each well. The mixture was allowed to stand at 25 $^{\circ}$ C for 15 min and absorbance was read at 540 nm. Total nitrite levels were calculated from a previously prepared sodium nitrite standard curve.

2.5. Gene expression analysis

Total RNA from target tissues (liver, head kidney and white skeletal muscle) was extracted with a MagMaxTM-96 for microarrays total RNA

isolation kit (Life Technologies, Carlsbad, CA, USA) after tissue homogenization in TRI reagent following manufacturers' instructions. RNA quantity and purity was determined by Nanodrop (Thermo Scientific) with absorbance ratios at 260 nm/280 nm of 1.9–2.1. Reverse transcription (RT) of 500 ng of total RNA was performed with random decamers using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) following manufacturers' instructions. RT reactions were incubated for 10 min at 25 $^{\circ}$ C and 2 h at 37 $^{\circ}$ C. Negative control reactions were run without reverse transcriptase.

Real-time quantitative PCR was carried out with the CFX96 ConnectTM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), using 96-well PCR array layouts designed for simultaneously profiling a panel of 31 genes for liver samples, and 29 genes for head kidney and muscle samples (summarized in Table 5). Overall, the genes comprised in the liver array covered different biological processes such as GH/IGF system (10), energy sensing and oxidative metabolism (4), respiration uncoupling (1), antioxidant defence and molecular chaperons (11) and cytoplasmic and lysosomal proteases (5). Transcripts analysed in muscle were associated with the GH/IGF system (10), energy sensing and oxidative metabolism (10), respiration uncoupling (1) and muscle growth and cell differentiation (8). Transcripts analysed in

Table 5
Genes included in the liver (†), head kidney (‡) and white muscle (#) pathway-focused PCR arrays.

Gene name/category	Symbol	Gene name/category	Symbol
<i>GH/IGF system</i>		<i>Muscle growth and cell differentiation</i>	
Growth hormone receptor I	<i>ghr-i^{†#}</i>	Myoblast determination protein 1	<i>myo1[#]</i>
Growth hormone receptor II	<i>ghr-ii^{†#}</i>	Myogenic factor MYOD2	<i>myo2[#]</i>
Insulin-like growth factor-I	<i>igf-i^{†#}</i>	Myogenic factor 5	<i>myf5[#]</i>
Insulin-like growth factor-II	<i>igf-ii^{†#}</i>	Myogenic factor 6	<i>myf6/mrf4/herculin[#]</i>
Insulin-like growth factor binding protein 1a	<i>igfbp1[†]</i>	Myostatin/Growth differentiation factor 8	<i>mstn/gdf-8[#]</i>
Insulin-like growth factor binding protein 2b	<i>igfbp2[†]</i>	Myocyte-specific enhancer factor 2A	<i>mef2a[#]</i>
Insulin-like growth factor binding protein 3	<i>igfbp3[#]</i>	Myocyte-specific enhancer factor 2C	<i>mef2c[#]</i>
Insulin-like growth factor binding protein 4	<i>igfbp4[†]</i>	Follistatin	<i>fst[#]</i>
Insulin-like growth factor binding protein 5b	<i>igfbp5b[#]</i>		
Insulin-like growth factor binding protein 6b	<i>igfbp6b[#]</i>	<i>Antioxidant defence and molecular chaperons</i>	
Insulin-like growth factor receptor I	<i>igfr1^{†#}</i>	Catalase	<i>cat[†]</i>
Insulin-like growth factor receptor II	<i>igfr2^{†#}</i>	Glutathione peroxidase 1	<i>gpx1[†]</i>
Insulin receptor	<i>insr^{†#}</i>	Glutathione peroxidase 4	<i>gpx4[†]</i>
		Glutathione reductase	<i>gr[†]</i>
<i>Energy sensing and oxidative metabolism</i>		Peroxioredoxin 3	<i>prdx3[†]</i>
Sirtuin 1	<i>sirt1[#]</i>	Peroxioredoxin 5	<i>prdx5[†]</i>
Sirtuin 2	<i>sirt2[#]</i>	Superoxide dismutase [Mn]	<i>Mn-sod/sod2[†]</i>
Sirtuin 5	<i>sirt5[#]</i>	Fatty acid binding protein, heart	<i>h-fabp[†]</i>
NADH-ubiquinone oxidoreductase chain 2	<i>nd2[#]</i>	Glucose-regulated protein, 170 kDa	<i>grp-170[†]</i>
NADH-ubiquinone oxidoreductase chain 5	<i>nd5[#]</i>	Glucose-regulated protein, 94 kDa	<i>grp-94[†]</i>
Cytochrome c oxidase subunit I	<i>coxi[#]</i>	70 kDa heat shock protein, mitochondrial	<i>mthsp70/grp-75/mortalin[†]</i>
Cytochrome c oxidase subunit II	<i>coxi[#]</i>		
Carnitine palmitoyltransferase 1A	<i>cpt1a^{†#}</i>	<i>Cytoplasmatic and lysosomal proteases</i>	
Citrate synthase	<i>cs^{†#}</i>	Calpain 1	<i>capn1[†]</i>
Proliferator-activated receptor gamma coactivator 1 alpha	<i>pgc1a^{†#}</i>	Calpastatin	<i>cast[†]</i>
Hypoxia inducible factor-1 alpha	<i>hif-1a[†]</i>	Cathepsin B	<i>ctsb[†]</i>
		Cathepsin D	<i>ctsd[†]</i>
		Cathepsin L	<i>ctsl[†]</i>
<i>Respiration uncoupling</i>		<i>Macrophages and monocytes chemokines</i>	
Uncoupling protein 1	<i>ucp1[†]</i>	Macrophage colony-stimulating factor 1 receptor 1	<i>csf1r1[‡]</i>
Uncoupling protein 3	<i>ucp3[#]</i>	C-C chemokine receptor type 3	<i>ccr3[‡]</i>
		C-C chemokine CK8/C-C motif chemokine 20	<i>ck8/ccl20[‡]</i>
<i>Interleukins and cytokines</i>		<i>Immunoglobulins</i>	
Interleukin-1 beta	<i>il-1β[‡]</i>	Immunoglobulin M	<i>IgM[‡]</i>
Interleukin-6	<i>il-6[‡]</i>	Immunoglobulin M membrane-bound form	<i>mIgM[‡]</i>
Interleukin-7	<i>il-7[‡]</i>	Immunoglobulin T	<i>IgT[‡]</i>
Interleukin-8	<i>il-8[‡]</i>	Immunoglobulin T membrane-bound form	<i>IgT-m[‡]</i>
Interleukin-10	<i>il-10[‡]</i>		
Interleukin 12 subunit beta	<i>il12[‡]</i>	<i>Antimicrobial peptide/Iron recycling</i>	
Interleukin-15	<i>il-15[‡]</i>	Hepcidin	<i>hepc[‡]</i>
Interleukin-34	<i>il-34[‡]</i>		
Tumor necrosis factor-alpha	<i>tnf a</i>	<i>Pattern recognition receptors</i>	
<i>Antiprotease</i>		Toll-like receptor 1	<i>tlr1[‡]</i>
Alpha-2-macroglobulin	<i>a2m[‡]</i>	Toll-like receptor 2	<i>tlr2[‡]</i>
		Toll-like receptor 5	<i>tlr5[‡]</i>
<i>T-cell markers</i>		Toll-like receptor 9	<i>tlr9[‡]</i>
Cluster of differentiation 3 epsilon chain	<i>cd3e[‡]</i>	Macrophage mannose receptor 1	<i>mrc1[‡]</i>
Cluster of differentiation 3 zeta chain	<i>cd3z[‡]</i>		
CD4-full	<i>cd4-full[‡]</i>		
Cluster of differentiation 8 alpha	<i>cd8a[‡]</i>		
Cluster of differentiation 8 beta	<i>cd8b[‡]</i>		
Zeta-chain-associated protein kinase 70	<i>zap70[‡]</i>		

the head kidney were interleukins and cytokines (9), macrophages and monocytes chemokines (3), immunoglobulins (4), antiprotease (1), antimicrobial peptide/iron recycling (1), T-cell markers (6) and pattern recognition receptors (5). Specific primer pair sequences are listed in

Sup. Table S3. Controls of general PCR performance were included on each array, being performed all the pipetting operations by means of the EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany). Briefly, RT reactions were diluted to convenient

concentrations and the equivalent of 660 pg of total input RNA was used in a 25 μ L volume for each PCR reaction. PCR-wells contained a 2 \times SYBR Green Master Mix (Bio-Rad) and specific primers at a final concentration of 0.9 μ M were used to obtain amplicons of 50–150 bp in length.

The program used for PCR amplification included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 60 s at 60 °C. The efficiency of PCR reactions was always higher between 90% and 100%, and negative controls without sample templates were routinely performed for each primer set. The specificity of reactions was verified by analysis of melting curves (ramping rates of 0.05 °C/sec over a temperature range of 55–95 °C), and linearity of serial dilutions of RT reactions. Fluorescence data acquired during the PCR extension phase were normalized using the delta–delta Ct method [29]. β -Actin was tested for gene expression stability using GeNorm software (M score = 0.21) and it was used as housekeeping gene in the normalization procedure. Fold-change calculations were done in reference to the mean response of CTRL fish. For comparing the mRNA gene expression level of a panel of genes in a given dietary treatment, all data values were in reference to the expression level of a specific gene in CTRL fish. In liver, gene expression was in reference to the expression level of *cpt1*, whereas in head kidney and white skeletal muscle was in reference of *il7* and *igfr2*, respectively, which were arbitrarily assigned a value of 1.

2.6. Calculations

Zootechnical performance measures were calculated as:

Feed conversion ratio (FCR) = apparent feed intake/weight gain, where wet weight gain is: FBW–IBW. FBW, final body weight; IBW, initial body weight.

- Daily growth index (DGI) = $100 \times ((\text{Final body weight})^{1/3} - (\text{Initial body weight})^{1/3})/\text{days}$
- Relative growth rate (RGR, % $\cdot\text{day}^{-1}$) = $(e^g - 1) \times 100$, where $g = (\ln(\text{Final body weight}) - \ln(\text{Initial body weight}))/\text{days}$
- Voluntary feed intake (VFI) = $100 \times \text{crude feed intake}/(\text{average body weight} \times \text{days})$, where ABW was calculated as: $(\text{IBW} + \text{FBW})/2$
- Protein efficiency ratio (PER) = $\text{weight gain}/\text{crude protein ingested}$
- Nutrient or energy gain (g or kJ $\cdot\text{kg ABW}^{-1}\cdot\text{day}^{-1}$) = $(\text{final carcass nutrient or energy content} - \text{initial carcass nutrient or energy content})/(\text{average body weight} \times \text{days})$, where ABW was calculated as: $(\text{IBW} + \text{FBW})/2$
- Nutrient retention (%) was calculated as: $100 \times \text{nutrient gain}/\text{nutrient intake}$.

2.7. Statistical analysis

Statistic evaluation of the data was accomplished by mixed effect ANOVA, using the SPSS statistical analysis software (SPSS ver. 23.0; Chicago, USA). The dependent variable was the corresponding response, the fixed factors were “treatment” and “sampling time” and the random factor was “tank” nested within treatment. A significance threshold of $p < 0.05$ was applied to all statistical tests. For data expressed in Table 2, a one way ANOVA Tukey HSD's post-test for pairwise comparisons between means of different groups were performed. All variables were checked for normality and homogeneity of variance, by using the Shapiro-Wilk and the Levene's test, respectively. For plasma ACH50, peroxidase, MCV, MCHC index, plasma bactericidal activity, nitric oxide, thrombocytes and monocyte counts, data were transformed by means of \log_{10} . For gene expression data, a \log_2 transformation was applied to all expression values. Unsupervised multivariate analysis by principle component analysis (PCA) was first performed on data as an unbiased statistical method to observe intrinsic

trends in the dataset, using EZ-INFO[®] v3.0 (Umetrics, Sweden). To achieve the maximum separation among the groups, supervised multivariate analysis by partial least-squares discriminant analysis (PLS-DA) was sequentially applied, using EZ-INFO[®] v3.0 (Umetrics, Sweden). Potential differential genes were selected according to the Variable Importance in the Projection (VIP) values. Variables with VIP > 1 were considered to be influential for the separation of samples in PLS-DA analysis [30–32].

3. Results

3.1. Growth performance

No differences were observed between diet groups in final body weight (FBW), daily growth index (DGI), relative growth rate (RGR) and voluntary feed intake (VFI) either after 15 or 92 days of feeding the experimental diets. Fish fed the SUP diet showed a tendency for higher feed conversion ratio (FCR) compared to those fed the CTRL diet, after 15 days of feeding, whereas this trend was not observed after the long-term feeding period (Table 2).

3.2. Haematological profile

In general, total WBC and RBC counts, as well as Hb, MCH and MCHC values, were similar between dietary treatments and sampling times, whereas Ht and MCV levels increased between first and final sampling points regardless of experimental diets (Table 3). While the concentration of peripheral thrombocytes remained unchanged throughout the trial, circulating lymphocytes, neutrophils and monocytes showed a decrease over time regardless of diet (Table 3). Moreover, peripheral monocyte concentration decreased in fish fed the SUP diet compared to those fed CTRL, regardless of feeding time.

3.3. Cortisol and immune parameters in plasma

Both plasma bactericidal activity and nitric oxide levels showed a decrease over time regardless of dietary treatments, whereas anti-protease activity showed the opposite pattern (Table 4). Cortisol, ACH50, peroxidase and protease values were not affected by either sampling point or dietary treatment.

3.4. Humoral innate immune parameters in mucus

Peroxidase activity decreased over time in skin mucus regardless of dietary treatments, whereas no differences were observed in ACH50, bactericidal and protease activities (Table 4).

3.5. Gene expression

Most of the analysed genes in liver (25 out of 31) showed differential expression (up- or down-regulated) between the two sampling points (Table 6). Tryptophan supplementation induced a down-regulation of *igfr1*, *igfr2* and *insr* after 15 days of feeding, but these differences were not maintained after long-term feeding. Significant differences were also observed in muscle gene expression at the two different sampling points (Table 7), with a clear trend of up-regulation of transcript levels (18 out of 21 differentially expressed genes) at 92 days after feeding. Regarding dietary effects, *igfbp3* expression was up-regulated in fish fed the SUP diet after the short-term feeding period, and *capn1* was down-regulated after 92 days of feeding the SUP diet. In head kidney tissue, 14 out of 29 genes were differentially expressed after 92 days of feeding regardless of dietary treatment (Table 8). Up-regulated genes were *il-34*, *ccr3*, *ck8/ccl20*, *thr9*, *sIgM*, *mIgM*, and *thr5*, whereas *il-1 β* , *il-8*, *sIgT*, *hepc* and *cd8b* were down-regulated after 92 days of feeding. Regarding a diet effect on health-related biomarkers in the head kidney, three transcripts (*cd3x*, *thr2* and *l-10*) were up-

Table 6
Hepatic gene expression in gilthead seabream fed dietary treatments during 15 and 92 days.

Biological Process	Gene symbol	15 Days		92 Days		Two-way ANOVA (p < 0.05)		
		CTRL	SUP	CTRL	SUP	Time	Diet	Time x diet
GH/IGF System	<i>ghr-i</i>	1.17 ± 0.22	1.37 ± 0.24	1.96 ± 0.18	2.31 ± 0.32	< 0.001	0.732	0.773
	<i>ghr-ii</i>	1.39 ± 0.11	1.46 ± 0.11	0.88 ± 0.09	0.78 ± 0.09	< 0.001	0.636	0.416
	<i>igf-i</i>	5.76 ± 0.69	5.55 ± 0.32	8.77 ± 1.17	8.17 ± 0.78	< 0.001	0.864	0.806
	<i>igf-ii</i>	2.01 ± 0.26	2.62 ± 0.27	4.71 ± 0.53	5.25 ± 1.02	< 0.001	0.579	0.352
	<i>igfbp1a</i>	0.06 ± 0.01	0.09 ± 0.02	0.04 ± 0.00	0.04 ± 0.00	< 0.001	0.373	0.217
	<i>igfbp2b</i>	2.12 ± 0.15	2.52 ± 0.19	1.42 ± 0.13	1.63 ± 0.15	< 0.001	0.330	0.863
	<i>igfbp4</i>	0.68 ± 0.06	0.63 ± 0.06	0.54 ± 0.04	0.49 ± 0.05	0.006	0.537	0.794
	<i>igfr1</i>	0.10 ± 0.01 ^b	0.05 ± 0.01 ^a	0.08 ± 0.01	0.07 ± 0.01	0.354	0.145	0.003
	<i>igfr2</i>	0.28 ± 0.03 ^b	0.12 ± 0.01 ^{a*}	0.22 ± 0.02	0.26 ± 0.03 [#]	0.012	0.275	< 0.001
	<i>insr</i>	0.96 ± 0.08 ^{b*}	0.47 ± 0.01 ^{a*}	0.69 ± 0.08 [#]	0.84 ± 0.06 [#]	0.139	0.246	< 0.001
Cytoplasmic and lysosomal proteases	<i>capn1</i>	0.15 ± 0.02	0.19 ± 0.02 [#]	0.12 ± 0.01	0.09 ± 0.01 [*]	< 0.001	0.957	0.013
	<i>cast</i>	0.28 ± 0.02	0.29 ± 0.02	0.53 ± 0.05	0.50 ± 0.05	< 0.001	0.867	0.626
	<i>ctsb</i>	1.76 ± 0.14	1.98 ± 0.20	1.86 ± 0.16	2.01 ± 0.15	0.523	0.668	0.884
	<i>ctsd</i>	0.17 ± 0.02	0.18 ± 0.02	1.03 ± 0.19	1.06 ± 0.14	< 0.001	0.780	0.939
	<i>ctsl</i>	6.74 ± 0.53	8.21 ± 0.65	11.50 ± 0.86	12.20 ± 1.30	< 0.001	0.499	0.293
Energy sensing and oxidative metabolism	<i>pgc1a</i>	0.32 ± 0.04	0.39 ± 0.04	0.17 ± 0.01	0.16 ± 0.02	< 0.001	0.584	0.162
	<i>cpt1a</i>	0.93 ± 0.06	0.90 ± 0.04	1.09 ± 0.09	1.16 ± 0.09	0.001	0.813	0.228
	<i>cs</i>	0.43 ± 0.03	0.49 ± 0.02	0.81 ± 0.06	0.89 ± 0.07	< 0.001	0.429	0.668
	<i>hif-1α</i>	0.55 ± 0.03	0.50 ± 0.03	0.36 ± 0.03	0.35 ± 0.02	< 0.001	0.464	0.525
Antioxidant effects	<i>mthsp70/grp-75</i>	0.53 ± 0.08	0.54 ± 0.04	0.70 ± 0.07	0.76 ± 0.09	0.005	0.704	0.831
	<i>grp-170</i>	1.24 ± 0.15	1.33 ± 0.18	1.13 ± 0.15	1.23 ± 0.14	0.602	0.429	0.907
	<i>grp-94</i>	3.82 ± 0.68	3.65 ± 0.58	1.47 ± 0.24	2.47 ± 0.52	0.013	0.574	0.117
	<i>cat</i>	10.86 ± 0.95	11.63 ± 1.33	13.15 ± 1.24	13.24 ± 0.67	0.051	0.744	0.924
	<i>gpx1</i>	1.08 ± 0.06	1.17 ± 0.06	0.96 ± 0.05	0.92 ± 0.08	0.006	0.914	0.263
	<i>gpx4</i>	4.08 ± 0.65	4.52 ± 0.75	13.82 ± 2.19	14.60 ± 0.81	< 0.001	0.521	0.978
	<i>gr</i>	0.24 ± 0.01	0.25 ± 0.02	0.35 ± 0.02	0.35 ± 0.03	< 0.001	0.996	0.824
	<i>prdx3</i>	0.45 ± 0.03	0.47 ± 0.04	0.68 ± 0.06	0.71 ± 0.05	< 0.001	0.817	0.994
	<i>prdx5</i>	0.29 ± 0.04	0.25 ± 0.03	1.13 ± 0.12	1.11 ± 0.09	< 0.001	0.624	0.674
	<i>Mn-sod/sod2</i>	0.80 ± 0.07	0.76 ± 0.06	0.77 ± 0.06	0.75 ± 0.07	0.822	0.620	0.952
	<i>h-fabp</i>	26.47 ± 2.04	27.89 ± 1.66	45.78 ± 4.31	58.29 ± 6.00	< 0.001	0.620	0.952
Respiration uncoupling	<i>ucp1</i>	15.18 ± 1.17	17.26 ± 1.08	8.77 ± 0.88	10.39 ± 0.74	< 0.001	0.248	0.746

Values are expressed as means ± SE (n = 9). P-values from two-way ANOVA (p ≤ 0.05). Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences among dietary treatments for the same time while symbols stand for significant differences between times for the same diet.

regulated after a short-term feeding period in fish fed the SUP diet compared to those the CTRL diet, although a general but non-significant trend to gene expression increase was observed for almost all analysed genes at 15 days when fed the SUP diet. This effect was not retained after 92 days of feeding.

In order to get a clearer picture of the time and diet effect on tissue gene expression, an overall multivariate analysis combining raw data from the three tissues (using PLS-DA) was performed to discriminate the tissues' molecular signatures of fish fed the experimental diets both at short and long-term feeding periods (Fig. 1). This approach showed that overall expression patterns can be summarized through 3 main components that explain 88.31% of total variance (Fig. 1A). Component 1 (50.06% of total variance, X-axis) was able to clearly separate fish sampled at early and late on the feeding trial. Dietary groups after 15 days of feeding evidenced a good separation along component 2 (24.36% of total variance, Y-axis) (Fig. 1B). Component 3 (13.89% of total variance, Y-axis) appeared to be also related to diet effect, contributing to further separate CTRL and SUP groups at early sampling (Fig. 1C). The variable importance projection (VIP) score of the genes after three components is presented in Fig. 1D. Biomarkers with a VIP > 1 that appeared after the first component (highlighted in yellow), were mostly represented by liver and muscle genes (22 out of 26). By contrast, genes explaining the most variance associated to component 2 (highlighted in blue) were mostly from head kidney (7 genes) and liver (4 genes). Only 6 new genes, with a representation of the 3 analysed tissues (3 from muscle, 2 from liver, 1 from head kidney), were reported as main contributors to variance when the

component 3 (highlighted in orange) was also considered. When the VIP contribution was ranked after the 3 components on the analysis, the highest contribution corresponded to hepatic *insr*, *igfr1* and *igfr2* genes, which were down-regulated in fish fed the SUP diet at short-period sampling (Table 6).

4. Discussion

The present study represents the first attempt to explore and compare the putative short and long-term effects of a functional diet, in the context of an extreme formulation (0% fishmeal) supplemented with tryptophan, on the growth and health condition of the gilthead seabream juveniles. It should be noted that the first weeks of the study were conducted at high temperature for gilthead seabream, what may elicit an increase in amino acid requirements [33], and both diets were just above the lysine and methionine requirements estimated by Peres and Oliva-Teles [20].

In the present study, no differences were observed between dietary treatments after a long-term feeding period, both at zootechnical, physiological and transcriptional level. Nevertheless, this holistic approach revealed interesting results about the impact of a supplemented diet at different sampling points. The fact that “time” seemed to be the main modulatory factor on the humoral immune parameters analysed underlines the sensitivity of some of these measures to contextual factors. For instance, plasma ACH50 levels tended to increase in fish fed the SUP diet after a short-feeding period. Machado et al. [22] and Azeredo et al. [34] also observed a tendency for increased plasma

Table 7
Muscle gene expression in gilthead seabream fed dietary treatments during 15 and 92 days.

Biological Process	Gene symbol	15 Days		92 Days		Two-way ANOVA (p < 0.05)		
		CTRL	SUP	CTRL	SUP	Time	Diet	Time x diet
GH/IGF System	<i>ghr-i</i>	3.16 ± 0.33	3.22 ± 0.35	9.99 ± 1.27	10.86 ± 0.83	< 0.001	0.669	0.620
	<i>ghr-ii</i>	4.72 ± 0.71	8.35 ± 3.11	3.97 ± 0.66	2.66 ± 0.36	0.012	0.928	0.104
	<i>igf-i</i>	0.16 ± 0.01	0.25 ± 0.08	0.29 ± 0.04	0.22 ± 0.04	0.089	0.729	0.167
	<i>igf-ii</i>	1.34 ± 0.12	2.00 ± 0.35	2.60 ± 0.24	2.27 ± 0.20	0.001	0.336	0.058
	<i>igfbp3</i>	3.69 ± 0.30 ^{a#}	5.42 ± 0.52 ^{b#}	1.80 ± 0.18 [†]	1.58 ± 0.10 [‡]	< 0.001	0.275	0.004
	<i>igfbp5b</i>	1.64 ± 0.11	2.09 ± 0.14	3.57 ± 0.38	3.23 ± 0.24	< 0.001	0.051	0.538
	<i>igfbp6b</i>	0.28 ± 0.03	0.34 ± 0.03	0.32 ± 0.04	0.28 ± 0.03	0.647	0.827	0.109
	<i>insr</i>	1.65 ± 0.16	2.22 ± 0.45	2.25 ± 0.19	2.62 ± 0.72	0.131	0.374	0.505
	<i>igfr1</i>	1.40 ± 0.09	1.72 ± 0.26	2.85 ± 0.20	3.13 ± 0.32	< 0.001	0.421	0.699
	<i>igfr2</i>	0.98 ± 0.10	1.48 ± 0.38	1.34 ± 0.09	2.01 ± 0.54	0.025	0.296	0.883
	Muscle growth & cell differentiation	<i>myod1</i>	10.86 ± 0.53	12.92 ± 1.88	13.90 ± 1.60	13.59 ± 1.17	0.136	0.635
<i>myo1</i>		2.04 ± 0.41	1.95 ± 0.23	2.28 ± 0.22	1.92 ± 0.25	0.347	0.908	0.277
<i>myf5</i>		0.47 ± 0.03	0.50 ± 0.04	0.48 ± 0.03	0.53 ± 0.12	0.854	0.625	0.890
<i>myf6</i>		0.45 ± 0.03	0.55 ± 0.04	0.75 ± 0.06	1.23 ± 0.41	< 0.001	0.074	0.712
<i>mstn</i>		2.16 ± 0.23	2.54 ± 0.34	6.38 ± 1.65	6.94 ± 1.88	< 0.001	0.749	0.972
<i>mef2a</i>		15.43 ± 1.14	19.37 ± 2.38	42.76 ± 3.10	48.54 ± 4.43	< 0.001	0.158	0.669
<i>mef2c</i>		5.94 ± 0.22	5.88 ± 0.51	12.08 ± 1.21	12.85 ± 1.51	< 0.001	0.819	0.595
<i>fst</i>		0.67 ± 0.08	0.88 ± 0.15	0.57 ± 0.07	0.55 ± 0.06	0.013	0.540	0.301
Energy sensing & oxidative metabolism	<i>sirt1</i>	0.37 ± 0.02	0.42 ± 0.05	0.56 ± 0.06	0.59 ± 0.10	0.002	0.638	0.682
	<i>sirt2</i>	0.48 ± 0.02	0.56 ± 0.07	0.75 ± 0.06	0.76 ± 0.05	< 0.001	0.350	0.549
	<i>sirt5</i>	1.03 ± 0.08	1.16 ± 0.17	1.16 ± 0.10	0.97 ± 0.12	0.809	0.373	0.220
	<i>cpt1a</i>	10.72 ± 0.37	12.32 ± 1.18	22.94 ± 1.91	22.87 ± 2.26	< 0.001	0.507	0.536
	<i>cs</i>	25.09 ± 1.58	29.77 ± 3.84	36.41 ± 2.49	35.23 ± 2.49	0.004	0.359	0.419
	<i>nd2</i>	44.81 ± 3.28	45.38 ± 5.75	88.01 ± 15.02	82.28 ± 6.00	< 0.001	0.849	0.887
	<i>nd5</i>	26.63 ± 1.85	30.03 ± 3.09	45.55 ± 7.20	36.86 ± 3.23	0.003	0.798	0.246
	<i>cox i</i>	239.75 ± 17.83	333.71 ± 48.73	320.82 ± 27.86	253.56 ± 20.87	0.758	0.827	0.048
	<i>cox ii</i>	123.85 ± 6.90	163.49 ± 27.34	146.09 ± 23.72	112.52 ± 8.34	0.349	0.997	0.094
	Respiration uncoupling	<i>ucp3</i>	14.43 ± 1.93	17.25 ± 3.13	29.40 ± 4.92	30.68 ± 3.38	< 0.001	0.520
<i>pgc1a</i>		0.58 ± 0.15	0.37 ± 0.06	2.47 ± 0.43	3.00 ± 0.61	< 0.001	0.727	0.249

Values are expressed as means ± SE (n = 9). P-values from two-way ANOVA (p ≤ 0.05). Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences among dietary treatments for the same time while symbols stand for significant differences between times for the same diet.

ACH50 values in European seabass and Senegalese sole juveniles fed a tryptophan supplemented diet for a period of 15 or 38 days, respectively. In the present study, the absence of an immune-priming effect, such as antigen recognition, may explain the lower response/variation in most humoral parameters and the health biomarkers panel analysed. In this context, Machado, et al. [22] showed that slight short-term feeding effects on the European seabass immune response can be enhanced upon stimulation with an inflammatory agent as observed by a general increase in innate immune parameters in fish fed diets supplemented with essential AA.

Simultaneous gene expression analysis using a customized PCR-array platform offers the possibility to identify over time and at a high level of confidence the most responsive tissues and biomarkers in fish fed different experimental diets. This was inferred from discriminant analysis (PLS-DA) integrating all expression data from liver, skeletal muscle and head kidney in fish fed CTRL and SUP diets, which showed the existence of three major groups in terms of gene expression: two groups corresponding to each diet group for the early sampled fish plus a merged group combining all fish from the late sampling. In this discriminant model, half of the total variance was explained by the first component (~50% of observed variance), corresponding to the effects of time on gene expression, whereas the second and third components (~40% of observed variance) mostly described the short-term diet effects. The sources of variance due to sampling time would reflect the increase of fish size and the change of experimental conditions throughout the 92-day feeding trial, with a decrease of daily water temperature from 25 to 18.5 °C (Fig. S1). Regarding the specific effects of diet, it is noteworthy that, though VIP analysis with the first three components highlighted that the top contributing genes were liver

markers of GH/IGF axis (*insr*, *igfr1*, *igfr2*), after the same analysis with the first two components points towards a high importance of head kidney biomarkers at the early sampling point. Therefore, the effect of dietary tryptophan supplementation is particularly important after a short-term feeding period, which seems particularly evident for some immune-related genes (*il-10*, *cd3x*, *tr12*).

The up-regulation of immune-related genes can be considered a beneficial effect as reported by other authors in response to administration of AA or derivatives. For instance, Cuesta, et al. [35] have reported the up-regulation of lymphocyte markers and other immune-relevant genes in the head kidney of gilthead seabream upon intraperitoneal injection of melatonin (synthesized from serotonin and Trp). Research in Japanese flounder, also revealed that TLR2 expression was up-regulated in blood leukocytes after treatments with Poly:I(C) and peptidoglycan [36]. Moreover, the *il-10* transcript, which is produced by activated monocytes (T cells) seems to be a crucial factor for some forms of peripheral tolerance and a major suppressor of immune system and inflammation [37]. This type of immune enhancement can be especially important in cases of stressful conditions (e.g. temperature changes, handling, crowding, transport), known to have immunosuppressive effects.

Stimulation of the immune status of gilthead seabream after 2 weeks of feeding with the SUP diet was concomitant with an early and transient decrease in feed conversion (higher FCR). This may be related to the down-regulated expression of hepatic markers (i.e. *igfr1*, *igfr2* and *insr*) of the GH/IGF system, which is highly responsive to nutritional and environmental stimuli [38]. IGFs directly stimulate cell proliferation, differentiation, and hypertrophy and inhibit muscle atrophy, with the effects of IGF1 on muscle being mediated by the specific binding

Table 8
Head kidney expression in gilthead seabream fed dietary treatments during 15 and 92 days.

Biological Process	Gene symbol	15 Days		92 Days		Two-way ANOVA (p < 0.05)		
		CTRL	SUP	CTRL	SUP	Time	Diet	Time x diet
Interleukins & Cytokines	<i>il-1β</i>	0.09 ± 0.02	0.11 ± 0.02	0.03 ± 0.00	0.03 ± 0.01	< 0.001	0.403	0.576
	<i>il-6</i>	0.02 ± 0.00*	0.04 ± 0.01	0.05 ± 0.01#	0.04 ± 0.01	0.104	0.580	0.015
	<i>il-7</i>	1.03 ± 0.15	1.17 ± 0.09	1.00 ± 0.08	0.80 ± 0.12	0.105	0.625	0.078
	<i>il-8</i>	0.05 ± 0.01	0.06 ± 0.01	0.03 ± 0.00	0.03 ± 0.01	0.013	0.842	0.487
	<i>il-10</i>	0.47 ± 0.03 ^a	0.66 ± 0.04 ^b	0.67 ± 0.05	0.52 ± 0.04	0.601	0.731	< 0.001
	<i>il-12</i>	0.06 ± 0.01	0.08 ± 0.01	0.05 ± 0.00	0.04 ± 0.00	0.137	0.993	0.077
	<i>il-15</i>	0.23 ± 0.02*	0.24 ± 0.02	0.33 ± 0.02 [#]	0.27 ± 0.02	< 0.001	0.693	0.021
	<i>il-34</i>	1.11 ± 0.12	1.26 ± 0.07	2.18 ± 0.15	2.02 ± 0.14	< 0.001	0.736	0.132
	<i>tnf-α</i>	0.14 ± 0.02	0.16 ± 0.01	0.17 ± 0.02	0.16 ± 0.01	0.361	0.659	0.220
Macrophages and monocytes chemokines	<i>csf1r1</i>	1.73 ± 0.19*	1.99 ± 0.11	2.90 ± 0.22 [#]	2.41 ± 0.15	< 0.001	0.958	0.015
	<i>ccr3</i>	4.85 ± 0.59	4.55 ± 0.38	5.71 ± 0.35	5.65 ± 0.49	0.012	0.873	0.996
	<i>ck8/ccl20</i>	0.36 ± 0.06	0.46 ± 0.04	0.62 ± 0.10	0.52 ± 0.06	0.019	0.620	0.047
Immunoglobulins	<i>slgM</i>	76.46 ± 7.40	65.68 ± 10.87	129.14 ± 16.21	103.47 ± 14.30	0.008	0.043	0.817
	<i>mlgM</i>	12.86 ± 1.24	13.73 ± 1.14	17.73 ± 1.17	15.12 ± 1.14	0.017	0.748	0.594
	<i>slgT</i>	0.67 ± 0.42	1.11 ± 0.72	4.81 ± 1.48	2.73 ± 0.85	0.140	0.701	0.463
	<i>mlgT</i>	9.16 ± 0.96	10.91 ± 1.67	8.35 ± 1.06	7.76 ± 0.70	0.011	0.728	0.124
Anti-protease	<i>a2m</i>	0.10 ± 0.04	0.12 ± 0.02	0.09 ± 0.02	0.09 ± 0.02	0.655	0.725	0.132
Antimicrobial peptide/iron recycling	<i>hepc</i>	67.75 ± 10.00	103.42 ± 17.42	10.91 ± 1.88	10.20 ± 1.69	< 0.001	0.349	0.202
T-cell markers	<i>cd3e</i>	2.33 ± 0.35	3.89 ± 0.70	3.04 ± 0.13	2.85 ± 0.25	0.567	0.420	0.150
	<i>cd3x</i>	2.00 ± 0.23 ^a	3.27 ± 0.49 ^b	2.62 ± 0.19	2.55 ± 0.18	0.601	0.137	0.028
	<i>cd4-full</i>	1.51 ± 0.23	2.59 ± 0.54	2.05 ± 0.13	1.84 ± 0.12	0.491	0.389	0.021
	<i>cd8a</i>	1.28 ± 0.24	2.06 ± 0.53	1.19 ± 0.10	0.98 ± 0.13	0.133	0.876	0.118
	<i>cd8b</i>	0.61 ± 0.16	1.14 ± 0.34	0.35 ± 0.04	0.28 ± 0.04	0.002	0.810	0.119
	<i>zap70</i>	1.55 ± 0.19	1.95 ± 0.20	2.01 ± 0.18	1.93 ± 0.13	0.108	0.590	0.130
Pattern recognition receptors	<i>tlr1</i>	1.15 ± 0.05	1.20 ± 0.07	1.26 ± 0.06	1.04 ± 0.08	0.617	0.041	0.059
	<i>tlr2</i>	1.44 ± 0.12 ^{a*}	1.95 ± 0.06 ^{b*}	3.04 ± 0.15 [#]	3.02 ± 0.18 [#]	< 0.001	0.104	0.007
	<i>tlr5</i>	0.32 ± 0.04	0.33 ± 0.02	0.48 ± 0.07	0.48 ± 0.04	0.001	0.463	0.796
	<i>tlr9</i>	0.25 ± 0.03 [*]	0.34 ± 0.04 [*]	0.79 ± 0.10 [#]	0.62 ± 0.05 [#]	< 0.001	0.666	0.050
	<i>mrc1</i>	5.18 ± 0.63	5.39 ± 0.47	5.83 ± 0.32	5.59 ± 0.57	0.373	0.889	0.552

Values are expressed as means ± SE (n = 9). P-values from two-way ANOVA (p ≤ 0.05). Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences among dietary treatments for the same time while symbols stand for significant differences between times for the same diet.

with IGF1 receptor (IGFR1) [39]. Montserrat et al. [40] reported that, in gilthead seabream myocytes, IGF2 (mediated by IGFR2) activates the MAPK/ERK and PI3K/AKT pathways in a stronger way than IGF1, suggesting that IGF2 is powerful in stimulating their muscle growth [41]. Likewise, insulin signalling via its receptor induce complex effects on metabolism, cell growth and differentiation, including macrophages and endothelial cells. The major tissues targeted by insulin's effects on metabolism include: i) muscle, where it promotes glucose uptake and protein synthesis, and ii) liver, where insulin promotes glucose utilization, suppresses glucose production, and promotes triglyceride synthesis [42]. Thus, the transient observed down-regulation of *insr* could negatively influence protein synthesis, and it would be consistent with the trend for a higher FCR in fish fed the SUP diet at an early stage. Overall, these observations support the idea that a wide panel of biomarkers helps to better explore in a consistent manner the modulation and effects of functional aquafeeds in terms of fish growth performance and its health status along time.

5. Conclusions

This study provides additional information about the effect of dietary supplementation with essential AA in aquafeeds, particularly regarding the supplementation with tryptophan both over short- and long-term feeding periods. It was demonstrated that, in the context of a challenging fishmeal-free diet, supplementation with tryptophan seems to improve gilthead seabream juveniles' immune status on a short-term basis without compromising long-term fish growth. Hence, we consider

that the use of functional feeds can be a promising approach for boosting fish immune status, particularly through a short-term feeding period before a predictable stressful event or disease outbreak, considering that these putative advantageous effects are not retained on a long-term basis. Nonetheless, further studies focusing on disease resistance and other stressors must be planned to improve the knowledge on the modulation of growth and immune status in fish through the dietary use of AA.

Ethics statement

CCMAR facilities and their staff are certified to house and conduct experiments with live animals (Group-C licenses by the Direção Geral de Alimentação e Veterinária, Ministério da Agricultura, Florestas e Desenvolvimento Rural, Portugal). The protocol was approved by the CCMAR Animal Welfare Committee.

Author contributions

LC, SE, JP-S, TSS and BC conceived the experiment and contributed with both reagents and goods. LR-P conducted the main experimental work. RA, SF-B, BR, JAM-S, JAC-G and JP-S assisted with analytical procedures and statistical analysis. LR-P directed most laboratory techniques and wrote the manuscript under the supervision of JAM-S, JAC-G, JP-S, BC and TSS. L-RP, JAM-S. All authors contributed to and approved the manuscript. The authors also acknowledge Rita Colen and Denise Schrama (UAlg – CCMAR), for their help and support

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Appendix A. Supplementary data

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