Health status in gilthead seabream (*Sparus aurata)* juveniles fed diets devoid of fishmeal and supplemented with *Phaeodactylum tricornutum*

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Abstract

To enhance fish general health, feeds can be supplemented with health promoting additives, reducing the need to use chemotherapeutics. Incorporation of marine algae biomasses in aquafeeds has been shown to improve fish immune status by enhancing innate immune response. This study intended to evaluate the effects of microalgae *Phaeodactylum tricornutum* incorporation in feed by two different processes, either as freeze-dried biomass or broken cell wall biomass, on fish health status and performance. Triplicate groups of gilthead seabream juveniles (13.3 ± 0.3 g) were either fed a control diet (CTRL) with an extreme (i.e. 0 % fishmeal), nutritionally balanced, formulation, or two experimental diets formulated as the CTRL with 1 % inclusion of *P. tricornutum* at the expense of wheat meal: BC diet contains *P. tricornutum* broken cells and WC diet microalgae whole cells. After 2 and 12 weeks of feeding, blood was collected for haematological procedures whereas plasma and mucus were sampled for immune parameters. Head-kidney, liver and white skeletal muscle were also collected for gene expression measurements.

Mucus bactericidal and alternative complement pathway activity increased when seabream juveniles were fed BC diet for 2 and 12 weeks, respectively. No major differences were observed in haematological nor plasma humoral parameters after 12 weeks irrespective of dietary treatment. Arrays of 29-31 genes were analyzed in the different tissues, revealing an early dietary effect (2 weeks) in a tissue-specific pattern. In liver, the major effect was found in the GH/IGF axis and in muscle there was a late

down-regulation of myostatin (*mstn*) gene, mainly due to WC diet, even though all fish had similar growth performance. Regarding the head-kidney, BC diet led to interleukin 6 (*il-6*) and alpha-2-macroglobulin (*a2m*) gene up-regulation. Hence, it seems that BC diet has a potential stimulatory effect that might be relevant as a prophylactic measure before a predictable stressful event.

Keywords

Gilthead seabream*; Phaeodactylum tricornutum*; fishmeal-free diet; feed additive; innate immunity; immunonutrition

1. Introduction

Fish production has strongly depended on fishmeal (FM) as the major protein source in aquafeeds, mainly because of its high nutritional value and balanced amino acid (AA) profile. Simultaneously, aquaculture has greatly expanded in the last decades, increasing the demand for marine resources. In order to reduce the industry's pressure over these raw materials, FM replacement has been a key point of research, more importantly in species that show high protein requirement [1] such as gilthead seabream (*Sparus aurata*). Often, this has been successfully achieved by partially replacing FM by plant protein (PP) feedstuffs [2-5]. However, total FM replacement by alternative protein sources can lead to impaired growth performance and immune status [6, 7], mostly because of an unbalanced amino acid profile [8]. Also, the presence of antinutritional factors (ANFs) in PP can damage the intestinal epithelium and promote enteritis in carnivorous fish species [9, 10].

Aquaculture intensification and sustainability led to new challenges for farmed species since there is a need to adapt not only to the inclusion of new dietary ingredients, but also to cope with the challenges arising from intensive fish production. Repeated exposure to stressful conditions caused by routine farming practices (high stocking densities, crowding, size sorting and transportation) [11-13] can lead to poor growth performance and immunocompromised fish, especially if nutritional requirements are not met. To avoid these negative effects, feeds are often supplemented with essential amino acids (EAA) [4, 14, 15] and in some cases specialized processing techniques are employed to neutralize ANFs [16, 17]. Concomitantly, fish feed additives are also used as alternative strategies to reduce and prevent adverse effects of extreme diet formulation and stress on aquaculture fish [13, 18, 19].

Marine organisms are a plentiful source of new biologically active compounds such as polysaccharides, polyphenols, functional peptides, or fatty acids, amongst others [20, 21]. These compounds can act as additives for the development of new functional feeds reported to have immunostimulating effects in fish [22-24]. Incorporation of marine algae extracts in aquafeeds has been shown to improve growth and survival of commensal bacteria in fish gastrointestinal tract, or even improving host immune status and enhancing innate immune responses (including increased lysozyme and alternative complement pathway activity, phagocytic and neutrophil activation in fish) [24-26].

Phaeodactylum tricornutum is a marine microalgae rich in polyunsaturated fatty acids (PUFA), particularly eicosapentaenoic acid (EPA), but also β-glucans and fucoxanthin [27-29]. PUFA are paramount to promote optimal growth and health of farmed fish [30]. Oral administration of PUFA can change the membrane fatty acid composition of phagocytic cells and enhance their phagocytic activity [31]. β-1,3 glucans are glucose polysaccharides produced by several organisms, namely algae which can activate and enhance fish immune response [32-34]. β-1,3 glucans show repeating patterns on their structure that are recognized in the gut by cell pattern recognition receptors (PRR), leading to the activation of the host´s innate immune cells [35]. Both *in vitro* and *in vivo* studies have shown fucoxanthin anti-inflammatory effects, by inhibiting the production of pro-inflammatory mediators including interleukin 1 β (IL-1β), tumor necrosis factor (TNF-α) and interleukin-6 (IL-6). Furthermore, fucoxanthin antioxidant effect was demonstrated through the inhibition of NFκB activation and stimulation of catalase and superoxide dismutase activity under an acute inflammatory response [36, 37].

P. tricornutum has already been used either as a FM alternative protein source or an additive to promote immunostimulation [26, 38]. However, more in-depth studies are required to explore the potential of *P. tricornutum* as an immunostimulant and health

promoter in animals, as a strategy to curtail the possible detrimental effects of very low FM dietary inclusion. One major issue to deal with, when microalgae is orally given to fish, is the cell wall, which restricts the access of gut enzymes to the cell components. Previous works indicate that the digestibility of several algae species is dependent on disrupting the algal cell wall by appropriate processes [39, 40]. This study intended to evaluate the effects of dietary supplementation with *P. tricornutum* incorporated in feed by two different processes, either as whole cells (intact cell wall) or broken cells (disrupted cell wall through high pressure) on health status and growth performance of gilthead seabream juveniles. To our knowledge, this is the first study where there is a comparison between the use of *P. tricornutum* intact biomass and biomass pre-treated to disrupt the cell wall and allow higher availability of its bioactive compounds.

2. Material & Methods

2.1. Phaeodactylum biomasses

Microalgae *Phaeodactylum tricornutum* (wild strain) biomass was produced by Fitoplancton Marino (Spain) in photobioreactors. One fraction of the intact biomass was freeze-dried and named Phaeodactylum whole-cells biomass (WC). A fraction of the same initial biomass was subjected to a mechanical process for cell disruption, which comprised high-pressure homogenization and bead milling steps. The exact details of the cell disintegration process are not disclosed due to industrial confidentiality. After freeze-drying, it originated the Phaeodactylum broken-cells biomass (BC). On a dry basis, the composition of biomasses was 34% crude protein, 10% crude lipid and 29% ash.

2.2. Diet composition

The study comprised three fishmeal-free diets. A control diet (CTRL) formulated with moderate levels of poultry meal (10%) and high levels of plant ingredients (soy protein concentrate, wheat gluten, corn gluten meal, soybean meal, guar meal, and rapeseed meal) as major protein sources. A blend of fish, soybean and rapeseed oils was used a major lipid source. This control formulation served as basis for the two additional diets, which comprised a 1% inclusion of *P. tricornutum* biomass, either as whole-cells (diet WC) or broken cells (diet BC). In both cases, algae biomasses were incorporated at the expenses of wheat. All diets were supplemented with selected crystalline amino acids and an inorganic phosphate source to avoid any nutritional deficiencies. Diets were isoproteic (crude protein, 50% dry matter) and presented similar levels of crude lipids (17.8-18.9% dry matter) and gross energy content (23.0-23.3 kJ g^{-1} dry matter) (Table 1).

Diets were manufactured by SPAROS Lda. (Olhão, Portugal). All powder ingredients were mixed accordingly to the target formulations in a double-helix mixer (model 500L, TGC Extrusion, France) and ground (below 250 µm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Diets, with a pellet size of 2.0 mm, were manufactured with a twin-screw extruder (model BC45, Clextral, France) with a screw diameter of 55.5 mm. Extrusion conditions: feeder rate (83 kg/h), screw speed (232 rpm), water addition in barrel 1 (300 ml/min), temperature barrel 1 (36-38ºC), temperature barrel 3 (107-111°C). Extruded pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). The blend of oils was added post-extrusion by vacuum coating (model PG-10VCLAB, Dinnissen, The Netherlands). Throughout the duration of the trial, experimental feeds were stored at room temperature.

2.3. Fish rearing conditions

The experiment was carried out in compliance with the Guidelines of the European Union Council (Directive 2010/63/EU) and Portuguese legislation for the use of laboratory animals. Fish were assigned to 1 m^3 tanks at Centre of Marine Sciences (CCMAR) facilities (Faro, Portugal). Seawater flow was kept at 2 $L.min^{-1}$ (mean temperature 23 ± 2.6 °C; mean salinity 34 ± 0.7 ‰) in a flow-through system with aeration (mean dissolved oxygen above 5 $mg.L^{-1}$). Water quality parameters were monitored daily and adjusted when necessary. Mortality was monitored daily. Diets were randomly assigned to triplicate groups of 150 gilthead seabream (initial body weight: 13.3 ± 0.3 g; ~2.0 $kg.m^{-3}$ initial stocking density) that were fed to visual satiety by hand, twice daily for 12 weeks.

2.4. Feeding trial and tissue sampling

The feeding trial lasted 12 weeks. Fish were weighed at the beginning and after 2 and 12 weeks, and feed consumption for each experimental unit was registered weekly. Eight and eighteen fish per tank were individually weighed and sampled after 2 and 12 weeks for blood, skin mucus and tissues (head-kidney, liver and white skeletal muscle), after sacrifice with a tricaine methanesulfonate lethal dose (200 $\mu g.L^{-1}$). Six fish from each tank were stored at −20 °C until analysis of proximate composition and amino acids content. Blood was collected from the caudal vein using heparinized syringes and centrifuged at 10,000 × *g* during 10 min at 4 ºC to obtain plasma samples. Skin mucus and tissue samples were immediately frozen at -80 ºC until further analysis.

2.5. Growth parameters and feed utilization

Calculations were done as follows: Daily growth index (DGI) (%/day) = $((Wf^{1/3} - Wi^{1/3})/days) \times 100$; Feed conversion ratio (FCR) = apparent feed intake (g/fish)/(Wf −

Wi); Protein efficiency ratio (PER) = (Wf − Wi)/crude protein intake; Daily intake (g/kg ABW/day) = dry matter or nutrient intake (g or mg)/((Wf + Wi)/2) (kg)/days; Nutrient retention: 100 × (FBW × final carcass nutrient content − IBW × initial carcass nutrient content)/nutrient intake.

2.6. Haematological procedures

The haematological profile consisted of total white (WBC) and red (RBC) blood cells counts, haematocrit (Ht) and haemoglobin (Hb; SPINREACT kit, ref. 1001230, Spain). The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were also calculated as follows: MCV (μm^3) = (Ht/RBC) × 10; MCH ($pg.cell^{-1}$) = (Hb)/ RBC × 10; MCHC ($g.100\ mL^{-1}$) = (Hb/Ht) × 100. For determination of RBC and WBC concentration, whole blood was diluted 1/20 (WBC), 1/200 (RBC) in HBSS with heparin (30 $U.ml^{-1}$) and cell counts were done in a Neubauer chamber. Blood smears were prepared from peripheral blood, air dried and stained with Wright's stain (Haemacolor; Merck) after fixation for 1 minute with formol–ethanol (10 % formaldehyde in ethanol). Neutrophils were labeled through the detection of peroxidase activity revealed by the Antonow´s technique described in Afonso et al. [41]. The slides were examined under oil immersion (1000×), and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes and neutrophils. The relative percentage and absolute value ($\times 10^4\ mL^{-1}$) of each cell type was calculated.

2.7. Innate humoral parameters

Peroxidase activity

Total peroxidase activity in plasma and mucus was measured following the procedure described by Quade and Roth [42]. Briefly, 10 μL of plasma and 20 μL of mucus were diluted with 140 and 130 μL, respectively, of HBSS without Ca^{2+} and Mg^{2+} in 96-well plates. Then, 50 μL of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB; Sigma) and 50 μL of 5 mM H_2O_2 were added to the wells. The reaction was stopped after 2 min by adding 50 μL of 2 M H_2SO_4 and the optical density (OD) was read at 450 nm in a Synergy HT microplate reader (Biotek). Wells without plasma or mucus were used as blanks. The peroxidase activity (units.mL^{-1} plasma or mucus) was determined defining that one unit of peroxidase produces an absorbance change of 1 OD.

Bactericidal activity

Plasma bactericidal activity was determined following the method of Machado et al. [43]. *Photobacterium damselae* subsp. *piscida* (Phdp), strain PP3, was used. Briefly, 20 μL of sample were mixed with 20 μL of Phdp (1×10^6 cfu.mL^{-1}) in duplicate in a U-shaped 96-well plate, that was then incubated for 2.5 h at 25 ºC (20 μL of TSB were added instead of plasma to 2 wells and served as positive control). Afterwards, 25 μL of 3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (1 mg.mL^{-1}; Sigma) were added to each well and incubated for 10 min at 25 ºC to allow the formation of formazan precipitates. Plates were then centrifuged at 2,000 × *g* for 10 min and the precipitate was dissolved in 200 μL of dimethyl sulfoxide (Sigma). The absorbance of the dissolved formazan was measured at 560 nm. Bactericidal activity is expressed as percentage, calculated from the difference between bacteria surviving compared to the number of bacteria from positive controls (100%).

Protease activity

The protease activity was determined in plasma and mucus as described by Ross et al. [44] with some modifications. Briefly, 100 µL of sodium bicarbonate buffer ($NaHCO_3$, 5 $mg.mL^{-1}$, pH 8.3) and 125 µL of azocasein solution (20 $mg.mL^{-1}$ in $NaHCO_3$, 5 $mg.mL^{-1}$, pH 8.3) were added to 10 µL of plasma, whereas for mucus 100 µL of sample was used and mixed with 100 µL of azocasein solution (20 $mg.mL^{-1}$ in $NaHCO_3$, 5 $mg.mL^{-1}$, pH 8.3) both reaction mixtures were incubated for 19 h at 30 °C. Finally, 250 µL of trichloroacetic acid were added to both reactions.Mixtures were centrifuged at 6,000 × *g* for 5 min at room temperature. Afterwards, 100 µL of the supernatant was transferred to a 96 well-plate and mixed with 100 µL of NaOH (40 $mg.mL^{-1}$). The OD was read at 450 nm in a Synergy HT microplate reader. Sodium bicarbonate buffer in place of plasma or mucus served as a blank, whereas the reference sample was a trypsin solution in place of plasma or mucus. Sample trypsin activity ratio was calculated as follows: (sample absorbance/reference absorbance) x 100. All analyses were conducted in duplicates.

Antiprotease activity

The anti-protease activity was determined as described by Ellis et al. [45] with some modifications. Briefly, 10 µL of plasma were incubated with the same volume of trypsin solution (5 $mg.mL^{-1}$ in $NaHCO_3$, 5 $mg.mL^{-1}$, pH 8.3) for 10 min at 22 °C in 1.5 mL. After incubation, 100 µL of phosphate buffer (NaH_2PO_4, 13.9 $mg.mL^{-1}$, pH 7.0) and 125 µL of azocasein solution (20 $mg.mL^{-1}$ in $NaHCO_3$, 5 $mg.mL^{-1}$, pH 8.3) were added and incubated for 1 h at 22 °C. Finally, 250 µL of trichloroacetic acid were added to the reaction mixture and incubated for 30 min at 22 °C. The mixture was centrifuged at 10,000 × *g* for 5 min at room temperature. Afterwards, 100 µL of the supernatant was transferred to a 96 well-plate and mixed with 100 µL of NaOH (40 $mg.mL^{-1}$). The OD

was read at 450 nm in a Synergy HT microplate reader. Phosphate buffer in place of plasma and trypsin served as blank, whereas the reference sample was phosphate buffer in place of plasma. Sample inhibition percentage of trypsin activity was calculated as follows: 100 – ((sample absorbance/Reference absorbance)x100). All analyses were conducted in duplicates.

Complement pathway (ACH_{50})

Alternative complement pathway (ACP) activity was estimated as described by Sunyer and Tort [46]. The following buffers were used: GVB (isotonic veronal buffered saline), pH 7.3, containing 0.1 % gelatin; EDTA-GVB, which is GVB with the addition of 20 mM EDTA; and Mg-EGTA-GVB, which is GVB with 10 mM $MgCl_2$ and 10 mM EGTA. Horse red blood cells (HRBC; Probiologica Lda, Portugal) were used for ACP determination. HRBC were washed four times in GVB and resuspended in GVB to a concentration of 2.5×10^8 cells.mL^{-1}. 10 µL of HRBC suspension were then added to 40 µL of serially diluted plasma or mucus in Mg-EGTA-GVB buffer. Samples were incubated at room temperature for 100 min with regular shaking. The reaction was stopped by adding 150 µL of cold EDTA-GVB. Samples were then centrifuged and haemolysis was estimated by measuring the OD of the supernatant at 414 nm in a Synergy HT microplate reader (Biotek). The ACH50 units were defined as the concentration of plasma giving 50% haemolysis of HRBC. All analyses were conducted in triplicates.

2.8. Gene expression

Total RNA from target tissues (liver, head-kidney, white skeletal muscle) was extracted using the MagMAX^{TM}-96 for microarrays total RNA isolation kit (Life Technologies, Carlsbad, CA, USA) after tissue homogenization in TRI reagent. RNA yield in all

tissues was 50–100 μg determined by Nanodrop (Thermo Scientific, Wilmington, DE, USA) 260 and 280 nm UV absorbance ratios (A260/280) of 1.9–2.1. Reverse transcription (RT) of 500 ng total RNA was performed with random decamers using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. RT reactions were incubated for 10 min at 25 ºC and 2 h at 37 ºC. Negative control reactions were run without reverse transcriptase. Real-time quantitative PCR was carried out on a CFX96 Connect™ 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 Supplemental Table S1). Genes comprised in the arrays were selected for their involvement in fish growth, antioxidant status and health performance. Specific primer pair sequences are listed in Supplemental Table S2.

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 obtain the equivalent concentration of 660 pg of total input RNA which were used in a 25 μL volume for each PCR reaction. PCR-wells contained a 2× SYBR Green Master Mix (Bio-Rad) and specific primers at a final concentration of 0.9 μM 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 than 90%, 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 0.5 °C/10 s 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 (Livak and Schmittgen, 2001). β-Actin (*actb*) 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 expression ratio between BC or WC and CTRL fish. For comparing the mRNA 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 white skeletal muscle and head kidney gene expression was in reference to *igfr2* and *il-7*, respectively, which was arbitrarily assigned a value of 1.

2.9. Data analysis

All results are expressed as mean ± standard error (mean ± SE). All residuals were tested for normality (Shapiro-Wilk´s test) and homogeneity of variance (Levene's test). When residuals did not meet the assumptions, data was transformed before analysis to account for this. Mixed-effect ANOVAs were performed, with "time" and "diet" (and their interaction) as fixed effects and "tank" as a random effect, followed by Tukey post-hoc tests. All statistical analyses were performed using the computer package SPSS for WINDOWS. The level of significance used was $P \leq 0.05$ for all statistical tests.

3. Results

3.1. Growth performance and nutrient intake and utilization

Growth performance and whole-body composition are presented in Table 2. At the end of the 12-week growth trial, final body weight (68.5 – 64.2 g) and growth performance indicators (DGI, FCR and PER) remained unaffected by dietary treatments. All fish showed similar final whole-body composition regardless of dietary treatment.

Nutrient intake and utilization are presented in Table 3. Fish fed CTRL diet showed higher lipid intake. Dry matter and protein intake remained similar among groups. Fish fed WC diet showed higher lipid retention. Protein retention was not affected by the dietary treatments. In addition, time effects (2 weeks *vs* 12 weeks) were observed in all parameters studied in Tables 2 and 3.

3.2. Haematological profile

Haematological parameters such as total WBC and RBC were not affected by dietary treatments (Table 4). Ht increased from 2 to 12 weeks (Table 4) whereas diet WC showed the highest values regardless of time. Hb concentration was higher in fish fed diet BC at 12 weeks when compared to CTRL. Furthermore, Hb concentration increased from 2 to 12 weeks for this dietary treatment (Table 4). MCV increased over time during the feeding trial (Table 4). MCH and MCHC were not affected by the dietary treatments, while MCH increased during the feeding trial only in fish fed BC (Table 4). Peripheral cell dynamics changed from 2 to 12 weeks, increasing for neutrophils and decreasing for lymphocytes and monocytes regardless of dietary treatment (Table 5). Thrombocytes concentration decreased on BC-fed fish after 12 weeks (Table 5).

3.3. Plasma & skin mucus immune parameters

Plasma humoral immune parameters, namely plasma bactericidal activity decreased from 2 to 12 weeks regardless of dietary treatment (Fig. 1A), whereas anti-protease activity (Fig. 1B), alternative complement pathway (ACH_{50}) (Fig. 1C) and peroxidase activity (Fig. 1E) increased over time. Plasma protease activity increased throughout the feeding trial only in WC-fed fish (Fig. 1D). Skin mucus bactericidal activity of BC-fed fish increased over time and at 12 weeks was higher than CTRL fed group (Fig. 2A). Mucus ACH_{50} of CTRL-fed fish increased over time from 2 to 12 weeks whereas BC- and WC-fed groups showed higher complement activity than CTRL at 2 weeks (Fig. 2B). Mucus protease activity remained unchanged throughout the feeding trial (Fig. 2C), while mucus peroxidase activity decreased throughout the feeding trial irrespective of dietary treatment (Fig. 1D).

3.4. Gene expression

From the pathway focused array of analyzed genes it was possible to determine a dietary effect with a tissue-specific pattern. Dietary effects were found for several genes related to the growth hormone/insulin growth factor system (GH/IGF) in liver (Table 6). Insulin-like growth factor II gene (*igf-ii*) was up-regulated in WC-fed fish at 2 weeks when compared to CTRL group. BC-fed fish showed a down-regulation of insulin-like growth factor receptor I and II genes (*igfr1, igfr2*) expression at 2 weeks relative to CTRL and WC-fed fish. Hepatic insulin receptor gene (*insr*) expression was down-regulated at 2 weeks in BC-fed fish compared to CTRL. On a different pathway (cytoplasmic and lysosomal activity) calpain 1 gene (*capn1*) expression was up-regulated in WC-fed fish. Finally, catalase gene (*cat*) expression was up-regulated in WC-fed fish after 2 weeks. Head-kidney gene expression (Table 7) also showed a

dietary effect with interleukin 6 (*il-6*) and alpha-2-macroglobulin (*a2m*) genes up-regulated at an early stage (2 weeks) in BC-fed fish. Muscle tissue (Table 8) showed only a down-regulation of myostatin gene (*mstn*) at 12 weeks in WC-fed fish. Finally, it was also possible to ascertain a clear time effect for several genes involved in different pathways, since most of the genes showed higher expression at 12 weeks especially in muscle (Table 8).

4. Discussion

In the present study, the potential beneficial effects of adding *P. tricornutum* to an extreme sustainability-driven diet formulation (i.e. 0% FM) were explored, either as whole freeze-dried biomass (WC diet) or processed broken cells (BC diet). In the past, *P. tricornutum* has been successfully tested as a FM replacement ingredient and immunostimulant when incorporated as whole cell biomass in fish feeds [26, 38, 47]. Sørensen et al. [26] reported that *P. tricornutum* can replace up to 6% of the FM in Atlantic salmon (*Salmo salar*) feeds without adverse effects on feed utilization and growth performance over a period of 82 days of feeding. Accordingly, in the current study the incorporation of 1% *P. tricornutum* did not negatively affect growth performance over the course of the trial (12 weeks).

Dietary protein in fish feeds has shifted in the last years from marine derived sources to terrestrial ones [48, 49]. This shift, although maintaining good growth performance, has the potential to negatively affect fish immune status and response to stressors [19, 50, 51]. In the current study, health status analysis was based on the haematological profile along with several humoral and cellular defense indicators after feeding dietary treatments. Haematology and peripheral cell dynamics were not strongly affected by the incorporation of 1 % *P. tricornutum*. However, BC-fed fish showed a decrease in total WBC which translated in a lower concentration of thrombocytes at 12 weeks. Although RBC counts did not differ between experimental groups, fish fed BC diet showed a significant increase in Hb concentration at 12 weeks. Higher Hb concentration might indicate an improved O_2 carrying capacity, increasing the animal energy producing potential in case of a stressful situation [52]. Non-specific humoral and cellular parameters were not affected by the supplementation with 1% *P. tricornutum* either as whole (WC) or processed (BC) freeze-dried biomass. However, previous studies in fish

revealed a tendency of dietary microalgae supplementation to stimulate or modulate the immune response. Consequently, a study done with gilthead seabream fed diets containing 10% *Navicula* sp. included either as whole freeze-dried biomass or as a silage preparation (SN) combined with *Lactobacillus sakei* (10^6 CFU g^{-1}), reported immunostimulant effects caused by dietary supplementation [53]. These authors found an increased leucocyte peroxidase, phagocytosis and complement activities in seabream fed the SN diet compared to those fed CTRL after 2 weeks of feeding. Those data appeared to support the use of processed microalgae (SN), where bioactive compounds are readily available for absorption and digestion. However, a probiotic effect cannot be ruled out since SN diet was combined with *L. sakei*, and lactic acid bacteria have already been described to enhance fish innate immune system [54-56].

Cerezuela et al. [38] reported a significant increase in serum complement, phagocytic and respiratory burst activity in gilthead seabream fed diets supplemented with 5 and 10% *P. tricornutum* whole biomass for 4 weeks. Immunostimulant effects were attributed to the presence of β-1,3-glucans. Glucans from *P. tricornutum* have a high degree of structural resemblance with laminarin, a beta glucan extracted from brown macroalgae which stimulates macrophage phagocytic activity [29, 57]. Along with systemic defenses, the skin mucus innate immune defenses were also evaluated in the present study. Pathogens are firstly recognized in mucosal tissues, leading to a local activation of innate immunity components that will in turn activate the overall physiological response [58]. In fish skin, mucus acts as a natural barrier against a wide array of stressors and as a source of lysozyme, complement, lectins and proteolytic enzymes. Contrarily to the humoral parameters, *P. tricornutum* incorporation elicited an early response in mucus innate immune components with BC and WC diet showing increased complement activity at 2 weeks. At 12 weeks BC fed fish also showed higher

bactericidal activity than CTRL. Several authors have tested different plant or herbal based immunostimulants effect on skin mucosal immunity [59-61]. In common carp fed palm fruit extract, skin mucus lysozyme and protease activity were elevated after eight weeks of feeding [61]. Similarly, Guardiola et al. [60], reported an increase of mucus peroxidase and protease activities, as well as an enhancement of the antioxidant status in animals fed diets with 10% fenugreek seeds. Although effects in mucosal immunity seem promising, it is important to keep in mind that in the present study fish were not stimulated by an inflammatory agent or a live bacteria challenge, in addition to the lower level of inclusion (1 %) used in the present study, which might explain the lack of response obtained for most of the humoral parameters.

Transcriptional changes of metabolic, health and growth biomarkers were analyzed in different tissues (i.e. liver, head-kidney and white skeletal muscle). This integrated approach allows an understanding of growth performance and health status, at molecular level, of fish in the given sampling points. From 2 to 12 weeks, several genes were modulated due to *P. tricornutum* dietary supplementation mostly at an early stage (2 weeks). Major impact was found in the GH/IGF system in liver, where Igfs play a key role on animal's growth and development, directly stimulating cell proliferation and differentiation [62, 63]. Previous studies in gilthead seabream juveniles indicated that the somatotropic axis can be affected by changes in feed protein source and level of essential fatty acids [7, 19, 64, 65]. WC-fed fish showed a hepatic upregulation of *igf-ii* at 2 weeks and the downregulation of *mstn* in muscle at the end of the growth trial. Muscle growth in fish depends on myocyte proliferation. Igf-II is a powerful proliferation factor in muscular tissue while Mstn was found to be a potent inhibitor of myoblast proliferation and fiber hypertrophy [66, 67]. Thus, present results point to a positive effect at the transcriptional level in the somatotropic axis from the *P.*

tricornutum WC-supplemented diet. Still, in the present study no tendency for increased final body weight was perceivable at 12 weeks. Sørensen et al. [26] also reported the absence of negative effects after feeding Atlantic salmon for 82 days with a diet where 6 % FM was replaced by *P. tricornutum* whole-cell biomass. However, it is important to point out that the level of microalgae incorporation was higher in the latter study in comparison to the present one. Both Igfs and insulin induce complex effects on metabolism. Igf-I acts primarily as a promoter of cell differentiation and growth and insulin as a regulator to maintain metabolic homeostasis [68, 69]. Still, physiological effects depend on specific binding to the homologous receptor. In gilthead seabream, Igfr-I is mainly expressed in muscle tissue but also in liver, promoting muscle growth and enhanced metabolism [66, 68]. BC-fed fish showed hepatic down regulation of *insr*, *igfr1* and *igfr2* mRNA transcripts, while muscle expression levels were similar among groups. Despite lower hepatic expression levels found in BC-fed fish, it was not possible to ascertain any growth or metabolic impairment between groups. In a different experiment, Ramos-Pinto et al. [19] fed gilthead seabream juveniles with a FM-free diet supplemented with tryptophan, revealing the same trend for early down-regulation of GH/IGF axis in liver without compromising growth performance and metabolism. Additionally, dietary supplementation with *P. tricornutum* whole cells induced changes in other hepatic biological processes such as upregulation of *capn1* at 2 weeks. Calpains are cytoplasmic proteases with a regulatory or signalling function in proteolysis, affecting intracellular protein turnover and muscle growth [70, 71]. Finally, *cat* was up-regulated in WC-fed fish at an early stage. Catalase is an antioxidant enzyme which plays a crucial role in maintaining cell homeostasis. *Phaeodactylum tricornutum* is rich in fucoxanthin, a carotenoid that acts as a ROS scavenger and promotes the translocation of nuclear factor-erythroid 2-related factor 2 (Nrf2). Once inside the

nucleus, Nrf2 regulates the gene expression of several antioxidant enzyme genes [72, 73].

The head-kidney was also evaluated to determine the effect of dietary treatments on the gene expression patterns of several relevant immune-related transcripts. Diet-related effects on gene expression were promoted by the broken cells diet at 2 weeks: BC group was fed the disrupted cell wall biomass, making microalgae cell contents more available to these fish namely β-glucans. In *P. tricornutum*, β-glucans are located inside the cell in specialized organelles termed vacuoles [29]. Once available, these polysaccharides are known to have immunostimulatory effects in fish [57]. At present, the mechanism by which β-glucans are recognized in fish is not fully elucidated, although it is thought that this recognition follows the same pattern as in higher vertebrates. These molecules are recognized by leucocyte surface receptors, mainly by C-type lectin (CLRs) and Toll-like receptors (TLRs), which activate the transcription of the proinflammatory cytokines IL-1β, TNF-α and IL-6 [57, 74]. BC-fed fish showed *il-6* and *a2m* up-regulation. Interleukin 6 is a pleiotropic cytokine, with both pro- and anti-inflammatory functions. During inflammatory processes, this cytokine is produced by activated cells inducing an acute phase response (APR) and the production of acute phase proteins (APP) [75, 76]. Alpha-2-macroglobulin is an APP that acts as a non-specific protease inhibitor involved in host defense mechanisms, inhibiting both endogenous and exogenous proteases.

The use of immunostimulants is generally beneficial for fish health status, but effects depend primarily on nutrient or nutraceutical bioavailability. In the present study, the physical process *P. tricornutum* cells underwent in BC diet seemed to improve this diet immunostimulatory effect when compared to WC diet. Effects occurred mainly at an early stage (2 weeks), which, when compared to previously reported results, suggests that they depend on dose and length of administration. In this study, the level of

incorporation was low (1% or 10 g/kg feed), which can partially explain the mild immunostimulatory effect reported. Nonetheless, results are promising and emphasize the pertinence of further evaluating the inclusion of *P. tricornutum* biomasses in the context of a short-term feeding period before a predictable stressful event or disease outbreak. In future works, different levels of supplementation higher than 1% should be tested, followed by an inflammatory insult, in order to evaluate fish immune response.

Ethics statement

CCMAR facilities and their staff are certified to house and conduct experiments with live animals (Group-C licences 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.

Acknowledgments

This work has been funded under the EU FP7 by the MIRACLES project No. 613588: Multi-product Integrated bioRefinery of Algae: from Carbon dioxide and Light Energy to high-value Specialties and by national funds through FCT - Foundation for Science and Technology within the scope of UIDB/04423/2020 and UIDP/04423/2020. The views expressed in this work are the sole responsibility of the authors and do not necessary reflect the views of the European Commission. B. Reis, L. Ramos-Pinto, B. Costas and S. Engrola were supported by the Portuguese Foundation for Science and Technology (PD/BDE/129262/2017, PD/BDE/114436/2016, IF/00197/2015 and IF/00482/2014, respectively).

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Table 1. Ingredients and proximate composition of experimental diets

	Dietary treatments		
	CTRL	**BC**	**WC**
Ingredients (%)			
Poultry meal[1]	10.00	10.00	10.00
Soy protein concentrate[2]	14.00	14.00	14.00
Wheat gluten[3]	10.44	10.44	10.44
Corn gluten meal[4]	11.00	11.00	11.00
Guar meal[5]	9.00	9.00	9.00
Soybean meal 48[6]	15.20	15.20	15.20
Rapeseed meal[7]	3.00	3.00	3.00
Wheat meal[8]	5.50	4.50	4.50
Fish oil[9]	9.20	9.20	9.20
Soybean oil[10]	3.00	3.00	3.00
Rapeseed oil[10]	3.00	3.00	3.00
Vitamin and mineral premix[11]	1.00	1.00	1.00
Binder[12]	0.20	0.20	0.20
Antioxidant[13]	0.20	0.20	0.20
Sodium propionate[14]	0.10	0.10	0.10
Monocalcium phosphate[15]	3.00	3.00	3.00
L-Histidine[16]	0.30	0.30	0.30
L-Lysine[16]	1.20	1.20	1.20
L-Threonine[16]	0.25	0.25	0.25
L-Tryptophan[16]	0.11	0.11	0.11
DL-Methionine[17]	0.30	0.30	0.30
Phaeodactylum (broken cells)[21]		1.00	
Phaeodactylum (whole cells)[22]			1.00
Proximate composition			
Dry matter (DM), %	93.9	95.6	95.8
Ash, % DM	7.0	8.5	8.5
Crude protein, % DM	49.9	50.4	50.1
Crude fat, % DM	18.9	17.8	18.2
Gross energy (kJ g^{-1} DM)	23.3	23.1	23.0

[1] Poultry meal: 62.4% crude protein (CP), 14.5% crude fat (CF), SAVINOR UTS, Portugal
[2] Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands
[3] VITEN: 82% CP, 2.1% CF, Roquette, France
[4] Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal
[5] Guar Korma: 55.3% CP, 7.8% CF, KFEED Ltd, Bulgary
[6] Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, CARGILL, Spain
[7] Defatted rapeseed meal: 32.7% CP, 4.1% CF, Ribeiro & Sousa Lda, Portugal
[8] Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal
[9] Sopropêche, France
[10] JC Coimbra, Portugal
[11] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/Kg diet): DL-alphatocopherol acetate, 100mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamine, 30 mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1 mg; nicotidin acid, 200 mg; folic acid, 15mg; ascorbic acid, 1000 mg; inositol, 500mg; biotin, 3 mg; calcium panthotenate, 100mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65 mg; 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; calcium carbonate, 1.86 g; excipient wheat middling's
[12] Kieselguhr, LIGRANA GmbH, Germany
[13] Paramega PX, Kemin Europe NV, Belgium
[14] PREMIX LDA., Portugal
[15] ALIPHOS MONOCAL, 22.7% P, ALIPHOS, Belgium
[16] Ajinomoto EUROLYSINE S.A.S., France
[17] Rhodimet NP99, ADISSEO, France
[18] Test Phaeodactylum biomasses: 34% CP, 10% CF, Fitoplancton Marino, Spain

Table 2. Growth performance and whole-body composition of gilthead seabream juveniles fed the dietary treatments for a 2 or 12 weeks period.

	Diets (2 weeks)			Diets (12 weeks)			ANOVA (P<0.05)		
Growth	**CTRL**	**BC**	**WC**	**CTRL**	**BC**	**WC**	**Diet*Time**	**Diet**	**Time**
Final body weight (FBW) (g)	20.07 ± 0.56	20.00 ± 0.38	19.84 ± 0.34	68.45 ± 2.57	66.52 ± 0.75	64.16 ± 1.63	ns	ns	<0.01
Daily growth index (DGI)	2.32 ± 0.17	2.77 ± 0.14	2.33 ± 0.08	1.87 ± 0.06	1.84 ± 0.02	1.79 ± 0.04	ns	ns	<0.01
Feed conversion ratio (FCR)	1.58 ± 0.06	1.57 ± 0.06	1.53 ± 0.03	1.41 ± 0.03	1.44 ± 0.01	1.46 ± 0.02	ns	ns	<0.01
Protein efficiency ratio (PER)	1.38 ± 0.06	1.33 ± 0.05	1.33 ± 0.02	1.55 ± 0.03	1.47 ± 0.01	1.46 ± 0.02	ns	ns	<0.01
Final whole-body composition (% ww)									
Moisture	69.42 ± 0.01	69.01 ± 0.71	68.91 ± 0.40	66.15 ± 0.51	66.58 ± 0.44	65.51 ± 0.23	ns	ns	<0.01
Protein	15.23 ± 0.16	15.63 ± 0.18	15.66 ± 0.26	17.04 ± 0.28	16.81 ± 0.30	17.03 ± 0.06	ns	ns	<0.01
Fat	9.93 ± 0.21	9.74 ± 0.50	9.83 ± 0.12	13.05 ± 0.71	12.82 ± 0.46	13.85 ± 0.21	ns	ns	<0.01
Ash	3.87 ± 0.24	4.24 ± 0.19	4.24 ± 0.19	1.21 ± 0.05	1.26 ± 0.05	1.21 ± 0.05	ns	ns	<0.01

Initial body weight - 13.2 ± 0.08 g. Initial composition of fish (% ww) – Moisture: 72.69; Protein: 14.11; Fat: 7.90; Ash: 4.51. Values represent mean ± standard error
ns – non significant

Table 3. Nutrient intake and utilization of gilthead seabream juveniles fed the dietary treatments for a 2 or 12 weeks period.

	Diets (2 weeks)			Diets (12 weeks)			ANOVA (P<0.05)			Post-hoc Tukey Diet		
Intake (g/kg ABW/day)	**CTRL**	**BC**	**WC**	**CTRL**	**BC**	**WC**	**Diet*Time**	**Diet**	**Time**	**CTRL**	**BC**	**WC**
Dry matter	41.76 ± 1.31	42.66 ± 1.46	41.53 ± 2.14	20.16 ± 0.09	20.57 ± 0.07	20.45 ± 0.07	ns	ns	<0.01	-	-	-
Protein	19.57 ± 0.62	20.53 ± 0.70	19.92 ± 1.03	9.45 ± 0.04	9.90 ± 0.03	9.81 ± 0.04	ns	ns	<0.01	-	-	-
Lipids	8.64 ± 0.27	7.74 ± 0.26	7.33 ± 0.38	4.17 ± 0.02	3.73 ± 0.01	3.61 ± 0.01	ns	<0.01	<0.001	A	B	B
Retention (% of intake)												
Protein	24.06 ± 0.24	24.64 ± 1.33	25.53 ± 0.58	27.54 ± 0.84	25.68 ± 0.61	26.03 ± 0.37	ns	ns	0.02	-	-	-
Lipids	43.55 ± 3.07	46.66 ± 5.12	50.45 ± 0.10	50.23 ± 3.76	54.70 ± 2.30	61.16 ± 0.60	ns	0.04	<0.01	B	AB	A

Values represent mean ± standard error. Different capital letters represent significant differences between diets regardless of time (P<0.05). ABW – average body weight.
ns – non significant.

Table 4. Haematocrit, haemoglobin, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cells (RBC) and white blood cells (WBC) in gilthead seabream juveniles fed the dietary treatments for a 2 or 12 weeks period.

	Diets (2 weeks)			Diets (12 weeks)			ANOVA (P<0.05)			Post-hoc Tukey Diet		
Haematology	**CTRL**	**BC**	**WC**	**CTRL**	**BC**	**WC**	**Diet*Time**	**Diet**	**Time**	**CTRL**	**BC**	**WC**
Haematocrit (%)	31.0 ± 1.32	32.2 ± 1.46	35.0 ± 1.55	36.4 ± 1.27	39.0 ± 1.02	39.4 ± 1.58	ns	0.02	<0.01	B	A.B	A
Haemoglobin (g/dL)	2.1 ± 0.17	2.0 ± 0.11*	2.5 ± 0.21	2.2 ± 0.16 ^{b}	3.1 ± 0.26$^{\#\ a}$	2.7 ± 0.22 ^{a,b}	0.04	0.05	0.01	-	-	-
MCV (µm^3)	114.2 ± 5.58	107.4 ± 5.51	114.6 ± 5.57	131.3 ± 8.24	134.9 ± 9.64	123.0 ± 5.50	ns	ns	<0.01	-	-	-
MCH (pg/cell)	7.6 ± 0.57	6.8 ± 0.67*	8.4 ± 0.66	7.9 ± 0.63	10.6 ± 0.74$^{\#}$	8.2 ± 0.55	0.01	ns	0.03	-	-	-
MCHC (g/100 mL)	6.6 ± 0.31	6.4 ± 0.48	7.3 ± 0.46	6.0 ± 0.32	8.0 ± 0.61	6.7 ± 0.53	ns	ns	ns	-	-	-
WBC (x10^4 /µL)	6.7 ± 0.84	8.5 ± 0.64*	6.1 ± 0.52	5.2 ± 0.42	4.8 ± 0.37$^{\#}$	5.2 ± 0.37	0.04	ns	<0.01	-	-	-
RBC (x10^6 /µL)	2.8 ± 0.13	3.0 ± 0.15	3.0 ± 0.12	2.8 ± 0.17	3.0 ± 0.21	3.3 ± 0.17	ns	ns	ns	-	-	-

Values represent mean ± standard error. Different superscript letters represent significant differences between diets within the same time (P<0.05). Different superscript symbols represent significant differences in time within the same diet (P<0.05). Different capital letters represent significant differences between diets regardless of time (P<0.05).

ns – non significant

Table 5. Absolute values of peripheral blood leucocytes (thrombocytes, lymphocytes, monocytes and neutrophils) in gilthead seabream juveniles fed the dietary treatments for a 2 or 12 weeks period.

	Diets (2 weeks)			Dies (12 weeks)			ANOVA (P<0.05)		
Peripheral blood leucocytes	**CTRL**	**BC**	**WC**	**CTRL**	**BC**	**WC**	**Diet*Time**	**Diet**	**Time**
Thrombocytes (x10^4 /µL)	4.1 ± 0.47	5.7 ± 0.42*	3.9 ± 0.35	3.9 ± 0.32	3.4 ± 0.28$^{\#}$	4.1 ± 0.26	0.03	ns	0.02
Lymphocytes (x10^4 /µL)	1.8 ± 0.31	2.0 ± 0.16	1.5 ± 0.24	0.4 ± 0.08	0.4 ± 0.04	0.4 ± 0.07	ns	ns	<0.001
Monocytes (x10^4 /µL)	0.2 ± 0.05	0.2 ± 0.07	0.2 ± 0.04	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.01	ns	ns	<0.001
Neutrophils (x10^4 /µL)	0.4 ± 0.13	0.5 ± 0.08	0.5 ± 0.08	0.8 ± 0.09	0.9 ± 0.12	0.7 ± 0.09	ns	ns	<0.001

Values represent mean ± standard error. Different superscript symbols represent significant differences in time within the same diet (P<0.05).
ns – non significant

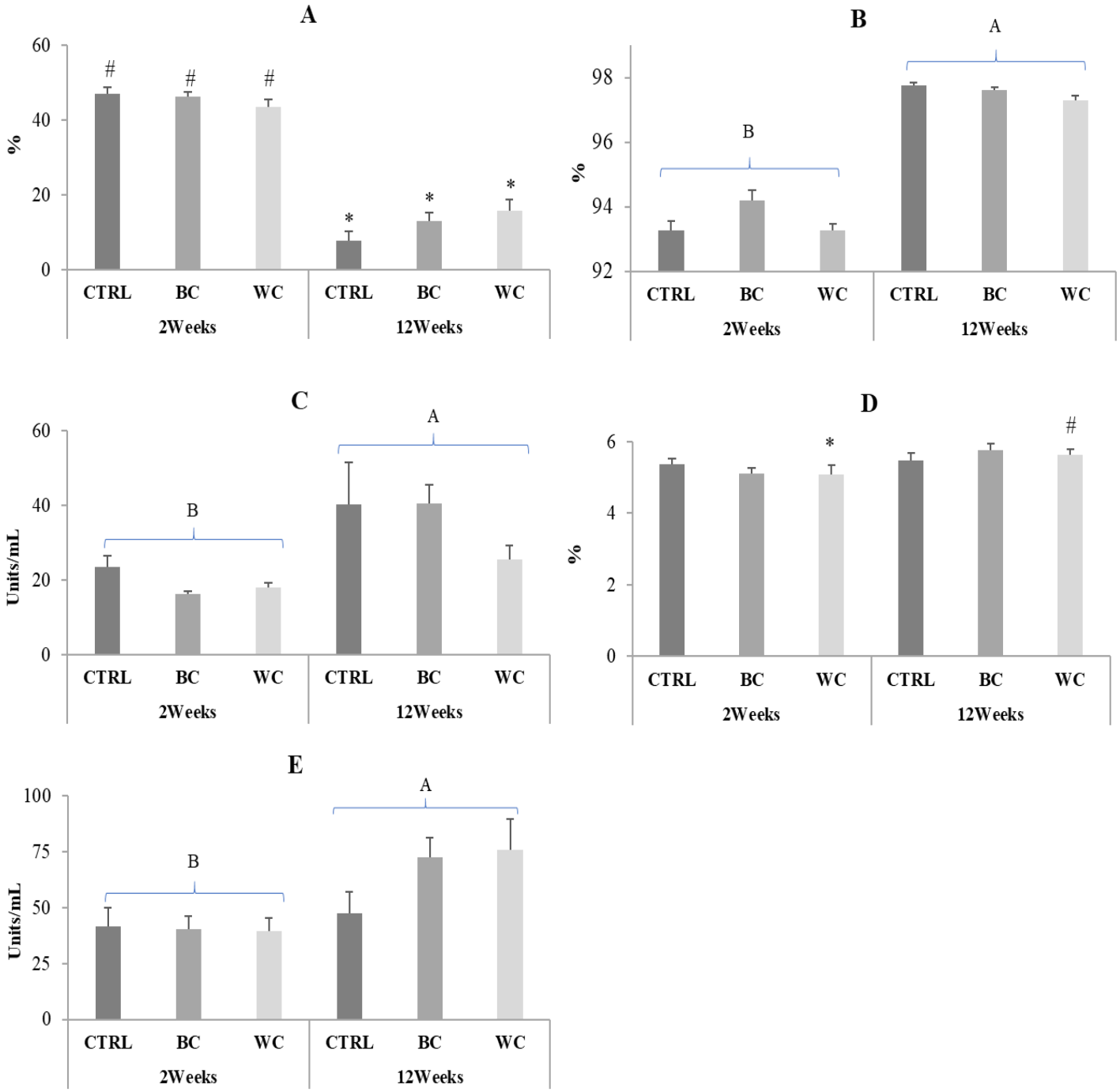

Fig. 1 - Plasma innate humoral parameters in gilthead seabream juveniles fed the dietary treatments for a 2 or 12 weeks period: Bactericidal activity (A); Anti-protease activity (B); Alternative complement pathway (C); Protease activity (D) and Peroxidase activity (E). Values represent mean ± standard error. Different superscript symbols represent significant differences in time within the same diet (P<0.05). Different capital letters represent significant differences between diets regardless of time (P<0.05).

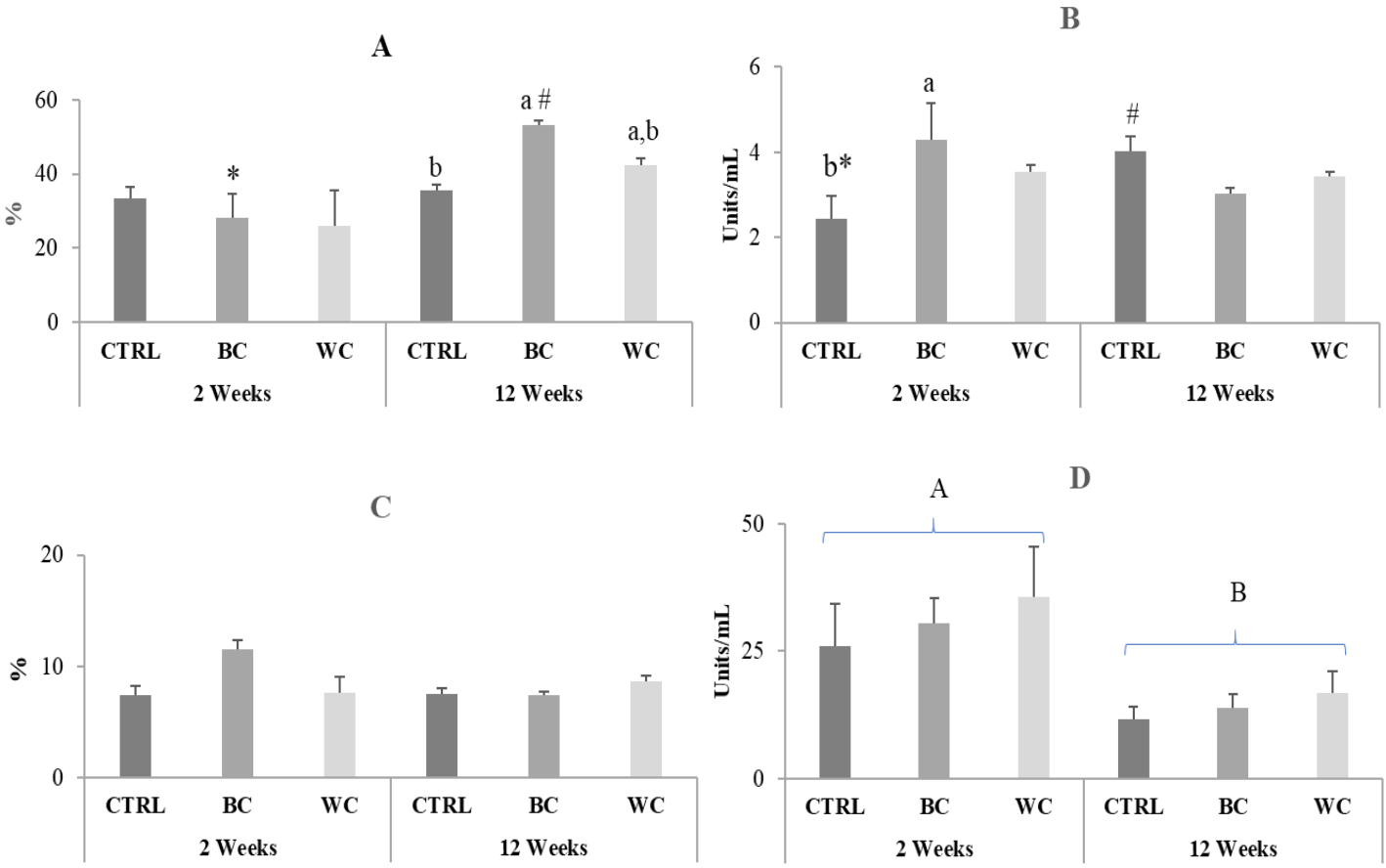

Fig. 2 - Mucus innate parameters in gilthead seabream juveniles fed the dietary treatments for a 2 or 12 weeks period: Bactericidal activity (A); Alternative complement pathway (B); Protease activity (C) and Peroxidase activity (D). Values represent mean ± standard error. Different superscript letters represent significant differences between diets within the same time (P<0.05). Different superscript symbols represent significant differences in time within the same diet (P<0.05). Different capital letters represent significant differences between diets regardless of time (P<0.05).

Table 6. Liver gene expression profile of gilthead seabream juveniles in response to the different dietary treatments at 2 and 12 weeks feeding time.

		Diets (2 weeks)			Diets (12 weeks)			ANOVA (P<0.05)		
Biological Process	**Genes**	**CTRL**	**BC**	**WC**	**CTRL**	**BC**	**WC**	**Diet*Time**	**Diet**	**Time**
GH/IGF Axis	*ghr-i*	1.17 ± 0.22	1.42 ± 0.20	1.38 ± 0.22	1.96 ± 0.18	2.39 ± 0.27	2.12 ± 0.23	ns	ns	<0.01
	ghr-ii	1.39 ± 0.11	1.63 ± 0.17	1.60 ± 0.12	0.88 ± 0.09	1.24 ± 0.18	0.99 ± 0.14	ns	ns	<0.01
	igf-i	5.75 ± 0.69	6.20 ± 0.83	6.79 ± 0.72	8.76 ± 1.24	7.64 ± 0.59	6.87 ± 0.64	ns	ns	<0.01
	igf-ii	2.01 ± 0.26*^{b}	2.62 ± 0.63*^{a,b}	4.33 ± 0.45^{a}	4.72 ± 0.53$^{\#}$	6.94 ± 1.32$^{\#}$	5.34 ± 0.74	0.02	ns	<0.01
	igfbp1a	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	ns	ns	<0.01
	igfbp2b	2.12 ± 0.15	2.28 ± 0.24	3.19 ± 0.32	1.42 ± 0.13	1.46 ± 0.10	1.45 ± 0.11	ns	ns	<0.01
	igfbp4	0.68 ± 0.06	0.61 ± 0.07	0.76 ± 0.07*	0.54 ± 0.05	0.62 ± 0.05	0.48 ± 0.04$^{\#}$	0.01	ns	<0.01
	igfr1	0.10 ± 0.01^{a}	0.04 ± 0.01^{b}	0.08 ± 0.01^{a}	0.08 ± 0.01	0.06 ± 0.00	0.07 ± 0.01	0.02	0.01	ns
	igfr2	0.28 ± 0.03^{a}	0.12 ± 0.01*^{b}	0.22 ± 0.04^{a}	0.22 ± 0.02	0.21 ± 0.02$^{\#}$	0.23 ± 0.04	0.01	ns	ns
	insr	0.96 ± 0.08^{a}	0.52 ± 0.06^{b}	0.77 ± 0.11^{a,b}	0.70 ± 0.08	0.65 ± 0.05	0.72 ± 0.07	0.01	ns	ns
Cytoplasmic & lysosomal protease activity	*capn1*	0.15 ± 0.01^{b}	0.19 ± 0.02*^{a,b}	0.27 ± 0.02*^{a}	0.12 ± 0.01	0.11 ± 0.01$^{\#}$	0.10 ± 0.01$^{\#}$	<0.01	ns	<0.01
	cast	0.28 ± 0.02	0.33 ± 0.02	0.26 ± 0.02	0.53 ± 0.05	0.54 ± 0.05	0.52 ± 0.04	ns	ns	<0.01
	ctsb	1.76 ± 0.14	2.03 ± 0.21	1.97 ± 0.25	1.86 ± 0.16	2.22 ± 0.16	2.16 ± 0.18	ns	ns	ns
	ctsd	0.17 ± 0.02	0.18 ± 0.02	0.17 ± 0.02	1.03 ± 0.19	1.24 ± 0.26	0.95 ± 0.25	ns	ns	<0.01
	ctsl	6.74 ± 0.53	7.89 ± 0.88	7.88 ± 0.84	11.50 ± 0.86	12.59 ± 1.05	11.67 ± 0.68	ns	ns	<0.01
Energy sensing and oxidative metabolism	*pgc1α*	0.32 ± 0.04	0.42 ± 0.05	0.59 ± 0.11	0.17 ± 0.01	0.16 ± 0.02	0.17 ± 0.03	ns	ns	<0.01
	cpt1a	0.93 ± 0.06	0.98 ± 0.07	1.14 ± 0.08	1.09 ± 0.09	1.15 ± 0.07	1.11 ± 0.10	ns	ns	ns
	cs	0.43 ± 0.03	0.49 ± 0.03	0.50 ± 0.04	0.80 ± 0.06	0.79 ± 0.05	0.85 ± 0.07	ns	ns	<0.01
	hif-1α	0.55 ± 0.03	0.56 ± 0.02	0.63 ± 0.04	0.36 ± 0.03	0.35 ± 0.01	0.36 ± 0.02	ns	ns	<0.01
Respiration uncoupling	*ucp1*	15.18 ± 1.17	15.30 ± 1.37	19.00 ± 1.06	8.77 ± 0.88	9.80 ± 1.13	9.43 ± 0.84	ns	ns	<0.01
Antioxidant defences	*mthsp70/grp-75*	0.53 ± 0.08	0.58 ± 0.06	0.60 ± 0.07	0.70 ± 0.08	0.68 ± 0.11	0.75 ± 0.09	ns	ns	0.03
	grp-170	1.24 ± 0.15	1.29 ± 0.23	0.98 ± 0.16	1.13 ± 0.15	1.24 ± 0.19	1.05 ± 0.10	ns	ns	ns
	grp-94	3.83 ± 0.68	4.88 ± 1.00	2.41 ± 0.38	1.47 ± 0.24	2.48 ± 0.43	1.65 ± 0.22	ns	ns	<0.01
	cat	10.86 ± 0.95^{b}	11.01 ± 0.52^{b}	15.71 ± 0.94^{a}	13.15 ± 1.24	13.45 ± 1.31	12.81 ± 0.79	0.03	ns	ns
	gpx1	1.08 ± 0.06	1.29 ± 0.12	1.28 ± 0.10	0.96 ± 0.05	1.13 ± 0.06	1.09 ± 0.10	ns	ns	0.02
	gpx4	4.08 ± 0.65	3.70 ± 0.40	5.75 ± 0.46	13.82 ± 2.19	14.18 ± 1.73	14.42 ± 2.09	ns	ns	<0.01
	gr	0.24 ± 0.01	0.28 ± 0.02	0.28 ± 0.02	0.35 ± 0.02	0.35 ± 0.02	0.35 ± 0.02	ns	ns	<0.01
	prdx3	0.45 ± 0.03	0.52 ± 0.04	0.53 ± 0.04	0.68 ± 0.06	0.72 ± 0.08	0.71 ± 0.05	ns	ns	<0.01
	prdx5	0.29 ± 0.04	0.29 ± 0.04	0.27 ± 0.03	1.13 ± 0.12	1.05 ± 0.04	1.18 ± 0.14	ns	ns	<0.01
	mn-sod / sod2	0.80 ± 0.07	0.92 ± 0.09	0.91 ± 0.09	0.77 ± 0.06	0.76 ± 0.09	0.83 ± 0.05	ns	ns	ns
	h-fabp	26.47 ± 2.04	26.17 ± 1.52	31.19 ± 3.05	45.78 ± 4.31	46.60 ± 3.54	51.95 ± 4.71	ns	ns	<0.01

Values represent mean ± standard error (n=9) (Raw data). Different superscript letters represent significant differences between diets within the same time (P<0.05). Different superscript symbols represent significant differences in time within the same diet (P<0.05).

ns – non significant

Table 7. Head-kidney gene expression profile of gilthead seabream juveniles in response to the different dietary treatments at 2 and 12 weeks feeding time

		Diets (2 weeks)			Diets (12 weeks)			ANOVA (P<0.05)		
Biological Process	**Genes**	**CTRL**	**BC**	**WC**	**CTRL**	**BC**	**WC**	**Diet*Time**	**Diet**	**Time**
Interleukins/cytokines	*il-1β*	0.09 ± 0.02	0.10 ± 0.02	0.09 ± 0.02	0.03 ± 0.00	0.04 ± 0.01	0.02 ± 0.00	ns	ns	<0.01
	il-6	0.02 ± 0.00*$^{a.b}$	0.04 ± 0.01^{a}	0.02 ± 0.00^{b}	0.05 ± 0.01$^{\#}$	0.04 ± 0.00	0.04 ± 0.01	0.03	ns	<0.01
	il-7	1.04 ± 0.15	1.46 ± 0.18*	1.10 ± 0.15	1.00 ± 0.08	0.75 ± 0.08$^{\#}$	0.66 ± 0.07	0.03	ns	<0.01
	il-8	0.05 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	ns	ns	<0.01
	il-10	0.47 ± 0.03	0.58 ± 0.05	0.67 ± 0.05	0.67 ± 0.05	0.54 ± 0.07	0.55 ± 0.09	ns	ns	ns
	il-12	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.02	0.05 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	ns	ns	ns
	il-15	0.23 ± 0.02	0.28 ± 0.03	0.24 ± 0.03	0.33 ± 0.02	0.22 ± 0.02	0.25 ± 0.03	ns	ns	ns
	il-34	1.11 ± 0.12	1.24 ± 0.09	1.13 ± 0.10	2.18 ± 0.15	1.90 ± 0.16	1.76 ± 0.20	ns	ns	<0.01
	tnf-α	0.14 ± 0.02	0.17 ± 0.09	0.15 ± 0.02	0.17 ± 0.02	0.13 ± 0.16	0.14 ± 0.01	ns	ns	ns
Macrophages/monocytes chemokines	*csf1r1*	1.73 ± 0.19*	2.14 ± 0.12	1.89 ± 0.08	2.90 ± 0.22$^{\#}$	2.58 ± 0.19	2.33 ± 0.18	0.02	ns	<0.01
	ccr3	4.85 ± 0.59	4.55 ± 0.31	4.21 ± 0.30	5.71 ± 0.35	4.83 ± 0.33	4.58 ± 0.56	ns	ns	ns
	ck8 / ccl20	0.36 ± 0.06	0.36 ± 0.05	0.41 ± 0.09	1.30 ± 0.72	0.48 ± 0.07	0.48 ± 0.06	ns	ns	<0.01
Immunoglobulins	*igm*	76.45 ± 7.40	78.78 ± 9.83	73.14 ± 8.04	129.14 ± 16.21	107.61 ± 16.79	96.53 ± 14.03	ns	ns	0.02
	igt	0.67 ± 0.42	3.10 ± 1.20	1.64 ± 0.93	4.82 ± 1.48	2.44 ± 0.72	3.58 ± 2.35	ns	ns	ns
	igt-m	9.16 ± 0.96	10.41 ± 1.23	11.33 ± 2.37	8.35 ± 1.06	7.06 ± 1.00	10.73 ± 2.53	ns	ns	ns
	migm	12.86 ± 1.24	14.55 ± 0.92	14.17 ± 1.39	17.73 ± 1.17	14.67 ± 2.01	18.09 ± 2.704	ns	ns	ns
Anti-protease	*a2m*	0.10 ± 0.04^{b}	0.20 ± 0.02^{a}	0.13 ± 0.01^{a,b}	0.09 ± 0.02	0.11 ± 0.03	0.08 ± 0.02	0.04	ns	0.03
Antimicrobial peptide/ Iron recycling	*hepc*	67.75 ± 10.00	70.28 ± 11.93	58.88 ± 5.06	10.91 ± 1.88	15.15 ± 5.27	7.31 ± 1.92	ns	ns	<0.01
T-cell markers	*cd3e*	2.33 ± 0.37	2.71 ± 0.16	3.15 ± 0.78	3.04 ± 0.13	2.71 ± 0.37	2.83 ± 0.33	ns	ns	ns
	cd3x	2.00 ± 0.23	2.41 ± 0.52	2.06 ± 0.45	2.62 ± 0.19	2.27 ± 0.31	2.47 ± 0.29	ns	ns	ns
	cd4-full	1.51 ± 0.23	2.21 ± 0.68	1.94 ± 0.66	2.05 ± 0.13	1.51 ± 0.19	1.92 ± 0.27	ns	ns	ns
	cd8a	1.28 ± 0.24	1.88 ± 0.60	1.57 ± 0.60	1.19 ± 0.10	0.89 ± 0.14	0.98 ± 0.16	ns	ns	ns
	cd8b	0.62 ± 0.16	0.57 ± 0.11	0.43 ± 0.05	0.35 ± 0.04	0.26 ± 0.04	0.28 ± 0.04	ns	ns	<0.01
	zap70	1.55 ± 0.19	1.62 ± 0.24	1.47 ± 0.22	2.01 ± 0.18	1.61 ± 0.19	1.95 ± 0.26	ns	ns	ns
Pattern recognition receptors	*tlr1*	1.16 ± 0.05	1.46 ± 0.13*	1.30 ± 0.10	1.26 ± 0.06	1.04 ± 0.07$^{\#}$	1.13 ± 0.10	0.03	ns	0.04
	tlr2	1.44 ± 0.12	1.91 ± 0.07	1.69 ± 0.08	3.05 ± 0.15	2.91 ± 0.28	3.17 ± 0.38	ns	ns	<0.01
	tlr5	0.32 ± 0.04	0.29 ± 0.02	0.27 ± 0.02	0.48 ± 0.07	0.45 ± 0.03	0.48 ± 0.04	ns	ns	<0.01
	tlr9	0.26 ± 0.03	0.27 ± 0.02	0.30 ± 0.04	0.79 ± 0.10	0.61 ± 0.09	0.69 ± 0.09	ns	ns	<0.01
	mrc1	5.18 ± 0.66	5.63 ± 0.44	5.17 ± 0.44	5.83 ± 0.32	5.10 ± 0.30	4.16 ± 0.31	ns	ns	ns

Values represent mean ± standard error (n=9) (Raw data). Different superscript letters represent significant differences between diets within the same time (P<0.05). Different superscript symbols represent significant differences in time within the same diet (P<0.05).

ns – non significant

Table 8. Skeletal muscle gene expression profile of gilthead seabream juveniles in response to the different dietary treatments at 2 and 12 weeks feeding time.

		Diets (2 weeks)			Diets (12 weeks)			ANOVA (P<0.05)		
Biological Process	**Genes**	**CTRL**	**BC**	**WC**	**CTRL**	**BC**	**WC**	**Diet*Time**	**Diet**	**Time**
GH/IGF axis	*ghr-i*	3.17 ± 0.33	3.85 ± 0.55	3.28 ± 0.30	9.99 ± 1.27	10.92 ± 0.97	9.02 ± 1.21	ns	ns	<0.01
	ghr-ii	4.73 ± 0.71	6.68 ± 1.20	4.96 ± 0.71	3.97 ± 0.66	3.58 ± 0.70	3.42 ± 0.53	ns	ns	0.01
	igf-i	0.16 ± 0.01	0.15 ± 0.03	0.16 ± 0.02	0.29 ± 0.04	0.23 ± 0.04	0.20 ± 0.04	ns	ns	<0.01
	igf-ii	1.34 ± 0.12	1.31 ± 0.18	1.30 ± 0.08	2.60 ± 0.24	2.42 ± 0.22	1.94 ± 0.21	ns	ns	<0.01
	igfbp3	3.69 ± 0.30$^{\#}$	3.61 ± 0.31$^{\#}$	4.13 ± 0.11$^{\#}$	1.80 ± 0.18*	1.36 ± 0.12*	1.28 ± 0.15*	0.03	ns	<0.01
	igfbp5b	1.64 ± 0.11	1.84 ± 0.33	1.40 ± 0.05	3.57 ± 0.38	2.90 ± 0.31	2.28 ± 0.18	ns	ns	<0.01
	igfbp6b	0.28 ± 0.03	0.20 ± 0.02	0.30 ± 0.04	0.32 ± 0.04	0.22 ± 0.03	0.19 ± 0.04	0.04	ns	ns
	insr	1.65 ± 0.16	1.73 ± 0.21	2.01 ± 0.25	2.25 ± 0.19	2.24 ± 0.22	1.82 ± 0.20	ns	ns	ns
	igfr1	1.40 ± 0.09	1.48 ± 0.21	1.31 ± 0.11	2.85 ± 0.20	2.98 ± 0.22	2.69 ± 0.28	ns	ns	<0.01
	igfr2	0.98 ± 0.10	1.21 ± 0.30	0.85 ± 0.09	1.34 ± 0.09	1.73 ± 0.35	1.29 ± 0.12	ns	ns	<0.01
Muscle growth and differentiation	*myod1*	10.87 ± 0.53	11.33 ± 1.46	11.46 ± 1.07	13.90 ± 1.60	12.61 ± 0.96	11.56 ± 0.65	ns	ns	ns
	myod2	2.04 ± 0.41	2.37 ± 0.30	2.39 ± 0.26	2.28 ± 0.22	2.01 ± 0.26	1.86 ± 0.35	ns	ns	ns
	myf5	0.47 ± 0.03	0.48 ± 0.06	0.47 ± 0.04	0.47 ± 0.03	0.35 ± 0.02	0.37 ± 0.03	ns	ns	0.01
	myf6	0.45 ± 0.03	0.47 ± 0.08	0.44 ± 0.03	0.75 ± 0.06	0.73 ± 0.05	0.65 ± 0.06	ns	ns	<0.01
	mstn	2.16 ± 0.23	2.93 ± 0.72	2.36 ± 0.28	6.38 ± 1.65^{a}	6.10 ± 1.29^{a}	2.03 ± 0.36^{b}	0.02	ns	<0.01
	mef2a	15.43 ± 1.14	16.90 ± 1.67	17.57 ± 1.69	42.76 ± 3.10	44.70 ± 3.73	41.50 ± 2.95	ns	ns	<0.01
	mef2c	5.94 ± 0.22	6.38 ± 0.76	6.05 ± 0.56	12.08 ± 1.21	12.30 ± 0.88	10.92 ± 0.98	ns	ns	<0.01
	fst	0.67 ± 0.08	0.74 ± 0.08	0.77 ± 0.11	0.57 ± 0.07	0.57 ± 0.06	0.54 ± 0.06	ns	ns	<0.01
Energy sensing & oxidative metabolism	*sirt1*	0.37 ± 0.02	0.43 ± 0.06	0.36 ± 0.03	0.56 ± 0.06	0.59 ± 0.04	0.54 ± 0.03	ns	ns	<0.01
	sirt2	0.48 ± 0.02	0.47 ± 0.06	0.46 ± 0.04	0.75 ± 0.06	0.74 ± 0.07	0.61 ± 0.04	ns	ns	<0.01
	sirt5	1.03 ± 0.08	1.19 ± 0.21	1.08 ± 0.10	1.16 ± 0.10	1.16 ± 0.14	1.07 ± 0.10	ns	ns	ns
	cpt1a	10.72 ± 0.37	12.81 ± 2.67	11.09 ± 0.86	22.94 ± 1.91	23.17 ± 2.22	16.58 ± 1.56	ns	ns	<0.01
	cs	25.09 ± 1.58	30.98 ± 5.76	26.85 ± 2.41	36.41 ± 2.49	40.95 ± 3.07	33.17 ± 2.77	ns	ns	<0.01
	nd2	44.81 ± 3.28	62.25 ± 16.12	47.24 ± 6.55	88.01 ± 15.03	86.45 ± 9.55	75.30 ± 8.90	ns	ns	<0.01
	nd5	26.63 ± 1.85	35.33 ± 7.91	26.71 ± 3.14	45.55 ± 7.20	42.46 ± 4.66	38.70 ± 4.04	ns	ns	<0.01
	cox i	239.75 ± 17.83	292.41 ± 64.62	282.61 ± 26.74	320.82 ± 27.86	318.17 ± 27.74	274.24 ± 30.86	ns	ns	ns
	cox ii	123.85 ± 6.90	170.12 ± 42.93	134.07 ± 13.09	146.09 ± 23.72	132.80 ± 10.66	125.64 ± 14.72	ns	ns	ns
Respiration uncoupling	*ucp3*	14.43 ± 1.93	11.73 ± 2.44	13.47 ± 1.99	29.40 ± 4.92	37.71 ± 3.50	30.81 ± 3.09	ns	ns	<0.01
	pgc1α	0.58 ± 0.15	0.91 ± 0.60	0.42 ± 0.10	2.47 ± 0.43	2.71 ± 0.65	3.66 ± 0.49	ns	ns	<0.01

Values represent mean ± standard error (n=9) (Raw data). Different superscript letters represent significant differences between diets within the same time (P<0.05). Different superscript symbols represent significant differences in time within the same diet (P<0.05).

ns – non significant

Supplementary Tables

Table S2. Forward (F) and reverse (R) primers used for real-time PCR in liver, head kidney and white muscle.

Gene Name	Symbol	Acc. No.		Primer sequences (5' → 3')
70 kDa heat shock protein, mitochondrial	*mthsp70/grp-75*	DQ524993	F	TCCGGTGTGGATCTGACCAAAGAC
			R	TGTTTAGGCCCAGAAGCATCCATG
Alpha-2-macroglobulin	*a2m*	AY358020	F	TCCTGGGTGACATTCTGGGT
			R	CCGTATGGCATCCTCAGCAG
ß-actin	*actb*	X89920	F	TCCTGCGGAATCCATGAGA
			R	GACGTCGCACTTCATGATGCT
C-C chemokineCK8 / C-C motif chemokine 20	*ck8/ccl20*	GU181393	F	CCGTCCTCATCTGCTTCATACT
			R	GCTCTGCCGTTGATGGAAC
C-C chemokine receptor type 3	*ccr3*	KF857317	F	CTACATCAGCATCACCATACGCATCCT
			R	TGGCACGGCACTTCTCCTTCA
Calpain 1	*capn1*	KF444899	F	CAGAACCACAACGCCGTGAAGTTT
			R	AGGCACTGGGCTTTAAGACTCTCG
Calpastatin	*cast*	KM522786	F	CCCAAACCCGAGCCCACCAT
			R	GACAAGAAGTCCAGAGCGTCTCCAGTA
Carnitine palmitoyltransferase 1A	*cpt1a*	JQ308822	F	GTGCCTTCGTTCGTTCCATGATC
			R	TGATGCTTATCTGCTGCCTGTTTG
Catalase	*cat*	JQ308823	F	TGGTCGAGAACTTGAAGGCTGTC
			R	AGGACGCAGAAATGGCAGAGG
Cathepsin B	*ctsb*	KJ524457	F	TGGTCGAGAACTTGAAGGCTGTC
			R	GGGTCTACTGCCATTCACAT
Cathepsin D	*ctsd*	AF03619	F	CACACTGGGAGACCTGCACTATGTCAATG
			R	ATTGCCAACTTGAAGTCCGTCCATACC
Cathepsin L	*ctsl*	KM522787	F	GGGAACGGATGACCAGCCTTGT
			R	CGGTGTCATTGGCAGAGTTGTAGTTG
CD4-full	*cd4-full*	AM489485	F	TCCTCCTCCTCGTCCTCGTT
			R	GGTGTCTCATCTTCCGCTGTCT
Citrate synthase	*cs*	JX975229	F	TCCAGGAGGTGACGAGCC
			R	GTGACCAGCAGCCAGAAGAG
Cluster of differentiation 3 epsilon chain	*cd3e*	MF175240	F	GGTGTGATGTTCGTCGTCTACAAGTG
			R	TGGCAGCGTGAGTGAGTCCT
Cluster of differentiation 3 zeta chain	*cd3x*	MF175235	F	ATGGCGGTCCAGACGAGGGTTTC
			R	ACCAGCGAGGACAGGACCAGCAG
Cluster of differentiation 8 alpha	*cd8a*	EU921630	F	GCAGCAACGGTAACACGAACG
			R	CCAGTATGAGCGGAGTACAGAACA
Cluster of differentiation 8 beta	*cd8b*	KX231275	F	CCGAAATGTGGAAGACTGGAACTC
			R	CCAGTATGAGCGGAGTACAGAACA
Cytochrome c oxidase subunit I	*coxi*	KC217652	F	GTCCTACTTCTTCTGTCCCTTCCTGTTCT
			R	AGGTTTCGGTCTGTAAGGAGCATTGTAATC
Cytochrome c oxidase subunit II	*coxii*	KC217653	F	ACTGCCTACACAGGACCTTGCC
			R	GTCTGCTTCCAGGAGACGGAATTGT
Fatty acid binding protein, heart	*h-fabp*	JQ308834	F	CTGGGTGTGGGCTTCGCTAC
			R	CTCTGTGTTCTTGATGGTGCTCTG

Follistatin	*fst*	AY544167	F	GGACCAGACAAACAACGCATATTG
			R	CATAGATGATCCCGTCGTTTCCAC
Glucose-regulated protein, 170 kDa	*grp-170*	JQ308821	F	CAGAGGAGGCAGACAGCAAGAC
			R	TTCTCAGACTCAGCATTTCCAGATTTC
Glucose-regulated protein, 94 kDa	*grp-94*	JQ308820	F	AAGGCACAGGCTTACCAGACAG
			R	CTTCAGCATCATCGCCGACTTTC
Glutathione peroxidase 1	*gpx1*	DQ524992	F	GAAGGTGGATGTGAATGGAAAAGATG
			R	CTGACGGGACTCCAAATGATGG
Glutathione peroxidase 4	*gpx4*	AM977818	F	TGCGTCTGATAGGGTCCACTGTC
			R	GTCTGCCAGTCCTCTGTCGG
Glutathione reductase	*gr*	AJ937873	F	TGCGTCTGATAGGGTCCACTGTC
			R	GTCTGCCAGTCCTCTGTCGG
Growth hormone receptor I	*ghr-i*	AF438176	F	ACCTGTCAGCCACCACATGA
			R	TCGTGCAGATCTGGGTCGTA
Growth hormone receptor II	*ghr-ii*	AY573601	F	GAGTGAACCCGGCCTGACAG
			R	GCGGTGGTATCTGATTCATGGT
Hepcidin	*hepc*	AM749960	F	ACTCCTGGAAGATGCCGTATGC
			R	AACTTACACCTCCTGCGTCCAC
Hypoxia inducible factor-1 alpha	*hif-1α*	JQ308830	F	CAGATGAGCCTCTAACTTGTGGAC
			R	TTAGCAAGAATGGTGGCAAGATGAG
Immunoglobulin M	*igm*	JQ811851	F	ACCTCAGCGTCCTTCAGTGTTTATGATGCC
			R	CAGCGTCGTCGTCAACAAGCCAAGC
Immunoglobulin M membrane-bound form	*migm*	KX599199	F	GCTATGGAGGCGGAGGAAGATAACA
			R	CAGCGTCGTCGTCAACAAGCCAAGC
Immunoglobulin T	*igt*	KX599200	F	GCTGTCAAGGTGGCCCCAAAAG
			R	CAACATTCATGCGAGTTACCCTTGGC
Immunoglobulin T membrane-bound form	*igt-m*	KX599201	F	AGACGATGCCAGTGAAGAGGATGAGT
			R	CGAAGGAGGAGGCTGTGGACCA
Insulin receptor	*insr*	KM522774	F	ACGGACAGCAAGAAGGCAGAGAATC
			R	CGAAGGAGGAGGCTGTGGACCA
Insulin-like growth factor binding protein 1a	*igfbp1a*	KM522771	F	ACGGACAGCAAGAAGGCAGAGAATC
			R	CCGTTCCAAGAGTTCACACACCAG
Insulin-like growth factor binding protein 2b	*igfbp2b*	AF377998	F	AGCGATGTGTCCTGAGATAGTGAG
			R	GCACCGTGGCGTGTAGACC
Insulin-like growth factor binding protein 3	*igfbp3**	MH577191 MH577192	F	ACA GTG CCG TCC ATC CAA
			R	GCT GCC CGT ATT TGT CCA
Insulin-like growth factor binding protein 4	*igfbp4*	KM658998	F	GGCATCAAACACCCGCACAC
			R	ATCCACGCACCAGCACTTCC
Insulin-like growth factor binding protein 5b	*igfbp5b*	MH577194	F	CGACAGGGCAGTCAAAGAAGCTAACC
			R	GTCTCGAAGGCATGTGAGCAGAAGG
Insulin-like growth factor binding protein 6b	*igfbp6b*	MH577196	F	GAT TGC TCA CTG CGG ATC
			R	GGA GGG ACA GAC CTT GAA
Insulin-like growth factor receptor I	*igfr1*	KM522775	F	TCAACGACAAGTACGACTACCGCTGCT

			R	CACACTTTCTGGCACTGGTTGGAGGTC
Insulin-like growth factor receptor II	*igfr2*	KM522776	F	ACATTCGGGCAGCACTCCTAAGAT
			R	CCAGTTCACCTCGTAGCGACAGTT
Insulin-like growth factor-I	*igf-i*	AY996779	F	TGTCTAGCGCTCTTTCCTTTCA
			R	AGAGGGTGTGGCTACAGGAGATAC
Insulin-like growth factor-II	*igf-ii*	AY996778	F	TGGGATCGTAGAGGAGTGTTGT
			R	CTGTAGAGAGGTGGCCGACA
Interleukin-1 beta	*il-1β*	AJ419178	F	GCGACCTACCTGCCACCTACACC
			R	TCGTCCACCGCCTCCAGATGC
Interleukin-6	*il-6*	EU244588	F	TCTTGAAGGTGGTGCTGGAAGTG
			R	AAGGACAATCTGCTGGAAGTGAGG
Interleukin-7	*il-7*	JX976618	F	CTATCTCTGTCCCTGTCCTGTGA
			R	TGCGGATGGTTGCCTTGTAAT
Interleukin-8	*il-8*	JX976619	F	CAGCAGAGTCTTCATCGTCACTATTG
			R	AGGCTCGCTTCACTGATGG
Interleukin-10	*il-10*	JX976621	F	AACATCCTGGGCTTCTATCTG
			R	GTGTCCTCCGTCTCATCTG
Interleukin 12 subunit beta	*il12*	JX976624	F	ATTCCCTGTGTGGTGGCTGCT
			R	GCTGGCATCCTGGCACTGAAT
Interleukin-15	*il-15*	JX976625	F	GAGACCAGCGAGCGAAAGGCATCC
			R	GCCAGAACAGGTTACAGGTTGACAGGAA
Interleukin-34	*il-34*	JX976629	F	TCTGTCTGCCTGCTGGTAG
			R	ATGCTGGCTGGTGTCTGG
Macrophage colony-stimulating factor 1 receptor 1	*csf1r1*	AM050293	F	TTGCGTGTGGTGAGGAAGGAAGGT
			R	AGCAGGCAGGGCAGCAGGTA
Macrophage mannose receptor 1	*mrc1*	KF857326	F	CTTCCGACCGTACCTGTACCTACTCA
			R	CGATTCCAGCCTTCCGCACACTTA
Myoblast determination protein 1	*myod1*	AF478568	F	ATGGAGCTGTCGGATATCTCTTTC
			R	GAAGCAGGGGTCATCGTAGAAATC
Myocyte-specific enhancer factor 2A	*mef2a*	KM522777	F	ATGGACGAGAGGAACAGGCAGGTTA
			R	GGCTATCTCACAGTCACATAGTACGCTCAG
Myocyte-specific enhancer factor 2C	*mef2c*	KM522778	F	TAGCAACTCCCACTCTACCAGGACAAG
			R	GGAATACTCGGCACCATAAGAAGTCG
Myogenic factor 5	*myf5*	JN034420	F	GCATGGTTGACAGCAACAGTCCAGTGT
			R	TGTCTTATCGCCCAAAGTGTCGTTCTTCAT
Myogenic factor 6	*myf6/mrf4/herculin*	JN034421	F	GCAGCAATGACAAACCAGAGAGACGGAACA
			R	TGTCTTATCGCCCAAAGTGTCGTTCTTCAT
Myogenic factor MYOD2	*myod2*	AF478569	F	CCAACTGCTCTGATGGCATGATGGATTTC
			R	GACCGTTTGCTTCTCCTGGACTCGTATG
Myostatin/Growth differentiation factor 8	*mstn/gdf-8*	AF258448	F	AAGAGCAGATCATCTACGGCAAGATCC
			R	TCAAGAGCATCCACAACGGTCTACCA
NADH-ubiquinone oxidoreductase chain 2	*nd2*	KC217558	F	TAGGTTGAATGACCATCGTA
			R	GGCTAAGGAGTTGAGGTT
NADH-ubiquinone oxidoreductase chain 5	*nd5*	KC217559	F	CCTAAACGCCTGAGCCCTGG

			R	GCTGTAAACGAGGTGGCTAGAAGG
Peroxiredoxin 3	*prdx3*	GQ252681	F	CCTAAACGCCTGAGCCCTGG
			R	ACCGTTTGGATCAATGAGGAACAGACC
Peroxiredoxin 5	*prdx5*	GQ252683	F	GAGCACGGAACAGATGGCAAGG
			R	TCCACATTGATCTTCTTCACGACTCC
Proliferator-activated receptor gamma coactivator 1 alpha	*pgc1α*	JX975264	F	CGTGGGACAGGTGTAACCAGGACTC
			R	TCCACATTGATCTTCTTCACGACTCC
Sirtuin 1	*sirt1*	KF018666	F	GGTTCCTACAGTTTCATCCAGCAGCACATC
			R	CCTCAGAATGGTCCTCGGATCGGTCTC
Sirtuin 2	*sirt2*	KF018667	F	GAACAATCCGACGACAGCAGTGAAG
			R	AGGTTACGCAGGAAGTCCATCTCT
Sirtuin 5	*sirt5*	KF018670	F	CAGACATCCTAACCCGAGCAGAG
			R	CCACGAGGCAGAGGTCACA
Superoxide dismutase [Mn]	*mn-sod/sod2*	JQ308833	F	CCTGACCTGACCTACGACTATGG
			R	AGTGCCTCCTGATATTTCTCCTCTG
Toll-like receptor 1	*tlr1*	KF857322	F	GGGACCTGCCAGTGTGTAAC
			R	AGTGCCTCCTGATATTTCTCCTCTG
Toll-like receptor 2	*tlr2*	KF857323	F	CATCTGCGACTCTCCTCTCTTCCT
			R	GCGTGGATAGAGTTGGACTTGAG
Toll-like receptor 5	*tlr5*	KF857324	F	TCGCCAATCTGACGGACCTGAG
			R	CAGAACGCCGATGTGGTTGTAAGAC
Toll-like receptor 9	*tlr9*	AY751797	F	GCCTTCCTTGTCTGCTCTTTCT
			R	GCCGTAGAGGTGCTTCAGTAG
Tumor necrosis factor-alpha	*tnf-α*	AJ413189	F	CAGGCGTCGTTCAGAGTCTC
			R	CTGTGGCTGAGAGCTGTGAG
Uncoupling protein 1	*ucp1*	FJ710211	F	GCACACTACCCAACATCACAAG
			R	CGCCGAACGCAGAAACAAAG
Uncoupling protein 3	*ucp3*	EU555336	F	AGGTGCGACTGGCTGACG
			R	TTCGGCATACAACCTCTCCAAAG
Zeta-chain-associated protein kinase 70	*zap70*	MF175239	F	TGGTGAAGGAGGAGATGATGAGG
			R	GCGAACGATGTAGCGGTTGT

(*) Acc. No. MH577191: *igfbp3a*; Acc. No. MH577192:*igfbp3b*. Primers used for *igfbp3* gene expression jointly amplify both *igfbp3a*and *igfbp3b*isoforms.

Table S1. Genes included in the liver (†), head kidney (‡) and white muscle (#) pathway-focused PCR arrays.

Gene name/category	Symbol
GH/IGF system	
Growth hormone receptor I	*ghr-i* †#
Growth hormone receptor II	*ghr-ii* †#
Insulin-like growth factor-I	*igf-i* †#
Insulin-like growth factor-II	*igf-ii* †#
Insulin-like growth factor binding protein 1a	*igfbp1a* †
Insulin-like growth factor binding protein 2b	*igfbp2b* †
Insulin-like growth factor binding protein 3	*igfbp3* #
Insulin-like growth factor binding protein 4	*igfbp4* †
Insulin-like growth factor binding protein 5b	*igfbp5b* #
Insulin-like growth factor binding protein 6b	*igfbp6b* #
Insulin-like growth factor receptor I	*igfr1* †#
Insulin-like growth factor receptor II	*igfr2* †#
Insulin receptor	*insr* †#
Energy sensing and oxidative metabolism	
Sirtuin 1	*sirt1* #
Sirtuin 2	*sirt2* #
Sirtuin 5	*sirt5* #
NADH-ubiquinone oxidoreductase chain 2	*nd2* #
NADH-ubiquinone oxidoreductase chain 5	*nd5* #
Cytochrome c oxidase subunit I	*coxi* #
Cytochrome c oxidase subunit II	*coxii* #
Carnitine palmitoyltransferase 1A	*cpt1a* †#
Citrate synthase	*cs* †#
Proliferator-activated receptor gamma coactivator 1 alpha	*pgc1α* †#
Hypoxia inducible factor-1 alpha	*hif-1α* †
Respiration uncoupling	
Uncoupling protein 1	*ucp1* †
Uncoupling protein 3	*ucp3* #

Gene name/category	Symbol
Muscle growth and cell differentiation	
Myoblast determination protein 1	*myod1* #
Myogenic factor MYOD2	*myod2* #
Myogenic factor 5	*myf5* #
Myogenic factor 6	*myf6/mrf4/ herculin* #
Myostatin/Growth differentiation factor 8	*mstn/gdf-8* #
Myocyte-specific enhancer factor 2A	*mef2a* #
Myocyte-specific enhancer factor 2C	*mef2c* #
Follistatin	*fst* #
Antioxidant defence and molecular chaperons	
Catalase	*cat* †
Glutathione peroxidase 1	*gpx1* †
Glutathione peroxidase 4	*gpx4* †
Glutathione reductase	*gr* †
Peroxiredoxin 3	*prdx3* †
Peroxiredoxin 5	*prdx5* †
Superoxide dismutase [Mn]	*mn-sod/sod2* †
Fatty acid binding protein, heart	*h-fabp* †
Glucose-regulated protein, 170 kDa	*grp-170* †
Glucose-regulated protein, 94 kDa	*grp-94* †
70 kDa heat shock protein, mitochondrial	*mthsp70/grp-75/mortalin* †
Cytoplasmatic and lysosomal proteases	
Calpain 1	*capn1* †
Calpastatin	*cast* †
Cathepsin B	*ctsb* †
Cathepsin D	*ctsd* †
Cathepsin L	*ctsl* †
Macrophages and monocytes chemokines	
Macrophage colony-stimulating factor 1 receptor 1	*csf1r1* ‡
C-C chemokine receptor type 3	*ccr3* ‡
C-C chemokine CK8 / C-C motif chemokine	*ck8/ccl20* ‡

Interleukins and cytokines	
Interleukin-1 beta	*il-1β* ‡
Interleukin-6	*il-6* ‡
Interleukin-7	*il-7* ‡
Interleukin-8	*il-8* ‡
Interleukin-10	*il-10* ‡
Interleukin 12 subunit beta	*il12* ‡
Interleukin-15	*il-15* ‡
Interleukin-34	*il-34* ‡
Tumor necrosis factor-alpha	*tnf-α* ‡

Immunoglobulins	
Immunoglobulin M	*igm* ‡
Immunoglobulin M membrane-bound form	*migm* ‡
Immunoglobulin T	*igt* ‡
Immunoglobulin T membrane-bound form	*igt-m* ‡

Antiprotease	
Alpha-2-macroglobulin	*a2m* ‡

Antimicrobial peptide/Iron recycling	
Hepcidin	*hepc* ‡

T-cell markers	
Cluster of differentiation 3 epsilon chain	*cd3e* ‡
Cluster of differentiation 3 zeta chain	*cd3x* ‡
CD4-full	*cd4-full* ‡
Cluster of differentiation 8 alpha	*cd8a* ‡
Cluster of differentiation 8 beta	*cd8b* ‡
Zeta-chain-associated protein kinase 70	*zap70* ‡

Pattern recognition receptors	
Toll-like receptor 1	*tlr1* ‡
Toll-like receptor 2	*tlr2* ‡
Toll-like receptor 5	*tlr5* ‡
Toll-like receptor 9	*tlr9* ‡
Macrophage mannose receptor 1	*mrc1* ‡