The growth promoting and immunomodulatory effects of a medicinal plant leaf extract obtained from *Salvia officinalis* and *Lippia citriodora* in gilthead seabream (*Sparus aurata*)

Ricardo Salomón[a, b], Joana Firmino[a, b], Felipe E. Reyes-López[c], Karl B. Andree[a], D. González-Silvera[d], M. Angeles Esteban[d], Lluis Tort[c], José C. Quintela[e], José M. Pinilla[e] Eva Vallejos-Vidal[c,f,*], Enric Gisbert[a, *]

[a] IRTA, Centre de Sant Carles de la Ràpita, Aquaculture Program, Carretera Poble Nou, km 5,5 43540 Sant Carles de la Ràpita, Spain

[b] PhD programme in Aquaculture, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

[c] Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

[d] Department of Cell Biology and Histology, Faculty of Biology, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, 30100, Murcia, Spain

[e] Natac Biotech, Calle Electrónica 7, 28923, Alcorcón, Madrid.

[f] Centro de Biotecnología Acuícola, Departamento de Biología, Facultad de Química y Biología, Universidad de Santiago de Chile, Santiago, Chile

[*]Corresponding authors:

E-mail address: enric.gisbert@irta.cat (E. Gisbert)

E-mail address: eva.vallejos@uab.es (Eva Vallejos-Vidal)

Abstract

In the present study, we evaluated the effects of a medicinal plant leaf extract (MPLE; 10%, ursolic acid, 3% other triterpenic compounds; 2% verbascoside and <1% polyphenols) obtained from *Lippia citriodora* and *Salvia officinalis* on somatic growth and immune responses in juvenile gilthead seabream (*Sparus aurata*). Fish (initial body weight = 26.0 ± 0.1 g) were fed two isoproteic (48% crude protein, 7% fishmeal), isolipidic (17% crude fat) and isoenergetic diets (21.7 MJ/kg), one of them containing 0.1% MPLE. Both diets were tested using four replicate tanks during 92 days. At the end of the trial, a significant increase in growth was observed in fish fed the diet containing the additive in comparison to fish fed the control diet (189.6 ± 2.5 g *vs.* 173.8 ± 4.1 g, respectively; $P < 0.05$). Specific growth rates (SGR) in fish fed the feed supplemented with 0.1% MPLE were significantly higher than in fish fed the control diet ($SGR_{0\text{-}92\ days\ (0.1\%\ MPLE\ diet)} = 2.26 \pm 0.01$ % day^{-1}, $SGR_{0\text{-}92\ days\ (control\ diet)} = 2.16 \pm 0.02$ % day^{-1}; $P < 0.05$). Feed conversion ratio (FCR) values in fish fed the control diet were higher than those in fish fed the MPLE diet ($FCR_{control\ diet} = 1.23 \pm 0.02$ *vs.* $FCR_{0.1\%\ MPLE\ diet} = 1.10 \pm 0.02$; $P < 0.05$). When evaluating non-specific immune plasmatic parameters, no significant variations were registered at the level of bacteriolytic and complement activities, nor IgM levels ($P > 0.05$). In order to evaluate the cellular immune competence of fish, an *ex vivo* assay with splenocytes primary cell culture (SPCC) from both dietary groups was conducted. SPCC were incubated with lipopolysaccharide (LPS) for 24 h and the expression of genes associated to several immune processes was evaluated (humoral immune response, pro- and anti-inflammatory cytokines, cell surface markers, and antioxidant enzymes). Particularly at 4 h post-exposure, dietary supplementation with 0.1% MPLE enhanced SPCC immune response to LPS by the up-regulation of genes involved in humoral immunity (*lys, IgM*), pro- (*tnf-α, il-1β*) and anti-inflammatory (*tgf-β1, il10*) cytokines, the leucocyte cell surface marker *cd4*, and antioxidative stress enzymes (*mn-sod, cat*). Therefore, a medicinal plant leaf extract (MPLE) obtained from *L. citriodora* and *S. officinalis* may be considered as efficient additive to be used in aquafeed since it does not induce a significant

immune reaction under basal conditions, but it provides immune protection after LPS treatment, together with increasing overall fish growth and improvement of feed efficiency values.

Keywords**:** additive, functional diet, immunity, pathogen-associated molecular pattern (PAMP), ursolic acid, verbascoside.

1. Introduction

Functional feeds are regarded as the future of the aquaculture industry. By preventive health management through feeding practices, aquatic animals can divert more energy to somatic growth and reduce biological energy reserves needed to fight disease or stress resistance. Nowadays, functional feeds include specific ingredients with specific functions or special product characteristics; therefore, providing solutions to recurrent problems in animal production cycles rather than only focusing on growth performance issues. A reality that affects the aquaculture industry and still unresolved, is the excessive use of antibiotics, regardless of the global strategy promoted by the Food and Agriculture Organization (FAO, 2016). In recent years, the increase in the use of antimicrobials has been reported due to intense worldwide fish farming and the spreading of several bacterial diseases (Defoirdt et al., 2011). However, antibiotic prophylaxis represents a high cost and leads to undesirable side effects such as bioaccumulation of drug residues, pollution, and increased antibiotic resistance among bacteria. A suitable solution to replace the excessive administration of antibiotics in the aquaculture industry is the use of additives such as immunostimulants that may be used in functional feeds to improve resistance to diseases by strengthening the innate immune defense mechanisms in aquatic animals (Dawood et al., 2018; Fuchs et al., 2015; Vallejos-Vidal et al., 2016; Wang et al., 2017). Among them, the use of immunostimulants from plant materials has been recognized as an ecofriendly approach for the control of pathogens and regulation of host health, as they possess medicinal properties that have

been reported to have a key role in enhancing fish immunity (Vaseeharan and Thaya, 2014). In this context, plant extracts or their by-products contain several active compounds, including phenols, polyphenols, alkaloids, terpenoids, lectines, and polypeptides, that have been shown to be effective alternatives to traditional prophylaxis and vaccines (Chakraborty and Hancz, 2011; Galina et al., 2009).

In this study, we evaluated the growth and immune response in juvenile gilthead sea bream (*Sparus aurata*) fed with a functional diet containing a medicinal plant leaf extract from sage (*Salvia officinalis*, Lamiaceae) and lemon verbena (*Lippia citriodora*, Verbenaceae). Extracts of sage are rich in phenolic compounds (e.g., coumarins, flavonoids, tannins) (Ghorbani and Esmaeilizadeh, 2017) and triterpenes, which are natural components found in a variety of common European plants and fruits, which are gaining attention for their functional benefits (Babalola and Shode, 2013). In traditional medicine, this plant has been reputed for its potential antitumor and antioxidant activities, anti-inflammatory properties and antiseptic effects (Ghorbani and Esmaeilizadeh, 2017; Jedinák et al., 2006). The extracts from the aromatic and medicinal plant lemon verbena contain a large quantity polyphenolic and triterpenic compounds, as well as verbascoside and its derivates (Mauriz et al., 2015; Quirantes-Piné et al., 2009). The above-mentioned compounds have reported beneficial pharmacological activities, including antioxidant, anti-inflammatory and antineoplastic properties in addition to numerous wound-healing and neuroprotective properties (Alipieva et al., 2014; Caturla et al., 2011; Funes et al., 2009). In fish, the anti-inflammatory activity of a triterpenic compound like ursolic acid was reported in zebrafish (*Danio rerio*) (Ding et al., 2015). Furthermore, a strong antiviral activity both *in vitro* and *in vivo* has recently been reported in rainbow trout (*Oncorhynchus mykiss*) (Li et al., 2019). However, none of these studies has used the strategy of a dietary administration to evaluate its applicability in aquaculture. By contrast, to the best of our knowledge, there are no antecedents of the verbascoside effect upon fish health.

The aim of this study was to evaluate a medicinal plant leaf extract (10%, ursolic acid, 3% other triterpenic compounds; 2% verbascoside and <1% polyphenols) from sage (*S. officinalis*) and lemon verbena (*L. citriodora*) as a feed additive, using gilthead seabream as a model species for marine aquaculture. This extract contains several compounds that are reputed in traditional medicine for their immunomodulatory properties, which if they also function in fish, would be beneficial in functional aquafeeds. Thus, we decided to test this phytochemical extract on growth performance and the systemic immune response through the evaluation of humoral immune parameters. The beneficial effects of the dietary administration of the medicinal plant leaf extract (MPLE) to a bacterial challenge were evaluated at the gene expression level in splenocytes by a short-term *ex vivo* stimulation with LPS, a broadly recognized pathogen-associated molecular pattern (PAMP).

2. Material and Methods

2.1 Fish and rearing conditions

A total of 300 gilthead seabream (body weight, BW = 5 – 8 g) were purchased from a commercial fish farm (Andromeda Group, Burriana, Spain) and transported by road (1 h) to IRTA facilities at Sant Carles de la Ràpita (Spain). Once there, fish were acclimatized for three weeks in 450 L tanks connected to a water recirculation system (IRTAmar™) at an initial density of 2 kg m^{-3}. Acclimation was conducted in the same experimental tanks (450 L) where the nutritional experiment was carried out. Water temperature (22-27 °C), oxygen (6.1 ± 0.2 mg l^{-1}) (OXI330, Crison Instruments), and pH (7.5 ± 0.01) (pHmeter 507, Crison Instruments, Barcelona, Spain), were daily controlled, whereas salinity (35‰) (MASTER-20 T; ATAGO Co. Ltd), as well as ammonia (0.13 ± 0.1 mg NH_4^+ l^{-1}) and nitrite (0.18 ± 0.1 mg NO_2^- l^{-1}) levels (HACH DR9000 Colorimeter, Hach®, Spain) were weekly monitored. Just before the start of the trial, all necessary animals (n = 280, 35 fish per tank) were individually measured in BW and standard length (SL) and distributed homogeneously among the eight experimental tanks.

124

125 *2.2 Experimental diets and fish sampling*

126 Experimental diets used in this trial were manufactured by SPAROS Lda (Portugal). Once
127 received and during the entire trial (92 days), they were stored in a refrigeration chamber at 4 °C
128 to avoid their oxidation. Two experimental diets with low fishmeal (FM) content (7% FM) were
129 tested: a control diet (48% protein, 17% lipids and energy: 21.7 MJ kg^{-1}) and the same diet but
130 supplemented with the MPLE additive obtained from *S. officinalis* and *L. citriodora* at 0.1% inclusion
131 (Table 1). This inclusion level was chosen according to previous results using similar compounds
132 (Gisbert et al., 2017). Sage and verbena leaf extracts (5 parts of sage : 1 part of verbena) were
133 produced by NATAC Biotech SL (Madrid, Spain) using water/ethanol extraction (plant leaf extract
134 ratio 5:1) and characterized as described in Arthur et al. (2011) and Wójciak-Kosior et al. (2013).
135 The biochemical composition in terms of the tested extract contained 73% carbohydrates, 2% crude
136 lipids, <1% crude proteins, 5% salts, 4% water, 10% ursolic acid, 3% other triterpenic compounds,
137 2% verbascoside and <1% polyphenols. Thus, the content in plant-derived bioactive compounds in
138 the experimental diet was 0.01% ursolic acid, 0.003% other triterpenic compounds, 0.002%
139 verbascoside and <0.001% polyphenols.

140 The trial lasted 92 days and each diet was tested by means of four replicate tanks. Diets were
141 distributed eight times per day by automatic feeders (ARVO-TEC T Drum 2000; Arvotec, Finland) at
142 the daily rate of 3.0% of the stocked biomass, which approached apparent satiation. One hour after
143 feed administration, uneaten pellets were recovered from the bottom of the tank, dried in an oven
144 (100 °C) and their dry weight used for estimating the amount of uneaten feed and calculate feed
145 intake. Sampling to monitor fish growth took place monthly from the nutritional trial in order to
146 adjust feeding rate and evaluate somatic growth performance. For that purpose, all fish in each
147 tank were netted, gently anaesthetized (tricaine methanesulfonate, MS-222, 50 mg l^{-1}) and their
148 BW (g) and standard length (SL, cm) determined. Fish growth was evaluated by means of the

following indices: Fulton's condition factor (K) = (BW_f / SL_f^3) × 100; specific growth rate in BW (SGR_{BW}, %) = [(ln BW_f − ln BW_i) × 100] / time (d); where BW_f and BW_i correspond to final and initial BW, and SL_f corresponds to final SL. Feed utilization was evaluated by the following formula: feed conversion ratio (FCR) = feed intake (g) / increase of fish biomass (g).

Proximate composition of the extract and experimental diets was determined as follows: crude fat was quantified gravimetrically after extraction in chloroform/methanol (2:1) and evaporation of the solvent under a stream of N followed by vacuum desiccation overnight (Folch et al., 1957); crude protein content was determined according to Lowry et al. (1951); ash contents were determined by keeping the sample at 500 to 600°C for 24 h in a muffle furnace (AOAC, 1990) and water content was estimated by sample drying at 120°C for 24 h. All chemical analyses were performed by duplicate.

2.3 Humoral immune parameters

After fish were measured, blood (ca. 1ml) was taken from anaesthetized fish (n = 5 fish per tank) by caudal puncture with lithium-heparinized syringes and immediately centrifuged (2,000 × *g* for 20 min at 4 °C) to separate plasma. Levels of immunoglobulin M (IgM) were measured by using the enzyme-linked immunosorbent assay (ELISA) (Wells et al., 1986). Aliquots of 100 µl of plasma (1/5 diluted with 50 mM carbonate-bicarbonate buffer, pH 9.6) were placed in flat-bottomed 96-well plates in triplicate and coated by overnight incubation at 4 °C. After three rinses with PBT buffer (20 mM Tris-HCl, 150 mM NaCl and 0.05% Tween 20, pH 7.3) the plates were blocked for 2 h at room temperature with blocking buffer containing 3% bovine serum albumin (BSA, Sigma) in PBT buffer, followed by three rinses with PBT buffer. The plates were then incubated for 1 h with 100 µl per well of mouse anti-gilthead seabream IgM monoclonal antibody (Aquatic Diagnostics Ltd.) (1/100 in blocking buffer), washed and incubated with the secondary antibody anti-mouse IgG-HRP (streptavidin horseradish-peroxidase) (1/1,000 in blocking buffer, Sigma). After exhaustive rinsing

with PBT buffer the plates were developed using 100 µL of a 0.42 mM solution of 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB, Sigma), which was prepared daily in a 100 mM citric acid/sodium acetate buffer (pH 5.4) containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min and stopped by the addition of 50 µL of 2M H_2SO_4 before the plates were read at λ = 450 nm in a plate reader (FLUOstar Omega, BMG Labtech). Negative controls consisted of samples without plasma, whose optical density (OD) values were subtracted for each sample value.

Natural haemolytic complement activity was measured in plasma according to Guardiola et al. (2018). The buffers used were: GVB (Isotonic veronal buffered saline), pH 7.3, containing 0.1% gelatin; EDTA-GVB, as the previous one but containing 20 mM EDTA; and Mg-EGTA-GVB, which is GVB with 10 mM Mg^{+2} and 10 mM EGTA. Rabbit red blood cells (RaRBC; Probiologica Lda, Portugal) were used for natural haemolytic complement determination. RaRBC were washed four times in GVB and resuspended in GVB to a concentration of 2.5 x 10^8 cells ml^{-1}. Twenty µl of RaRBC suspension were then added to 40 µl of serially diluted plasma in Mg-EGTA-GVB buffer. The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 40 µL of distilled water or Mg-EGTA-GVB buffer to 20 µl samples of RaRBC, respectively. Samples were incubated at room temperature for 100 min with regular shaking every 20 min. The reaction was stopped by adding 150 µl of cold EDTA-GVB. Samples were then centrifuged (400 x *g* for 5 min at 22 °C) and the extent of haemolysis was estimated by measuring the optical density of the supernatant at λ = 414 nm in a microplate reader (Synergy HT, Switzerland). The degree of haemolysis (Y) was calculated and the lysis curve for each specimen was obtained by plotting Y = (1-Y)-1 against the volume of plasma added (µl) on a log-log scaled graph. The volume of plasma producing 50% haemolysis (ACH_{50}) was determined and the number of ACH50 units/ mL obtained for each experimental fish sample.

The fish pathogen *Vibrio anguillarum* was used in the bactericidal assay. The strain was grown from 1 mL of stock culture that had been previously frozen at -80 °C. The bacteria cells were cultured for 48 h at 25 °C in Triptic Soy Agar (TSA, Difco Laboratories), and then inoculated in Triptic

200 Soy Broth (TSB, Difco Laboratories), both supplemented with NaCl to a final concentration of 1%
201 (w/v). Bacteria in TSB medium were then cultured at the same temperature, with continuous
202 shaking (100 rpm) for 24 h. Exponentially growing bacteria were resuspended in sterile PBS and
203 adjusted to 10^8 colony forming units per mL (CFU ml^{-1}).

204 Bactericidal activity was determined following the method of Stevens and Kehrli (Stevens et al.,
205 1991) with some modifications. Samples of 20 µL of plasma were added (in six replicates) to the
206 wells of a flat-bottomed 96-well plate. PBS solution was added to some wells instead of the plasma
207 (positive control). Aliquots of 20 µL of the previously cultured bacteria were added and the plates
208 were incubated for 5 h at 25 °C. Then, 25 µl of MTT (1 mg ml^{1}) were added to each well and the
209 plates were incubated again for 10 min at 25ºC to allow the formation of formazan. Plates were
210 then centrifuged (2,000 x *g* for 10 min) and the precipitates dissolved in 200 µl of DMSO were
211 transferred to a new flat-bottom 96-well plate. The absorbance of the dissolved formazan was
212 measured at λ = 570 nm. Bactericidal activity was expressed as percentage of non-viable bacteria,
213 calculated as the difference between absorbance of surviving bacteria compared to the absorbance
214 of bacteria from positive controls (100%).

215

216 *2.4 Ex vivo immune stimulation of splenocytes with LPS*

217 In order to evaluate the immunomodulatory effect of the tested additive when fish come in
218 contact with a pathogenic organism, an *ex vivo* assay was conducted. For this purpose, the spleen
219 was used because of its key role as a secondary lymphoid tissue and, therefore, its specific capacity
220 to activate the immune response in face of a widely recognized pathogen-associated molecular
221 pattern (PAMP) like lipopolysaccharide (LPS).

222 At the end of the nutritional trial, six specimens from each experimental group (biological
223 replicates) were sacrificed with an overdose of anesthetic (>150 mg l^{-1}, MS-222) and their spleens
224 removed. The *ex vivo* protocol and the dose of LPS used was similar to that described by
225 Campoverde et al. (Campoverde et al., 2017). In brief, the spleen of each fish was passed through

a 100 µm nylon mesh cell strainer (SefarNytal PA-13xxx/100, Spain) in Leibovitz L15 medium (Gibco) supplemented with 1:1000 penicillin-streptomycin (Gibco, catalogue number 15140-122) and 2% foetal calf serum (Gibco, catalogue number 10270-098). The resulting cell suspension was collected and centrifuged (at 400 x *g* for 10 min at room temperature). Then, the supernatant was discarded and replaced with 10 ml of Leibovitz L15 medium. The cell suspension was again centrifuged and supernatants removed and replaced with 30 ml of media. Cells were distributed to 12-well microtiter plates in 5 mL aliquots (2 wells per fish; methodological replicates). The obtained splenocyte primary cell cultures (SPCC) were incubated with a bacterial-type PAMP, LPS (Sigma, #L3129-100 MG). For this purpose, LPS was dissolved in sterile PBS. A LPS dose (50 µg ml^{-1}; Campoverde et al., 2017) was added to evaluate its effect upon the SPCC from control diet ($SPCC_{CD}$+LPS) and from 0.1% MPLE diet ($SPCC_{MPLE}$+LPS). The assay was carried out on microtiter plates (Greiner Bio-One, Spain). LPS-free samples were obtained incubating the SPCC from control ($SPCC_{CD}$+PBS) and 0.1% MPLE diets ($SPCC_{MPLE}$+PBS) with 250 µl of PBS. In order to evaluate the expression profile of immune genes, splenocytes were harvested at 4, 12 and 24 h after LPS exposure, centrifuged at 400 x *g* for 10 min at room temperature, and the supernatant discarded. Splenocytes with no stimuli were harvested immediately prior to the beginning of the treatment (time zero). After cell centrifugation, the pellet was immediately suspended in 1.5 ml of RNAlater® (Sigma-Aldrich, Spain), incubated overnight at 4 °C, then stored at -80ºC for further gene expression analyses.

2.5 RNA extraction and cDNA synthesis

Spleen total RNA was extracted using the QIAGEN RNeasy® Mini Kit following the manufacturer's recommendations. The amount of isolated RNA was determined by spectrophotometry with an ND-2000 NanoDrop (Thermo Scientific™) and its quality was evaluated by means of agarose gel electrophoresis (2%) according to Masek et al. (2005). Once the quality of the extracted RNA was verified, single-stranded cDNA was synthesized in order to evaluate their expression profile. For

cDNA synthesis, 1 µg of total RNA was reverse transcribed using a high capacity cDNA reverse transcription kit (QuantiTect® Reverse Transcription Kit) in a final reaction volume of 20 µl according to the instructions provided by the manufacturer.

2.6 Gene expression analyses by real-time PCR (qPCR)

The gilthead sea bream SPCC treatments were analyzed by qRT-PCR in order to evaluate the modulation of a set of immune-related genes. The screening included the analysis of humoral response (lysozyme [*lys*]; immunoglobulin M [*IgM*]), pro-inflammatory (*interleukin 1 beta* [*il-1β*]; *tumor necrosis factor alpha* [*tnf-α*]) and anti-inflammatory cytokines (*il-10*; *transforming growth factor beta 1* [*tgfβ1*]), the surface cell marker *cd4*, and antioxidant enzymes (manganese superoxide dismutase [*mn-sod*]; catalase [*cat*]). Two different reference genes (*β-actin*; *18S*) were assessed using the BestKeeper software (Pfaffl et al., 2004) to elucidate which one had less variation. Thus, *β-actin* was included as the reference gene for expression analyses. The specific primer set for each gene are detailed in Table 2.

The primer amplification efficiency (E) for all the genes included in this analysis was determined using a reference pool containing 1 µl of each sample included in this study. Based on the value of the slope of the regression line obtained, E was calculated according to the formula described in Pffaffl (Pfaffl, 2001) and E values reported in Table 2. Quantitative PCR reactions were performed with 2.5 µl iTaq universal SYBR green supermix (Bio-Rad Laboratories), 0.1 µl forward and reverse primers (final concentration of 500 nM at the reaction volume) and 1.3 µl of miliQ H_2O using 1:4 cDNA dilution from all the cDNA stock samples. The thermal conditions used were 3 min at 95 °C of pre-incubation followed by 40 cycles at 95 °C for 30 s and 60 °C for 30 s. An additional temperature ramping step from 65 to 95 °C was included to produce the melting curves and thus, verify the amplification of a unique single product on all samples. All the reactions were performed in duplicate using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). Quantification was done according to the Pfaffl method (Pfaffl, 2001) corrected for efficiency of

each primer set obtained. The normalized relative expression (NRE) value for each diet (control diet; 0.1% MPLE diet) and experimental condition (PBS; LPS) was calculated using the time zero (calibrator) and normalized to the β-actin (reference gene) expression. The results were expressed as mean expression values obtained at 0, 4, 12, and 24 hours of treatment (n = 6 fish per diet, experimental condition, and time-point assessed).

2.7 Statistical analysis

Differences in somatic growth and fish condition between both diets (control; 0.1% MPLE) were evaluated by means of a t-test. Two-way ANOVA test was used to determine differences in gene expression between dietary groups (factor 1) and sampling times (factor 2). Prior to ANOVA analyses, all data were checked for normality and homogeneity of variances. When statistical significances were found between groups ($P < 0.05$), a post-hoc Tukey test was conducted. Results in growth performance parameters and gene expression values are expressed as the mean ± SD (standard deviation). All statistical analyses were performed using Graph Pad Prism V.6.1. Software (GraphPad Software, San Diego, USA).

2.8 Ethics statement

The experiment complied with the Guiding Principles for Biomedical Research Involving Animals (EU2010/63), the guidelines of the Spanish laws (law 32/2007 and RD 53/2013), and authorized by the Ethical Committee of the Institute for Research and Technology in Food and Agriculture (Spain) for the use of laboratory animals.

3. Results

3.1 Somatic growth performance and feed utilization parameters

At the end of the 92-days trial, survival was similar among groups with values ranging between 98.0 to 99.0% ($P > 0.05$). Gilthead seabream fed the diet containing 0.1% MPLE were 8.3% heavier than those fed the control diet (189.6 ± 2.5 g *vs.* 173.8 ± 4.1 g, respectively; $P < 0.05$). Similarly, SGR values in fish fed the 0.1% MPLE diet were higher than those recorded in the control group (SGR = 2.26 ± 0.001 % day^{-1} *vs*. 2.16 ± 0.018 % day^{-1}, respectively; $P < 0.05$). No significant differences in $SL_{f,}$ and K and were found between both groups (Table 3; $P > 0.05$). Values of FCR were lower in fish fed the 0.1% MPLE diet than in those fed the control diet (Table 3; $P < 0.05$).

3.2 Non-specific humoral immune parameters

At the end of the feeding trial, there were no significant differences in the IgM levels, either bacteriolytic nor complement activities among gilthead seabream specimens fed both diets (Table 4; $P > 0.05$).

3.3 Gene expression in splenocytes incubated with LPS (ex vivo trial)

Normalized relative expression (NRE) for each gene from different experimental groups are presented in the Supplementary file 1. Regarding the humoral immune response, at 4 h post-exposure (hpe), *lys* in $SPCC_{MPLE}$+LPS was significantly higher than in the $SPCC_{CD}$+LPS (Fig. 1a; $P < 0.05$). At 12 hpe, *lys* expression levels in $SPCC_{MPLE}$+LPS increased in comparison to the same treatment at 4 hpe, while these values were significantly higher than those observed from the same group, but just incubated with PBS ($SPCC_{MPLE}$+PBS) ($P < 0.05$). The same effect, although at a lower magnitude, was observed between $SPCC_{CD}$ +LPS and $SPCC_{CD}$ +PBS. At 24 hpe, *lys* levels decreased in $SPCC_{CD}$+LPS and $SPCC_{MPLE}$+LPS compared to 12 hpe ($P < 0.05$); thus, decreasing to similar values recorded prior to LPS stimulation ($P < 0.05$).

Regarding *IgM*, $SPCC_{CD}$+LPS remained stable throughout the study (from time zero to 24 hpe) ($P > 0.05$). However, the $SPCC_{MPLE}$+LPS showed higher *IgM* levels compared to $SPCC_{CD}$+LPS (Fig. 1b, $P < 0.05$). On the other hand, at 12 and 24 hpe no differences in *IgM levels* were detected in none of the diets and treatments evaluated ($P > 0.05$). Collectively, the expression of *lys* and *IgM* suggest that the activation of the humoral immune response in $SPCC_{MPLE}$+LPS is perceived at 4 hpe, while in fish fed the control diet the response was characterized by a delayed activation of response (*lys*) or even no effect (*IgM*).

The expression of the pro-inflammatory cytokines *il-1β* and *tnfα* was also determined. A significant ten-fold increase of *il-1β* was registered in $SPCC_{MPLE}$+LPS at 4 hpe compared to $SPCC_{MPLE}$+PBS (Fig. 1c; $P < 0.05$). In fish fed the control diet, a significant increase was also observed in the expression of *il-1β* in $SPCC_{CD}$+LPS at 4 hpe compared to $SPCC_{CD}$+PBS. Importantly, at 4 hpe the *il-1β* expression value was also higher in $SPCC_{MPLE}$ +LPS when it was compared to $SPCC_{CD}$ +LPS ($P < 0.05$). The expression of *il-1β* diminished at 12 hpe in all the treatments evaluated, although it was still significantly higher in $SPCC_{MPLE}$+LPS in comparison to $SPCC_{MPLE}$+PBS. By contrast, *il-1β* levels in SPCC from fish fed the control diet were similar at 12 hpe when comparing the LPS and PBS treatments. No differences were registered at 24 hpe between both evaluated treatments ($P < 0.05$).

The pro-inflammatory cytokine *tnf-α* showed increased expression at 4 hpe in $SPCC_{MPLE}$ +LPS, as well as in $SPCC_{CD}$+LPS, though the magnitude of increase was higher in fish fed the additive ($P < 0.05$; Fig. 1d). At 12 hpe, in both dietary groups *tnf-α* expression decreased with regard to 4 hpe. In particular, *tnf-α* levels in $SPCC_{CD}$+LPS were similar to those recorded at basal level. By contrast, *tnf-α* levels in $SPCC_{MPLE}$+LPS were still significantly higher than those recorded at the beginning of the LPS exposure ($P < 0.05$). At 24 hpe, *tnf-α* expression returned to basal expression levels ($P > 0.05$). The pro-inflammatory *il-1β* and *tnf-α* data provided more evidence of activation and significantly higher immune response occurring in $SPCC_{MPLE}$+LPS.

The leukocyte membrane marker *cd4* showed a significant increase only at 4 hpe in $SPCC_{MPLE}$+LPS compared to $SPCC_{MPLE}$+PBS ($P < 0.05$; Fig. 1e), but also compared to $SPCC_{CD}$+LPS evaluated at the same time-point. This increase in $SPCC_{MPLE}$+LPS at 4 hpe was also observed in a time-dependent manner compared to 0 hpe. No differences were observed for the other time-points and treatments assessed ($P > 0.05$). This data suggested a correlation between the activation of the pro-inflammatory response and the CD4+ immune cell populations associated to the MPLE dietary additive.

Expression analysis of anti-inflammatory cytokines (*il-10*; *tgfβ1*) demonstrated levels for *il-10* remained stable throughout the 24 h-study and they were not affected by the exposure of SPCC to LPS in fish group fed the control diet ($P > 0.05$; Fig. 1f). However, at 4 hpe a significant increase was recorded in $SPCC_{MPLE}$+LPS compared to $SPCC_{CD}$+LPS ($P < 0.05$). Similarly, this up-regulation of the $SPCC_{MPLE}$+LPS was also registered concerning the $SPCC_{MPLE}$+LPS. At 12 hpe, *il-10* levels in $SPCC_{MPLE}$+LPS was still higher compared to $SPCC_{MPLE}$+PBS and $SPCC_{CD}$+LPS. At 24 hpe, no differences on the expression of *il-10* were detected ($P > 0.05$). The expression of *tgfβ1* in $SPCC_{MPLE}$+LPS increased compared to both $SPCC_{MPLE}$+PBS and $SPCC_{CD}$+LPS, whereas expression levels reached basal values at 12 hpe in $SPCC_{MPLE}$+LPS ($P > 0.05$). Thus, the same expression pattern observed at 4 hpe of anti-inflammatory (*il*-10 and *tgfβ1*) *and* pro-inflammatory actors of the humoral and cytokine responses, suggests that a coordinated and also intimate control of immune response takes place in $SPCC_{MPLE}$+LPS and whose response was not perceived in $SPCC_{CD}$+LPS.

The expression of anti-oxidative stress enzymes (*mn-sod*; *catalase*) was also evaluated. The level of *mn-sod* was significantly up-regulated at 4 hpe in $SPCC_{MPLE}$+LPS compared to $SPCC_{MPLE}$ +PBS (Fig. 1h; $P < 0.05$). Importantly, the expression in $SPCC_{MPLE}$ +LPS was also higher than in $SPCC_{CD}$ +LPS ($P < 0.05$). After 4 hpe, the expression values in $SPCC_{MPLE}$+LPS progressively decreased at 12 hpe and 24 hpe. However, *mn-sod* levels in $SPCC_{MPLE}$+LPS at 24 hpe were still higher than those recorded at 0 h ($P < 0.05$). A similar trend was observed in $SPCC_{CD}$+LPS. However, the highest significant expression

peak of *mn-sod* in $SPCC_{CD}$+LPS was only registered at 12 hpe ($P < 0.05$), then returned to basal expression levels at 24 hpe ($P > 0.05$).

On the other hand, the expression profile of catalase (*cat*) had a similar trend as was observed for mn-*sod*. Levels of *cat* in SPCC from fish fed the control diet ($SPCC_{CD}$+LPS; $SPCC_{CD}$+PBS) remained stable throughout the 24 h-study ($P > 0.05$; Fig. 1i). The highest expression in *cat* was registered in $SPCC_{MPLE}$+LPS at 4 hpe, values that were significantly higher than those recorded in $SPCC_{MPLE}$+PBS, $SPCC_{CD}$+LPS and $SPCC_{CD}$+PBS ($P < 0.05$). In $SPCC_{MPLE}$+LPS, *cat* expression decreased at 12 hpe ($P < 0.05$) and remained constant at 24 hpe ($P > 0.05$). In sum, the antioxidant gene expression profile suggests that a tight control of the oxidative process is produced in $SPCC_{MPLE}$+LPS at the same time that the peak in immune response activation (4 hpe) was registered.

Altogether, our results suggested that SPCC from gilthead seabream fed the 0.1% MPLE ($SPCC_{MPLE}$+LPS) showed an earlier activation and higher magnitude immune response than the observed response of the fish fed the control diet. This response seemed to be intimately regulated by both anti-inflammatory and anti-oxidant mechanisms.

4. Discussion

In this study, the effect of a functional diet formulated with low fishmeal levels (7%) and supplemented with 0.1% medicinal plant leaf extract from sage (*S. officinalis*) and lemon verbena (*L. citriodora*) was evaluated in terms of growth performance, non-specific humoral immune response parameters, and the expression profile of genes related to several immune processes including humoral response, pro- and anti-inflammation, and antioxidant enzymes in an *ex vivo* assay using SPCC. Our results showed that 0.1% MPLE increased the body weight and improved feed utilization (FCR) with no effects on the plasma immune parameters in gilthead seabream. Importantly, when isolated splenocytes were incubated with LPS (*ex vivo* conditions) their immune

399 response was activated earlier in those fish fed the 0.1% MPLE, and accompanied by regulatory
400 mechanisms at both anti-inflammatory and anti-oxidant levels.

401 Although functional diets in aquaculture are not considered as a primary strategy for promoting
402 somatic growth, several studies have shown an improvement in growth performance when fish are
403 fed these kinds of diets (Vallejos-Vidal et al., 2016; Wang et al., 2017). Under present experimental
404 conditions, the supplementation of a basal diet with low FM levels with 0.1% MPLE increased
405 growth performance compared to the control diet. In particular, fish fed the diet containing the
406 plant extracts were 8.3% heavier than the control group. Similar results were observed in rainbow
407 trout (*O. mykiss*) fed with dietary inclusion of sage oils (Sönmez et al., 2014) and post-weaned
408 piglets fed with a lemon verbena additive (Pastorelli et al., 2012). These results might be partially
409 attributed to the potential growth-promoting effects of polyphenolic compounds like verbascoside
410 (Chakraborty and Hancz, 2011). However, these results may also be attributed to triterpenoid
411 compounds, among which the ursolic acid, which has been reported to promote muscular growth
412 by hypertrophy of skeletal muscular fibers in mice (Kunkel et al., 2012) and rainbow trout
413 (Fernández-Navarro et al., 2006). These results in terms of growth are of special relevance due to
414 the low content of FM (7%), representing 75% of FM replacement in tested diets; thus, supporting
415 the change of the aquaculture industry towards compound diets less dependent on wild fishery-
416 derived raw materials (Froehlich et al., 2018).

417 In addition to evaluating the potential growth-promoting effects of the tested plant extract used
418 in this study, the authors wanted to screen their potential immunomodulatory effects (Vallejos-
419 Vidal et al., 2016). For this purpose, different humoral immune parameters were evaluated in
420 plasma at the end of the nutritional study, as well as the immune competence of splenocytes when
421 exposed to a PAMP, like bacterial LPS, by means of an *ex vivo* assay. The evaluation of plasmatic
422 immune parameters (bacteriolytic and complement activities, and IgM levels) revealed no
423 significant immunostimulant effect of the tested additive, although other studies on feed additives

derived from medicinal plants have reported increases in the activities of the above-mentioned parameters (Awad and Awaad, 2017; Harikrishnan et al., 2011; Vaseeharan and Thaya, 2014). Some authors have shown that the use of additives does not always have the expected immunological response, since the administration of natural additives showed counter-productive results (distress situation) due to the bio-energetic cost of prolonged and enhanced immune responses (Álvarez-Rodríguez et al., 2018). Furthermore, the lack of transversal standardized experimental dietary evaluation procedures impedes any comparison between the obtained results and those from the literature (Vallejos-Vidal et al., 2016). At first sight, it may seem that the tested compounds did not modulate the immunity in gilthead seabream. Thus, from these results it could be presumed that 0.1% MPLE had no effect upon the immunity. On the other hand, the results of the *ex vivo* study using SPCC stimulated with LPS, as described below, showed a stimulatory effect. These results may not be surprising taking into consideration that the activation of the immune response represents an important increase in energy expenditure; thus, affecting the energy budget of the organism (Aída et al., 2016). Based on these antecedents, our results suggested that the tested additive from MPLE administered at 0.1% during 92 days did not modify the status of immune homeostasis. One possible reason is that systemic humoral immune of the fish could adapt to the supplemented feed without major energetic consequences, because 92 days can be considered a long time for determining immunostimulation. Nevertheless, this basal conditioning was modified and apparently potentiated in the presence of a pathogenic *stimulus*, whereas under normal conditions humoral non-specific immune systems were not enhanced. Thus, new studies using other additive concentrations or shorter administration times could bring some additional light to this complex issue.

The evaluation of the systemic immune response of gilthead seabream using an *ex vivo* trial with SPCC exposed to LPS assessed changes in gene expression of a repertoire of classical immune gene markers (Vallejos-Vidal et al., 2016). In particular, the expression of *lys* and *IgM* were up-regulated at 4 hpe in SPCC from gilthead seabream fed the 0.1% MPLE diet and remained stable

450 until 12 hpe, whereas they returned to basal levels (0 h) at 24 hpe. A similar trend in *lys* expression
451 patterns were found in SPCC from when compared to the control group, although the magnitude
452 of increase in gene expression after LPS exposure in SPCC over the control group was significantly
453 lower than SPCC fed the diet containing the medicinal plant extract. Lysozyme and IgM play an
454 important role as defense molecules of the immune response. In particular, lysozyme is important
455 in mediating protection against microbial invasion (Saurabh and Sahoo, 2008). IgM is the most
456 common immunoglobulin in plasma and mucus and the key player in the orchestration of the
457 systemic immune memory responses in teleosts (Parra et al., 2015). Several authors have reported
458 increased values in the plasmatic non-specific immune response after the activation of the immune
459 system with plant-derived immunostimulants. For instance, tilapia (*Oreochromis niloticus*) fed a
460 diet supplemented with the Chinese herb *Astragalus radix,* which is rich in polyphenols, showed a
461 significant increase of lysozyme in serum (Yin et al., 2006), whereas Akrami et al. (Akrami et al.,
462 2015) found an increase in serum lysozyme activity in beluga sturgeon (*Huso huso*) fed a diet
463 supplemented with garlic. On the other hand, lower levels of liver lysozyme were found in gilthead
464 sea bream fed the diets supplemented with maslinic acid, a triterpenic olive-derived (Reyes-Cerpa
465 et al., 2018). These results were not in agreement with our findings, since even though we tried to
466 analyze lysozyme in our plasma samples, values were below detection levels in both groups (data
467 not presented), which supported the above-mentioned hypothesis that the tested additives had an
468 immune homeostatic effect.

469 Regarding IgM, there was an increase in IgM levels in the spleen of mice when polyphenolic
470 compounds were administered (Oršolić et al., 2005), whereas triterpenes were found to act
471 similarly (Jie et al., 1984). Regarding fish, Reyes-Cerpa et al. (2018) reported that Atlantic salmon
472 (*Salmo salar*) fed functional diets, containing different medicinal plants rich in phenolic compounds,
473 demonstrated an up-regulation of *IgM* in the spleen that was confirmed by increases in B
474 lymphocyte-produced antibodies in the serum. The above-mentioned results are in agreement with
475 those obtained in our study, suggesting a potential adjuvant effect of the MPLE that may be

476 responsible for antibody production when SPCC were stimulated with LPS (Reyes-Cerpa et al.,
477 2018). As it was previously mentioned, the gene expression patterns observed for *lys* and *IgM* in
478 SPCC after their exposure to LPS did not match with the plasmatic levels of these proteins in fish
479 fed the 0.1% MPLE. These differences could be related to the absence (because of the end of
480 nutritional trial) or presence of LPS (*ex vivo*) and its intrinsic capacity to activate the expression of
481 immune-related genes (Shepherd et al., 2018). The present results suggested that the
482 administration of 0.1% MPLE potentiates the splenocytes humoral immune response in a time and
483 magnitude-dependent manner.

484 The pro-inflammatory response plays a key role in the success of control and eradication of
485 pathogens. The current study revealed that MPLE increased the expression levels of both *il-1β* and
486 *tnf-α*. IL-1 is an early secreted pro-inflammatory cytokine responsible for a cascade of effects on
487 different members of this citokyne family that leads to signal transduction and activation of the
488 nuclear factor (NF)-kB pathway (Engelsma et al., 2002). In addition, *tnf-α* is one of the immune
489 genes initially expressed at an early stage of infection in fish having a key role in the activation of
490 macrophages/phagocytes and enhancing their microbial killing activity; thus, promoting leucocyte
491 proliferation and migration (Hayden and Ghosh, 2014; Zou and Secombes, 2016). Two major classes
492 of leukocytes are the $CD4^+$ and $CD8^+$ leukocytes; so named because of the presence on their
493 respective cell surface of these specific markers. Among these, the $CD4^+$ leukocytes are referred to
494 in some literature as "helper" T-lymphocytes because they aid in the regulation/activation of
495 response by $CD8^+$ cells, or "natural killer" T-lymphocytes, through their secretion of many types of
496 cytokines; among them IL1-β. Accordingly, our finding of an increase in the expression of *cd4*
497 suggested that the pro-inflammatory response is promoting the proliferation of CD4+ leukocyte
498 cells in 0.1% MPLE-fed fish. The immuno-stimulatory activity of *il-1β* and *tnf-α* in response to a
499 bacterial challenge was previously shown in carp (*Cyprinus carpio*) injected with recombinant *il-1*,
500 resulting in an enhancement of agglutinating antibody titers against *Aeromonas hydrophila* (Yin and
501 Kwang, 2000). Similarly to our results*, il-1β* was up-regulated in trout (*O. mykiss*) in a dose-

dependent manner in phagocytes from head kidney exposed to LPS (Zou et al., 2000) and carp (Engelsma et al., 2006) confirming its role in the regulation of the inflammatory response, as well as modulating the expression of *il-17* family members, which are important for antibacterial defense (Zou and Secombes, 2016). Under the present *ex vivo* experimental conditions, the increase of *il-1β* in SPCC of gilthead sea bream fed with the tested additive and exposed to LPS may also be attributed to the coordinated response with the up-regulation of *tnf-α*. It has been reported that in rainbow trout head kidney leukocytes and monocytes/macrophages treated with recombinant TNF-α triggered the expression of a number of immune genes associated with inflammation, including *il-1β*, *il-8*, *il-17C* and *cox-2*, and genes involved in antimicrobial responses (Zou et al., 2003). Thus, the up-regulated expression of *il-1β* and *tnf-α* could be the result of a coordinated immune response mechanism favored by the administration of the 0.1% MPLE as a dietary additive. It is worth noting that our results showed a differential response in the up-regulation of *il-1β* and *tnf-α* between the MPLE and control diets. Collectively, these results suggested that splenocytes from gilthead seabream fed the 0.1% MPLE had an increased pro-inflammatory immune activity that could likely be mediated by the proliferation of $CD4^+$ leucocyte cells.

When assessing the immune condition, the evaluation of genes associated to the anti-inflammatory response is important since they regulate and reduce the expression of pro-inflammatory cytokines (Reyes-Cerpa et al., 2013) when necessary, to prevent collateral damage to host tissues and avoid wasting bioenergetic resources (Moore et al., 2001). IL-10 is an anti-inflammatory cytokine and suppresses immune responses (Zou and Secombes, 2016) through its regulatory effect upon pro-inflammatory cytokines, as it has been shown in *in vitro* studies with goldfish (*Carassius auratus*) monocytes activated with heat-killed *Aeromonas salmonicida* then incubated with IL-10 (Grayfer et al., 2011). The regulatory role of IL-10 has also been reported in LPS-activated immune cell populations (neutrophils and macrophages) in carp (Piazzon et al., 2015). Additionally, we found an up-regulation of *tgf-β1*. Previous antecedents in teleost fish have proposed an important role for this cytokine in the control of the pro-inflammatory process and

528 the resolution of pathogenic infective processes (Reyes-Cerpa et al., 2014; Reyes-López et al., 2015).
529 The augmentation of expression of *tgf-β1* could be mediated by IL-1, as it has been reported in
530 primary head kidney-derived macrophages (Castro et al., 2011), and appears to be mediated via
531 the NF-B and MAPK signaling pathways (Yang et al., 2014). The regulation by *tgf-β1* of the LPS-
532 induced pro-inflammatory response in grass carp (*Ctenopharyngodon idella*), has been previously
533 reported (Wei et al., 2015). Present results were in agreement with those obtained by Zhan et al.
534 (2015) where *tgf-β1* expression increased in the head kidney and spleen of tilapia challenged with
535 *Streptococcus agalactiae* and stimulated by LPS. Thus, the up-regulation of both anti-inflammatory
536 cytokines measured in this study, *il-10* and *tgfβ1*, confirmed the anti-inflammatory properties of
537 verbascoside (Alipieva et al., 2014) and ursolic acid (Baricevic et al., 2001), while at the same time
538 stimulating some pro-inflammatory responses, such that it is likely that a balanced immune
539 response was maintained. These data suggested that the splenocytes from fish fed the 0.1% MPLE
540 diet exerted a tight control of the immune response to LPS by means of the up-regulation of anti-
541 inflammatory cytokines at the same time-point (4 hpe) where the pro-inflammatory response
542 peaked. Overall, immune protection was thereby established, and potentially improved, with a
543 general homoeostasis being maintained.

544 Reactive oxygen species (ROS) compose an important defense mechanism involved in the
545 activation of the immune response including the activation of T cells (Chen et al., 2018). However,
546 the imbalance of ROS, which can be a cause of oxidative stress, has been associated to aberrant
547 immunity (Chen et al., 2018). Thus, several cellular self-protective mechanisms against this
548 potential damage should also be tightly regulated during an immune response to prevent collateral
549 damage. In this way, *mn-sod* and *cat* are two enzymes involved in the cellular defense against
550 uncontrolled oxidative processes and catalyze the reduction of superoxide radicals and H_2O_2 (Otto
551 and Moon, 1996). To minimize the damaging effects of ROS, these two antioxidant enzymes have
552 related functions and are considered as the first line of defense against oxygen toxicity due to their
553 inhibitory effects on oxygen radical formation (Li et al., 2009; Pandey et al., 2003). Furthermore,

554 the presence of phenolic compounds in sage and lemon verbena have been reported to be
555 responsible for the high antioxidant and antibacterial capacity of these medicinal plants (Bulfon et
556 al., 2014; Funes et al., 2009). Results from the current study were in agreement with the above-
557 mentioned findings, as changes in levels of *mn-sod* and *cat* expression by SPCC exposed to LPS
558 occurred as a response by fish fed the 0.1% MPLE diet. These data suggested that fish fed with 0.1%
559 MPLE had an increased *redox* capacity related to the presence of triterpenic (Rufino-Palomares et
560 al., 2011) and polyphenolic (Sönmez et al., 2014) compounds, protecting against reactive oxygen
561 species and stimulation of the antioxidant defenses of the organism (John et al., 2001).

562 Despite the potential benefits of the tested MPLE obtained from sage and lemon verbena in
563 terms of growth performance and immunostimulatory properties reported in the current study,
564 verbascoside extracted from *Kigelia africana* has been reported to promote genotoxicity in human
565 lymphocytes (Santoro et al., 2008). However, our study demonstrated that the long administration
566 of a feed additive containing verbascoside at low levels (0.002%) had no toxic effects in gilthead sea
567 bream. These results were in agreement to other studies in different animal models that reported
568 that this compound posed no risk on animal health (Etemad et al., 2015, 2016; Perucatti et al., 2018
569 among others).

570

571 **5. Conclusions**

572 In summary, present data suggest that the inclusion of a medicinal plant leaf extract obtained
573 from sage and lemon verbena at 0.1% in diets with low FM levels not only promoted somatic growth
574 and reduced FCR values in gilthead seabream, but also enhanced their systemic immune response
575 as indicated by changes in gene expression of a repertoire of markers in an *ex vivo* trial using SPCC
576 exposed to LPS. However, the above-mentioned effects were not seen in bacteriolityc, complement
577 activities, and/or IgM levels in plasma, which may indicate, in comparison to other
578 immunostimulants, a very tight control of the immune status mediated by the tested compounds

579 (immune homeostasis), that functions well with the host strategy to save energy for metabolic
580 purposes when no real immune response is needed. Altogether, the up-regulation of genes
581 involved in non-specific immune response (*lys*, *IgM*), as well as pro- (*tnf-α*, *il-1β*) and anti-
582 inflammatory (*tgf-β1*, *il10*) cytokines, surface T-cell marker *cd4*, and antioxidative stress enzymes
583 (*mn-sod*, *cat*) indicated that the tested feed additive, rich in triterpenic and polyphenolic
584 compounds, mainly ursolic acid and verbascoside, has immunomodulatory properties that can be
585 useful for incorporation in aquafeeds.

586

587 **Acknowledgements**

588 This work has been financed through the DIETAplus project of the JACUMAR (Junta de Cultivos
589 Marinos, MAPAMA; Spanish government), which is co-funded with FEMP funds (EU), as well as by
590 the ERC (European Research Council) in MedAID project (Mediterranean Aquaculture Integrated
591 Development; Grant Agreement nb. 727315) and Fundación Seneca de la Región de Murcia (Grupo
592 de Excelencia grant no. 19883/GERM/15). Héctor R. Salomón Figueredo is supported by a PhD grant
593 from the government of Paraguay (BECAL). Joana P. Firmino has been subsidized by the Industrial
594 PhD program of the Generalitat de Catalunya and TECNOVIT-FARMFAES. Eva Vallejos-Vidal was
595 granted with DICYT-USACH Postdoctoral fellowship (Nb. 022043IB).

596

597 **References**

598 Aída, O.A., Herrera, M.L., Flores-Martínez, J.J., Welch, K.C., 2016. Metabolic cost of the activation
599 of immune response in the fish-eating myotis (*Myotis vivesi*): The effects of inflammation
600 and the acute phase response. PLoS One 11, 1–14.
601 https://doi.org/10.1371/journal.pone.0164938

602 Akrami, R., Gharaei, A., Mansour, M.R., Galeshi, A., 2015. Effects of dietary onion (*Allium cepa*)

603 powder on growth, innate immune response and hemato-biochemical parameters of beluga
604 (*Huso huso* Linnaeus, 1754) juvenile. Fish Shellfish Immunol. 45, 828–834.
605 https://doi.org/10.1016/j.fsi.2015.06.005

606 Alipieva, K., Korkina, L., Orhan, I.E., Georgiev, M.I., 2014. Verbascoside - A review of its
607 occurrence, (bio)synthesis and pharmacological significance. Biotechnol. Adv. 32, 1065-1076.
608 https://doi.org/10.1016/j.biotechadv.2014.07.001

609 Álvarez-Rodríguez, M., Pereiro, P., Reyes-López, F.E., Tort, L., Figueras, A., Novoa, B., 2018.
610 Analysis of the long-lived responses induced by immunostimulants and their effects on a
611 viral infection in Zebrafish (*Danio rerio*). Front. Immunol. 9, 1575.
612 https://doi.org/10.3389/fimmu.2018.01575

613 Arthur, H., Joubert, E., De Beer, D., Malherbe, C.J., Witthuhn, R.C., 2011. Phenylethanoid
614 glycosides as major antioxidants in *Lippia multiflora* herbal infusion and their stability during
615 steam pasteurisation of plant material. Food Chem. 127, 581-588.
616 https://doi.org/10.1016/j.foodchem.2011.01.044

617 Awad, E., Awaad, A., 2017. Role of medicinal plants on growth performance and immune status in
618 fish. Fish Shellfish Immunol. 67, 40–54. https://doi.org/10.1016/j.fsi.2017.05.034

619 Babalola, I.T., Shode, F.O., 2013. Ubiquitous ursolic acid : a potential pentacyclic triterpene natural
620 product. J. Pharmacogn. Phytochem. 2, 214–222.

621 Baricevic, D., Sosa, S., Della Loggia, R., Tubaro, A., Simonovska, B., Krasna, A., Zupancic, A., 2001.
622 Topical anti-inflammatory activity of *Salvia officinalis* L. leaves: the relevance of ursolic acid.
623 J. Ethnopharmacol. 75, 125–132. https://doi.org/10.1016/S0378-8741(00)00396-2

624 Bulfon, C., Volpatti, D., Galeotti, M., 2014. In vitro antibacterial activity of plant ethanolic extracts
625 against fish pathogens. J. World Aquac. Soc. 45, 545–557.
626 https://doi.org/10.1111/jwas.12151

627 Campoverde, C., Milne, D.J., Estévez, A., Duncan, N., Secombes, C.J., Andree, K.B., 2017. Ontogeny
628 and modulation after PAMPs stimulation of β-defensin, hepcidin, and piscidin antimicrobial
629 peptides in meagre (*Argyrosomus regius*). Fish Shellfish Immunol. 69, 200–210.
630 https://doi.org/10.1016/j.fsi.2017.08.026

631 Castro, R., Zou, J., Secombes, C.J., Martin, S.A.M., 2011. Cortisol modulates the induction of
632 inflammatory gene expression in a rainbow trout macrophage cell line. Fish Shellfish
633 Immunol. 30, 215–223. https://doi.org/10.1016/j.fsi.2010.10.010

634 Caturla, N., Funes, L., Pérez-Fons, L., Micol, V., 2011. A randomized, double-blinded, placebo-
635 controlled study of the effect of a combination of lemon verbena extract and fish oil omega-
636 3 fatty acid on joint management. J. Altern. Complement. Med. 17, 1051–1063.
637 https://doi.org/10.1089/acm.2010.0410

638 Chakraborty, S.B., Hancz, C., 2011. Application of phytochemicals as immunostimulant,
639 antipathogenic and antistress agents in finfish culture. Rev. Aquac. 3, 103–119.
640 https://doi.org/10.1111/j.1753-5131.2011.01048.x

641 Chen, Y., Zhou, Z., Min, W., 2018. Mitochondria, oxidative stress and innate immunity. Front.
642 Physiol. 9, 1–10. https://doi.org/10.3389/fphys.2018.01487

643 Dawood, M.A.O., Koshio, S., Esteban, M.Á., 2018. Beneficial roles of feed additives as
644 immunostimulants in aquaculture: a review. Rev. Aquac. 10, 950–974.
645 https://doi.org/10.1111/raq.12209

646 Defoirdt, T., Sorgeloos, P., Bossier, P., 2011. Alternatives to antibiotics for the control of bacterial
647 disease in aquaculture. Curr. Opin. Microbiol. 14, 251–258.
648 https://doi.org/10.1016/j.mib.2011.03.004

649 Ding, Y.J., Sun, C.Y., Wen, C.C., Chen, Y.H., 2015. Nephroprotective role of resveratrol and ursolic
650 acid in aristolochic acid intoxicated zebrafish. Toxins (Basel) 7, 97–109.

https://doi.org/10.3390/toxins7010097

Engelsma, M.Y., Huising, M.O., Van Muiswinkel, W.B., Flik, G., Kwang, J., Savelkoul, H.F.J., Verburg-Van Kemenade, B.M.L., 2002. Neuroendocrine-immune interactions in fish: A role for interleukin-1. Vet. Immunol. Immunopathol. 87, 467–479. https://doi.org/10.1016/S0165-2427(02)00077-6

Engelsma, M.Y., Stet, R.J.M., Schipper, H., Verbung-van Kemenade, B.M.L., 2006. Regulation of interleukin 1 beta RNA expression in the common carp, *Cyprinus carpio* L. Mol. Immunol. 43, 1653–1664. https://doi.org/10.1016/j.molimm.2005.09.024

FAO, 2016. The FAO action plan on antimicrobial resistance 2016-2020. Br. Med. J. 317, 25.

Fernández-Navarro, M., Peragón, J., Esteban, F.J., de la Higuera, M., Lupiáñez, J.A., 2006. Maslinic acid as a feed additive to stimulate growth and hepatic protein-turnover rates in rainbow trout (*Onchorhynchus mykiss*). Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 144, 130–140. https://doi.org/10.1016/j.cbpc.2006.07.006

Froehlich, H.E., Jacobsen, N.S., Essington, T.E., Clavelle, T., Halpern, B.S., 2018. Avoiding the ecological limits of forage fish for fed aquaculture. Nat. Sustain. 1, 298–303. https://doi.org/10.1038/s41893-018-0077-1

Fuchs, V.I., Schmidt, J., Slater, M.J., Zentek, J., Buck, B.H., Steinhagen, D., 2015. The effect of supplementation with polysaccharides, nucleotides, acidifiers and Bacillus strains in fish meal and soy bean based diets on growth performance in juvenile turbot (*Scophthalmus maximus*). Aquaculture 437, 243–251. https://doi.org/10.1016/j.aquaculture.2014.12.007

Funes, L., Fernández-arroyo, S., Laporta, O., Pons, A., Roche, E., Segura-carretero, A., 2009. Correlation between plasma antioxidant capacity and verbascoside levels in rats after oral administration of lemon verbena extract. Food Chem. 117, 589–598. https://doi.org/10.1016/j.foodchem.2009.04.059

675 Galina, J., Yin, G., Ardó, L., Jeney, Z., 2009. The use of immunostimulating herbs in fish. An
676 overview of research. Fish Physiol. Biochem. 35, 669–676. https://doi.org/10.1007/s10695-
677 009-9304-z

678 Ghorbani, A., Esmaeilizadeh, M., 2017. Pharmacological properties of *Salvia officinalis* and its
679 components. J. Tradit. Complement. Med. 7, 433–440.
680 https://doi.org/10.1016/j.jtcme.2016.12.014

681 Gisbert, E., Andree, K.B., Quintela, J.C., Calduch-Giner, J.A., Ipharraguerre, I.R., Pérez-Sánchez, J.,
682 2017. Olive oil bioactive compounds increase body weight, and improve gut health and
683 integrity in gilthead sea bream (*Sparus aurata*). Br. J. Nutr. 117, 351–363.
684 https://doi.org/10.1017/S0007114517000228

685 Grayfer, L., Hodgkinson, J.W., Hitchen, S.J., Belosevic, M., 2011. Characterization and functional
686 analysis of goldfish (*Carassius auratus* L.) interleukin-10. Mol. Immunol. 48, 563–571.
687 https://doi.org/10.1016/j.molimm.2010.10.013

688 Guardiola, F.A., Saraiva-Fraga, M., Cuesta, A., Esteban, M.A., 2018. Changes in natural haemolytic
689 complement activity induced by stress in gilthead seabream (*Sparus aurata* L.). Fish Shellfish
690 Immunol. 78, 317–321. https://doi.org/10.1016/j.fsi.2018.04.056

691 Harikrishnan, R., Balasundaram, C., Heo, M.S., 2011. Impact of plant products on innate and
692 adaptive immune system of cultured finfish and shellfish. Aquaculture 317, 1–15.
693 https://doi.org/10.1016/j.aquaculture.2011.03.039

694 Hayden, M.S., Ghosh, S., 2014. Regulation of NF-κB by TNF family cytokines. Semin. Immunol. 26,
695 253–266. https://doi.org/10.1016/j.smim.2014.05.004

696 Jedinák, A., Mučková, M., Košťálová, D., Maliar, T., Mašterová, I., 2006. Antiprotease and
697 antimetastatic activity of ursolic acid isolated from *Salvia officinalis*. Zeitschrift fur
698 Naturforsch. - Sect. C J. Biosci. 61, 777–782.

699 Jie, Y.H., Cammisuli, S., Baggiolini, M., 1984. Immunomodulatory effects of *Panax Ginseng* C.A.
700 Meyer in the mouse. Agents Actions 15, 386–391. https://doi.org/10.1007/BF01972376

701 John, S., Kale, M., Rathore, N., Bhatnagar, D., 2001. Protective effect of vitamin E in dimethoate
702 and malathion induced oxidative stress in rat erythrocytes. J. Nutr. Biochem. 12, 500–504.
703 https://doi.org/10.1016/S0955-2863(01)00160-7

704 Kunkel, S.D., Elmore, C.J., Bongers, K.S., Ebert, S.M., Fox, D.K., Dyle, M.C., Bullard, S.A., Adams,
705 C.M., 2012. Ursolic acid increases skeletal muscle and brown fat and decreases diet-induced
706 obesity, glucose intolerance and fatty liver disease. PLoS One 7.
707 https://doi.org/10.1371/journal.pone.0039332

708 Li, B.-Y., Hu, Y., Li, J., Shi, K., Shen, Y.-F., Zhu, B., Wang, G.-X., 2019. Ursolic acid from *Prunella*
709 *vulgaris* L. efficiently inhibits IHNV infection *in vitro* and *in vivo*. Virus Res. 273, 197741.
710 https://doi.org/10.1016/j.virusres.2019.197741

711 Li, Z.H., Xie, S., Wang, J.X., Sales, J., Li, P., Chen, D.Q., 2009. Effect of intermittent starvation on
712 growth and some antioxidant indexes of *Macrobrachium nipponense* (De Haan). Aquac. Res.
713 40, 526–532. https://doi.org/10.1111/j.1365-2109.2008.02123.x

714 Masek, T., Vopalensky, V., Suchomelova, P., Pospisek, M., 2005. Denaturing RNA electrophoresis
715 in TAE agarose gels. Anal. Biochem. 336, 46–50. https://doi.org/10.1016/j.ab.2004.09.010

716 Mauriz, E., Vallejo, D., Tuñón, M.J., Rodriguez-López, J.M., Rodríguez-Pérez, R., Sanz-Gómez, J.,
717 García-Fernández, M.C., 2015. Effects of dietary supplementation with lemon verbena
718 extracts on serum inflammatory markers of multiple sclerosis patients. Nutr. Hosp. 31, 764–
719 771. https://doi.org/10.3305/nh.2015.31.2.8319

720 Moore, K.W., de Waal Malefyt, R., Coffman, R.L., O'Garra, A., 2001. Interleukin -10 and the
721 interleukin -10 receptor. Annu. Rev. Immunol. 19, 683–765.
722 https://doi.org/10.1146/annurev.immunol.19.1.683

723 Oršolić, N., Terzić, S., Šver, L., Bašić, I., 2005. Polyphenolic compounds from propolis modulate
724 immune responses and increase host resistance to tumour cells. Food Agric. Immunol. 16,
725 165–179. https://doi.org/10.1080/09540100500258484

726 Otto, D.M.E., Moon, T.W., 1996. Endogenous antioxidant systems of two teleost fish, the rainbow
727 trout and the black bullhead, and the effect of age. Fish Physiol. Biochem. 15, 349–358.
728 https://doi.org/10.1007/BF02112362

729 Pandey, S., Parvez, S., Sayeed, I., Haque, R., Bin-Hafeez, B., Raisuddin, S., 2003. Biomarkers of
730 oxidative stress: A comparative study of river Yamuna fish *Wallago attu* (Bl. & Schn.). Sci.
731 Total Environ. 309, 105–115. https://doi.org/10.1016/S0048-9697(03)00006-8

732 Parra, D., Reyes-Lopez, F.E., Tort, L., 2015. Mucosal immunity and B cells in teleosts: Effect of
733 vaccination and stress. Front. Immunol. 6, 1–12. https://doi.org/10.3389/fimmu.2015.00354

734 Pastorelli, G., Rossi, R., Corino, C., 2012. Influence of *Lippia citriodora* verbascoside on growth
735 performance, antioxidant status, and serum immunoglobulins content in piglets. Czech J.
736 Anim. Sci. 57, 312–322. https://doi.org/10.17221/6006-CJAS

737 Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR.
738 Nucleic Acids Res. 29, e45. https://doi.org/10.1093/nar/29.9.e45

739 Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable
740 housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--
741 Excel-based tool using pair-wise correlations. Biotechnol. Lett. 26, 509–15.
742 https://doi.org/10.1023/B:BILE.0000019559.84305.47

743 Piazzon, M.C., Savelkoul, H.F.J., Pietretti, D., Wiegertjes, G.F., Forlenza, M., 2015. Carp Il10 has
744 anti-inflammatory activities on phagocytes, promotes proliferation of memory T cells, and
745 regulates B cell differentiation and antibody secretion. J. Immunol. 194, 187–199.
746 https://doi.org/10.4049/jimmunol.1402093

747 Quirantes-Piné, R., Funes, L., Micol, V., Segura-Carretero, A., Fernández-Gutiérrez, A., 2009. High-
748 performance liquid chromatography with diode array detection coupled to electrospray
749 time-of-flight and ion-trap tandem mass spectrometry to identify phenolic compounds from
750 a lemon verbena extract. J. Chromatogr. A 1216, 5391–5397.
751 https://doi.org/10.1016/j.chroma.2009.05.038

752 Reyes-Cerpa, S., Maisey, K., Reyes-López, F., Toro-Ascuy, D., Sandino, A.M., Imarai, M., 2013. Fish
753 cytokines and immune response, in: new advances and contributions to fish biology. InTech,
754 pp. 3–58. https://doi.org/http://dx.doi.org/10.5772/53504

755 Reyes-Cerpa, S., Reyes-López, F., Toro-Ascuy, D., Montero, R., Maisey, K., Acuña-Castillo, C.,
756 Sunyer, J.O., Parra, D., Sandino, A.M., Imarai, M., 2014. Induction of anti-inflammatory
757 cytokine expression by IPNV in persistent infection. Fish Shellfish Immunol. 41, 172–182.
758 https://doi.org/10.1016/j.fsi.2014.08.029

759 Reyes-Cerpa, S., Vallejos-Vidal, E., José Gonzalez-Bown, M., Morales-Reyes, J., Pérez-Stuardo, D.,
760 Vargas, D., Imarai, M., Cifuentes, V., Spencer, E., María Sandino, A., Reyes-López, F.E., 2018.
761 Effect of yeast (*Xanthophyllomyces dendrorhous*) and plant (Saint John's wort, lemon balm,
762 and rosemary) extract based functional diets on antioxidant and immune status of Atlantic
763 salmon (*Salmo salar*) subjected to crowding stress. Fish Shellfish Immunol. 74, 250–259.
764 https://doi.org/10.1016/j.fsi.2017.12.061

765 Reyes-López, F.E., Romeo, J.S., Vallejos-Vidal, E., Reyes-Cerpa, S., Sandino, A.M., Tort, L.,
766 Mackenzie, S., Imarai, M., 2015. Differential immune gene expression profiles in susceptible
767 and resistant full-sibling families of Atlantic salmon (*Salmo salar*) challenged with infectious
768 pancreatic necrosis virus (IPNV). Dev. Comp. Immunol. 53, 210–221.
769 https://doi.org/10.1016/j.dci.2015.06.017

770 Rufino-Palomares, E., Reyes-Zurita, F.J., Fuentes-Almagro, C.A., de la Higuera, M., Lupiáñez, J.A.,
771 Peragón, J., 2011. Proteomics in the liver of gilthead sea bream (*Sparus aurata*) to elucidate

772 the cellular response induced by the intake of maslinic acid. Proteomics 11, 3312–3325.
773 https://doi.org/10.1002/pmic.201000271

774 Santoro, A., Bianco, G., Picerno, P., Aquino, R.P., Autore, G., Marzocco, S., Gazzerro, P., Lioi, M.B.,
775 Bifulco, M., 2008. Verminoside- and verbascoside-induced genotoxicity on human
776 lymphocytes: Involvement of PARP-1 and p53 proteins. Toxicol. Lett. 178, 71–76.
777 https://doi.org/10.1016/j.toxlet.2008.02.006

778 Saurabh, S., Sahoo, P.K., 2008. Lysozyme: An important defence molecule of fish innate immune
779 system. Aquac. Res. 39, 223–239. https://doi.org/10.1111/j.1365-2109.2007.01883.x

780 Shepherd, B.S., Spear, A.R., Philip, A.M., Leaman, D.W., Stepien, C.A., Sepulveda-Villet, O.J.,
781 Palmquist, D.E., Vijayan, M.M., 2018. Effects of cortisol and lipopolysaccharide on expression
782 of select growth-, stress- and immune-related genes in rainbow trout liver. Fish Shellfish
783 Immunol. 74, 410–418. https://doi.org/10.1016/j.fsi.2018.01.003

784 Sönmez, A.Y., Bilen, S., Alak, G., Hisar, O., Yanık, T., Biswas, G., 2014. Growth performance and
785 antioxidant enzyme activities in rainbow trout (*Oncorhynchus mykiss*) juveniles fed diets
786 supplemented with sage, mint and thyme oils. Fish Physiol. Biochem. 41, 165–175.
787 https://doi.org/10.1007/s10695-014-0014-9

788 Stevens, M.G., Kehrli, M.E., Canning, P.C., 1991. A colorimetric assay for quantitating bovine
789 neutrophil bactericidal activity. Vet. Immunol. Immunopathol. 28, 45–56.
790 https://doi.org/10.1016/0165-2427(91)90042-B

791 Vallejos-Vidal, E., Reyes-López, F., Teles, M., MacKenzie, S., 2016. The response of fish to
792 immunostimulant diets. Fish Shellfish Immunol. 56, 34-69.
793 https://doi.org/10.1016/j.fsi.2016.06.028

794 Vaseeharan, B., Thaya, R., 2014. Medicinal plant derivatives as immunostimulants: An alternative
795 to chemotherapeutics and antibiotics in aquaculture. Aquac. Int. 22, 1079–1091.

796 https://doi.org/10.1007/s10499-013-9729-3

797 Wang, W., Sun, J., Liu, C., Xue, Z., 2017. Application of immunostimulants in aquaculture: current
798 knowledge and future perspectives. Aquac. Res. 48, 1–23.
799 https://doi.org/10.1111/are.13161

800 Wei, H., Yin, L., Feng, S., Wang, X., Yang, K., Zhang, A., Zhou, H., 2015. Dual-parallel inhibition of IL-
801 10 and TGF-β1 controls LPS-induced inflammatory response via NF-κB signaling in grass carp
802 monocytes/macrophages. Fish Shellfish Immunol. 44, 445–452.
803 https://doi.org/10.1016/j.fsi.2015.03.023

804 Wells, R.M.G., McIntyre, R.H., Morgan, A.K., Davie, P.S., 1986. Physiological stress responses in big
805 gamefish after capture: Observations on plasma chemistry and blood factors. Comp.
806 Biochem. Physiol. 84A, 565–571. https://doi.org/10.1016/0300-9629(86)90366-X

807 Wójciak-Kosior, M., Sowa, I., Kocjan, R., Nowak, R., 2013. Effect of different extraction techniques
808 on quantification of oleanolic and ursolic acid in *Lamii albi* flos. Industrial Crops and Products
809 44, 373-377. https://doi.org/10.1016/j.indcrop.2012.11.018

810 Yang, X., Wei, H., Qin, L., Zhang, S., Wang, X., Zhang, A., Du, L., Zhou, H., 2014. Reciprocal
811 interaction between fish TGF-β1 and IL-1β is responsible for restraining IL-1β signaling
812 activity in grass carp head kidney leukocytes. Dev. Comp. Immunol. 47, 197–204.
813 https://doi.org/10.1016/j.dci.2014.07.023

814 Yin, G., Jeney, G., Racz, T., Xu, P., Jun, X., Jeney, Z., 2006. Effect of two Chinese herbs (*Astragalus*
815 *radix* and *Scutellaria radix*) on non-specific immune response of tilapia, *Oreochromis*
816 *niloticus*. Aquaculture 253, 39–47. https://doi.org/10.1016/j.aquaculture.2005.06.038

817 Yin, Z., Kwang, J., 2000. Carp interleukin-1β in the role of an immuno-adjuvant. Fish Shellfish
818 Immunol. 10, 375–378. https://doi.org/10.1006/fsim.1999.0241

819 Zhan, X. liang, Ma, T. yang, Wu, J. ying, Yi, L. yuan, Wang, J. yuan, Gao, X. ke, Li, W. sheng, 2015.

Cloning and primary immunological study of TGF-β1 and its receptors TβR I /TβR II in tilapia (*Oreochromis niloticus*). Dev. Comp. Immunol. 51, 134–140. https://doi.org/10.1016/j.dci.2015.03.008

Zou, J., Holland, J., Pleguezuelos, O., Cunningham, C., Secombes, C.J., 2000. Factors influencing the expression of interleukin-1β in cultured rainbow trout (*Oncorhynchus mykiss*) leucocytes. Dev. Comp. Immunol. 24, 575–582. https://doi.org/10.1016/S0145-305X(99)00085-3

Zou, J., Peddie, S., Scapigliati, G., Zhang, Y., Bols, N.C., Ellis, A.E., Secombes, C.J., 2003. Functional characterisation of the recombinant tumor necrosis factors in rainbow trout, *Oncorhynchus mykiss*. Dev. Comp. Immunol. 27, 813–822. https://doi.org/10.1016/S0145-305X(03)00077-6

Zou, J., Secombes, C.J., 2016. The function of fish cytokines. Biology (Basel) 5, 23. https://doi.org/10.3390/biology5020023

833

834 Table 1. List of ingredients and proximal composition of experimental diets.

Ingredients, %	Control diet	MPLE diet
Fishmeal LT70	7.0	7.0
Soy protein concentrate	21.0	21.0
Pea protein concentrate	12.0	12.0
Wheat gluten	12.0	12.0
Corn gluten	12.0	12.0
Soybean meal 48	5.0	5.0
Wheat meal	10.4	10.4
Fish oil (SAVINOR)	15.0	15.0
Vitamin and mineral Premix PV01	1.0	1.0
Soy lecithin - Powder	1.0	1.0
Binder (guar gum)	1.0	1.0
MCP	2.0	2.0
L-Lysine	0.3	0.3
L-Tryptophan	0.1	0.1
DL-Methionine	0.2	0.2
MPLE	-	0.1
Proximate composition		
Crude protein, %	48.37	48.37
Crude fat, %	17.19	17.21
Fiber, %	1.52	1.52
Ash, %	5.88	5.88
Gross Energy, MJ/kg	21.62	21.62

835 Abbreviation: MPLE, medicinal plant leaf extract obtained from sage (*Salvia officinalis*) and lemon

836 verbena (*Lippia citriodora*).

837

838

839

840 Table 2. Sequence of primers used in real-time PCR analysis.

Gene name	Acronym	Accession no.	Sequence 5'→3'	Amplification efficiency (%)
β-Actin	*β-actin*	X89920	FW: TCCTGCGGAATCCATGAGA RV: GACGTCGCACTTCATGATGCT	1.99
Lysozyme	*lys*	AM749959.1	FW: TCATCGCTGCCATCATCTCC RV: TGTTCCTCACTGTCCCATGC	1.96
Immunoglobulin M	*igm*	JQ811851.1	FW: GATCGTGACATCGTCTGAGG RV: TGTTGGGTTGTGGTTGTAGG	2.01
Interleukin 1β	*il1β*	AJ277166.2	FW: TCAGCACCGCAGAAGAAAAC RV: TAACACTCTCCACCCTCCAC	1.99
Tumour necrosis factor alpha	*tnf-α*	AJ413189	FW: CAGGCGTCGTTCAGAGTCTC RV: CTGTGGCTGAGAGGTGTGTG	1.99
CD4 molecule	*cd4*	AM489485.1	FW: TAGCGGAAAGTGGAGGTGTG RV: GCCTGGGGTGTCTCATCTTC	2.00
Interleukin 10	*il10*	JX976621.1	FW: GAGCGTGGAGGAATCTTTCAA RV: GATCTGCTGGATGGACTGC	2.01
Transforming growth factor Beta 1	*tgfβ1*	AF424703.1	FW: AGACCCTTCAGAACTGGCTC RV: ACTGCTTTGTCTCCCCTACC	1.95
Manganese superoxide dismutase	*mn-sod*	JQ308833.1	FW: CCTGACCTGACCTACGACTATGG RV: AGTGCCTCCTGATATTTCTCCTCTG	1.97
Catalase	*cat*	JQ308823	FW: TGGTCGAGAACTTGAAGGCTGTC RV: AGGACGCAGAAATGGCAGAGG	2.01

841

842

843 Table 3. Survival, growth performance and feed efficiency parameters in gilthead seabream (*Sparus*
844 *aurata*) fed experimental diets. Values are expressed as the mean ± SD (*n* = 4 tanks). Different
845 letters denote statistical significant differences among groups (t-test, $P < 0.05$).

846

	Control diet	**Control diet + 0.1% MPLE**
Survival (%)	98.0 ± 1.0	99.0 ± 0.8
BW_i (g)	26.0 ± 0.2	26.0 ± 0.2
BW_f (g)	173.8 ± 8.2 a	189.6 ± 5.0 b
SL_f (cm)	18.8 ± 0.32	19.2 ± 0.20
Fulton's condition factor (K)	2.65 ± 0.04	2.68 ± 0.03
SGR (% day^{-1})	2.16 ± 0.004 a	2.26 ± 0.002 b
FCR	1.23 ± 0.04 b	1.10 ± 0.04 a

847 Abbreviation: MPLE, medicinal plant leaf extract obtained from sage (*Salvia officinalis*) and lemon
848 verbena (*Lippia citriodora*).

849

850

851

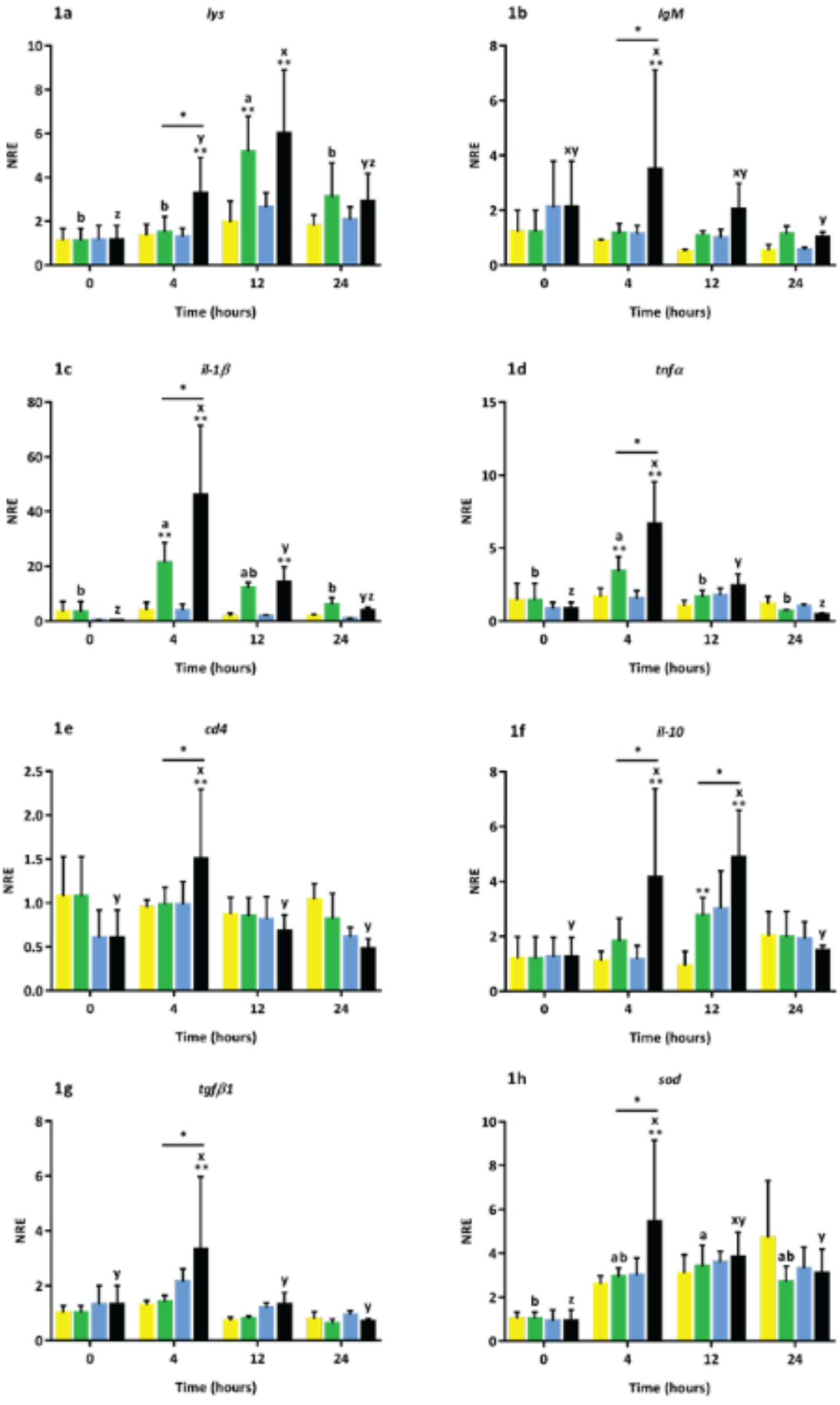
852

853 **Table 4.** Levels of protein immunoglobulin M (IgM) and complement and bacteriolytic activities
854 measured in gilthead seabream (*Sparus aurata*) plasma fed experimental diets. Data are expressed
855 as mean ± SEM [n = 4; calculated from the mean of each tank (n =5 fish per tank)]. No significant
856 differences were registered (t-test; $P > 0.05$)

	Control diet	**Control diet + 0.1% MPLE**
Protein IgM (Δ O.D. at λ = 450 nm)	0.55 ± 0.06	0.66 ± 0.06
Complement activity (Δ O.D. at λ = 540 nm)	94.85 ± 50.8	122.5 ± 50.0
Bacteriolytic activity (ACH_{50} U ml^{-1})	81.16 ± 0.66	80.39 ± 0.32

857 Abbreviation: MPLE, medicinal plant leaf extract obtained from sage (*Salvia officinalis*) and lemon
858 verbena (*Lippia citriodora*); O.D. = optical density; ACH_{50} = volume of plasma producing 50%
859 haemolysis ($P > 0.05$).
860
861

Figure 1. Normalized relative expression (NRE) of immune-related genes in gilthead seabream (*Sparus aurata*) after 92 days of feeding with experimental diets.. The expression of *lys*, *cd4*, *IgM*, *il-1ᵦ*, *tnf-α*, *il-10*, *tgfᵦ1*, *sod* and *cat* was evaluated in splenocytes primary cell culture (SPCC) isolated from gilthead sea breams at 4, 12 and 24 h after exposure to PBS or LPS. Yellow bar: PBS-treated splenocytes from gilthead sea bream fed with control diet ($SPCC_{CD}$+PBS). Green bars: LPS-treated splenocytes from gilthead sea bream fed with control diet ($SPCC_{CD}$+LPS). Blue bars: PBS-treated splenocytes from gilthead sea bream fed with 0.1% UA-VB diet ($SPCC_{MPLE}$+PBS). Black bars: LPS-treated splenocytes from gilthead sea bream fed with 0.1% UA-VB diet ($SPCC_{MPLE}$+LPS). The time 0 h corresponds to the basal state prior to the beginning of the treatment. Statistical analysis: Two-way ANOVA with Tukey's post hoc test. Asterisk (*) represents significant differences between LPS treatments at the same time-point evaluated; (**) represents significant differences between cells treated with PBS and LPS within the same diet and time-point evaluated; different letters (a, b and c) represent significant differences between the control diet and different post-exposure times with LPS ($P < 0.05$). Different letters (x, y and z) represent significant differences between the 0.1% UA-VB diet at different post-exposure times with LPS ($P < 0.05$). Abbreviations: MPLE, medicinal plant leaf extract obtained from sage (*Salvia officinalis*) and lemon verbena (*Lippia citriodora*); *il-1ᵦ*, *interleukin 1 beta*; *tnf-α*, *tumor necrosis factor alpha; il-10*, *interleukin 10*; *tgfᵦ1*, *transforming growth factor beta 1*; *cd4*, *cluster of differentiation 4*; *mn-sod*, *manganese superoxide dismutase*; *cat*: *catalase*.


1a
lys
1b
lgM
1c
il-1β
1d
tnfα
1e
cd4
1f
il-10
1g
tgfβ1
1h
sod
1i
cat
NRE
Time (hours)
0
4
12
24
SPCC_+PBS