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Olive oil bioactive compounds increase body weight, and improve gut health and integrity in gilthead sea bream (*Sparus aurata*)

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Abstract

An olive oil bioactive extract (OBE) rich in bioactive compounds like polyphenols, triterpenic acids, long-chain fatty alcohols, unsaturated hydrocarbons, tocopherols and sterols was tested (0, 0·08, 0·17, 0·42 and 0·73 % OBE) in diets fed to sea bream (*Sparus aurata*) (initial weight: 5·4 (SD 1·2) g) during a 90-d trial (four replicates). Fish fed diets containing 0·17 and 0·42 % OBE were 5 % heavier (61·1 (SD 1·6) and 60·3 (SD 1·1) g, respectively) than those of the control group (57·0 (SD 0·7) g), although feed conversion ratio and specific feed intake did not vary. There were no differences in lipid peroxidation (LPO) levels, catalase, glutathione reductase and glutathione *S*-transferase activities in the intestine and liver, although there was a tendency of lower intestinal and hepatic LPO levels in fish fed OBE diets. No differences in villus size were found among treatments, whereas goblet cell density in the control group was on average14·3 % lower than in fish fed OBE diets. The transcriptomic profiling of intestinal markers, covering different biological functions like (i) cell differentiation and proliferation, (ii) intestinal permeability, (iii) enterocyte mass and epithelial damage, (iv) IL and cytokines, (v) pathogen recognition receptors and (vi) mitochondria function, indicated that among the eighty-eight evaluated genes, twenty-nine were differentially expressed (0·17 % OBE diet), suggesting that the additive has the potential of improving the condition and defensive role of the intestine by enhancing the maturation of enterocytes, reducing oxidative stress, improving the integrity of the intestinal epithelium and enhancing the intestinal innate immune function, as gene expression data indicated.

Key words: Functional feeds: Gilthead sea bream: Gut health: Olive oil

The Mediterranean diet has been widely reported to be a model of healthy eating for its contribution to a favourable health status and a better quality of life[1]. Olive oil, along with fruits, vegetables and fish, is an important constituent of this diet, and is considered a major factor in preserving a healthy and relatively disease-free population[2]. Olive oil contains a high level of MUFA, as well as multiple minor components with biological properties[3]. The saponiable fraction of olive oil is primarily composed of TAG, partial glycerides, esters of fatty acids or free fatty acids and phosphatides, which represent nearly 98 % of the oil chemical composition, whereas its unsaponifiable fraction is dominated by minor compounds such as tocopherols, phytosterols, carotenoids (*β*-carotene and lutein), triterpenic alcohols (uvaol and erythrodiol), pentacyclic triterpenes (oleanolic and maslinic acid) and phenolic compounds (i.e. tyrosol, hydroxytyrosol, oleocanthal, oleuropein)[4,5].

A plethora of studies have reported health benefits of olive oil and its minor components in humans, especially in preventing and/or reducing hypercholesterolaemia, serum lipoprotein levels and atherosclerosis, hypertension, CVD and thrombotic risk, oxidation and oxidative stress, obesity and type 2 diabetes, inflammatory processes and cancer[4,6,7]. Although there is information about the use of olive oil as substitute of fish oil in aqua feeds[8], published evidence about the use of olive oil and its derivatives as feed additives is scarce, with most of the available information being focused on maslinic acid with contradictory results regarding the potential effects of this triterpenic compound in promoting fish growth performance[9–11].

Supporting fish growth was one of the main objectives of feed producers and fish farmers during the last decades when the efficiency of nutrient conversion and assimilation was one

Abbreviations: BW, body weight; BW_f, final body weight; C_t, cycle threshold; LPO, lipid peroxidation; OBE, olive oil bioactive extract; SL_f, final standard length.

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of their main targets of the industry; however, the development of this sector, as well as the intensification of production and strong competition among producers, has forced the sector to reduce production costs for improved economies of scale and find ways to create a competitive edge. In this context, maintaining fish health and welfare is a concern in aquaculture, particularly in the light of the potential effects of climate change and super intensification of the production. Moreover, new management strategies are needed to support growth and health[12]. Thus, the use of functional feeds may be regarded as the future of aquaculture; by embracing nutritional strategies to address specific stresses, environmental situations, life stage requirements and pathologies, the industry can optimise animal performance as well as operational efficiency when health improvements lead to reduced production losses. Thus, in the present study, we decided to test the potential benefits of an olive oil bioactive extract (OBE) (unsaponifiable fraction) rich in polyphenols, triterpenic acids, long-chain fatty alcohols, unsaturated hydrocarbons, tocopherols and sterols in terms of growth, feed utilisation, and gut health and integrity in gilthead sea bream (*Sparus aurata*).

Methods

Experimental diets

A control diet was formulated with high levels of marine-derived protein sources to contain 53% crude protein, 18% crude fat and 20·9 MJ/kg gross energy and fulfil the nutritional requirements of juvenile sea bream[13]. Based on this basal formulation, four additional diets were produced by adding at the expense of fish oil an olive oil containing 9% triterpenic acids, 2% polyphenols, 2% long-chain fatty alcohols and 1% sterols (OBE, OLEA OS-15 FBP; ProNutra Solutions S.L.) (Table 1). Diets were manufactured by Sparos Lda. Main ingredients were ground (below 250 μm) in a micropulverizer hammer mill (Hosokawa Micron). Powder ingredients and oils were then mixed according to the target formulation in a paddle mixer (RM90; Mainca). All diets were manufactured by temperature controlled extrusion (pellet sizes: 0·8 and 1·5 mm) by means of a low-shear extruder (P55; Italplast). Upon extrusion, all feed batches were dried in a convection oven (OP 750-UF; LTE Scientific) for 4 h at 45°C. Samples of each diet were analysed for proximate composition analysis (Table 1).

Animals, experimental conditions and general procedures

Gilthead sea bream fingerlings were obtained from a fish farm (Piscicultura Marina Mediterránea SL), transported by road to the IRTA-SCR facilities and acclimated for 10 d to new husbandry and water conditions in a 2 m^3 circular fiberglass tank. During this period, fish were fed twice a day with Microbaq 15 (Dibaq SA) at 2% of the stocked biomass. Before the onset of the trial, all fish were anaesthetised (tricaine methanesulfonate, MS-222, 150 mg/l), individually weighted (body weight (BW)) and measured for standard length (SL) to the nearest 0·1 g and 1 mm, respectively; and then distributed into twenty fiberglass cylindrical tanks of 400 litres (seventy-five fish per tank, $BW_i = 5{\cdot}4$ (SD 1·2) g).

Water temperature and pH (pH meter 507; Crison Instruments), salinity (MASTER-20T; ATAGO Co. Ltd) and dissolved O_2 (OXI330; Crison Instruments) were 22·1 (SD 0·2)°C, 7·8 (SD 0·1), 36 mg/l and 6·8 (SD 0·3) mg/l, respectively. Water flow rate in experimental tanks was maintained at approximately 9·0 l/min via a recirculation system (IRTAmar®) that maintained adequate water quality (total ammonia and nitrite were ≤0·10 and 0·4 mg/l, respectively) through UV, biological and mechanical filtration. Photoperiod followed natural changes according to the season of the year (January–April; latitude 40°37'41'N). Each diet was tested with four replicates for 90 d. Diets were distributed eight times per d by automatic feeders (ARVO-TEC T Drum 2000; Arvotec) at the rate of 3·3% of the stocked biomass, which approached apparent satiation.

Sampling to monitor fish growth took place monthly from the onset of the feeding period. For that purpose, all fish in each tank were netted, anaesthetised and their wet BW and SL determined. At the end of the trial (90 d), all fish from each tank were measured for their final BW (BW_f, g) and final standard length (SL_f, cm), as well as for determining final size distribution in BW_f. In addition, seventy-two specimens (fasted overnight) per experimental group (eighteen per tank) were killed with an overdose of anaesthetic for assessing the histological organisation of the of the intestinal mucosa, the activity of antioxidative stress enzymes in the intestine and liver (five per tank), proximate carcass composition (five per tank) and gene expression analysis of markers of intestinal integrity and health (eight per tank). Fish growth and feed utilisation from different experimental groups was evaluated by means of the following indices: Fulton's condition factor ($K = (BW_f/SL_f^3) \times 100$; specific growth rate in BW (SGR_{BW}, %) = $((\ln BW_f - \ln BW_i) \times 100)$/time (d); feed conversion ratio (FCR, g/g) = $F/(B_f - B_i)$ and apparent specific feed intake (SFI, %) = SGR × FCR, where F was the total feed intake during the experimental period considered (g) and, B_i and B_f were the initial and final biomass (g).

All animal experimental procedures were conducted in compliance with the experimental research protocol approved by the Committee of Ethics and Animal Experimentation of the Institut de Recerca i Tecnologia Agroalimentàries and in accordance with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

Proximate composition, lipid peroxidation and antioxidative stress enzymes in liver and intestine

For determining the body proximate composition of fish and feed, samples were homogenised (Ultra-Turrax T25 basic, IKA©; Werke), and small aliquots were dried (120°C for 24 h) to estimate water content. The total fat content in samples was quantified gravimetrically after extraction in chloroform–methanol (2:1) and evaporation of the solvent under a stream of N_2 followed by vacuum desiccation overnight[14]. Protein content was determined according to Lowry *et al.*[15]. Ash contents were determined by keeping the sample at 500–600°C for 24 h in

Table 1. Ingredient list and proximate chemical composition (in DM) of experimental diets

	Diets				
Ingredients (g/100 g feed)	A	B	C	D	E
Fishmeal 70LT*	33·00	33·00	33·00	33·00	33·00
Fishmeal 65†	6·00	6·00	6·00	6·00	6·00
CPSP 90‡	7·50	7·50	7·50	7·50	7·50
Squid meal§	7·50	7·50	7·50	7·50	7·50
Soyabean protein concentrate‖	5·00	5·00	5·00	5·00	5·00
Wheat gluten¶	8·00	8·00	8·00	8·00	8·00
Maize gluten**	2·20	2·20	2·20	2·20	2·20
Micronised soyabean meal††	5·00	5·00	5·00	5·00	5·00
Wheat meal‡‡	5·00	5·00	5·00	5·00	5·00
Pea starch§§	4·00	4·00	4·00	4·00	4·00
Concentrated olive oil (OLEA OS-15 FBP)	–	0·08	0·17	0·42	0·73
Fish oil‖‖	13·16	13·08	13·01	12·74	12·43
Vitamin and mineral premix¶¶	1·20	1·20	1·20	1·20	1·20
Vitamin E***	0·10	0·10	0·10	0·10	0·10
Soya lecithin	0·30	0·30	0·30	0·30	0·30
Binder (guar gum)	1·00	1·00	1·00	1·00	1·00
Antioxidant (dry form)†††	0·20	0·20	0·20	0·20	0·20
Composition (%)‡‡‡					
CP	53·1	53·1	53·0	53·1	53·0
Fat	18·4	18·3	18·4	18·4	18·4
Ash	10·7	10·7	10·8	10·7	10·6
Fibre	0·6	0·6	0·6	0·6	0·6
Nitrogen free extractive matter	8·5	9·6	9·6	9·5	9·7
Moisture	7·7	7·7	7·6	7·7	7·7
GE (MJ/kg)§§§	21·4	21·4	21·4	21·4	21·4

CP, crude protein; CF, crude fat.
* Peruvian fishmeal LT: 71 % CP, 11 % CF, Exalmar.
† Fair average quality fishmeal: 62 % CP, 12 %CF, COFACO.
‡ Soluble fish-protein concentrate (CPSP 90): 84 % CP, 12 % CF, Sopropêche.
§ Super prime squid meal: 80 % CP, 3·5 % CF, Sopropêche.
‖ Soycomil© Soy Protein concentrate: 65 % CP, 1 % CF, ADM.
¶ VITEN: 85·7 % CP, 1·3 % CF, ROQUETTE.
** 61 % CP, 6 % CF, COPAM.
†† Micronised soyabean meal: 51·7 % CP, 2·1 % CF, Sorgal SA.
‡‡ CP 11·6 %, CF 1·4 %, Casa Lanchinha.
§§ CP 0·3 %, CF <0·1 %, Cosucra.
‖‖ Marine oil omega 3: Henry Lamotte Oils GmbH.
¶¶ PVO40·01 premix for marine fish, PREMIX Lda. Vitamins (mg/kg diet): DL-*α* tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 6.88 mg; DL-cholecalciferol, 0.050 mg; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; B_{12}, 0·1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): cobalt sulphate, 2·5 mg; copper sulphate, 1·1 mg; ferric citrate, 0·2 g; potassium iodide, 5 mg; manganese sulphate, 15 mg; sodium selenite, 0·2 mg; zinc sulphate, 40 mg; magnesium hydroxide, 0·6 g; potassium chloride 1·1 g; sodium chloride, 0·5 g; calcium carbonate, 4 g.
*** LUTAVIT E 50, ENSOL.
††† Paramega PX, Kemin Europe NV.
‡‡‡ Based on analysis of three samples per diet.
§§§ Gross energy content was estimated by using the following: total carbohydrate × 17·2 J/kg, fat × 39·5 J/kg, and protein × 23·5 J/kg.

a muffle furnace[16]. All chemical analyses were performed in triplicate per fish and feed samples.

Approximately 100 mg of tissue per sample was homogenised for 5 min in eight volumes (v/w) of 0·15 M KCl–KOH, 1 mM-EDTA (pH 7·5) buffer and then subjected to sonication (Vibra-Cell©; Sonics) for 1·5 min at 0–4°C. Homogenised samples were centrifuged at 10 200 ***g*** for 5 min at 4°C, and the supernatant collected for analytical determinations. Quantification of lipid peroxidation (LPO) in the intestine and liver was conducted using the thiobarbituric acid reactive substances method described by Solé *et al.*[17]. In brief, LPO was measured using 200 µl of the homogenate mixed with 650 µl of methanol, 1-methyl-2-phenylindole (solution stock of 10·3 mM) in acetonitrile–methanol (1:3, v/v) and 150 µl of 37 % HCl. This mixture was incubated (40 min, 45°C), cooled on ice and centrifuged at 21 000 ***g*** for 10 min to remove protein precipitates. Absorbance was read at 586 nm, and the amount of peroxidised lipids (in nmol malondialdehyde/100 g tissue, w/w) was evaluated by means of a calibration curve made of a standard solution (10 mM 1,1,3,3-tetramethoxypropane). Homogenised samples, prepared for the determination of LPO, were used to measure activity of antioxidant enzymes. Catalase (CAT, *EC* 1.11.1.6) activity was measured by the decrease in absorbance at 240 nm ($e = 43{\cdot}6\ mM^{-1} \times cm^{-1}$) using 50 mM-$H_2O_2$ as substrate[18]. Glutathione *S*-transferase (GST, *EC* 2.5.1.18) was assayed by the formation of glutathione chlorodinitrobenzene adduct at 340 nm ($e = 9{\cdot}6\ mM^{-1} \times cm^{-1}$), using 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM glutathione as substrates[19]. Glutathione reductase (GR, *EC* 1.8.1.7) activity was determined by measuring the oxidation of NADPH at 340 nm ($e = 6{\cdot}22\ mM^{-1} \times cm^{-1}$), using 20 mM glutathione disulphide and 2 mM NADPH as substrates[20]. Enzyme activities were expressed as specific enzyme activities (nmol/minper mg protein), and soluble protein determined by the Bradford method[21]. All assays were conducted in triplicate

at 25°C, and absorbance read using a spectrophotometer (Tecan™ Infinite M200; Techan Group Ltd).

Histological organisation of the intestine

For histological purposes, fragments of liver, mid and posterior intestine from twenty fish per dietary treatment were dissected and fixed in 4 % buffered formaldehyde (pH = 7·4), dehydrated in a graded series of ethanol, cleared with xylene, embedded in paraffin and cut in serial sections (3–5 μm thick). Sections were stained with Masson's trichrome stain for general histological descriptions, whereas slides were stained with Periodic Acid Schiff for goblet cell identification (neutral mucins produced in intestinal goblet cells stain in magenta). All sections were observed under a light microscope (Leica DM LB; Leica Microsystems) and photographed (Olympus DP70 Digital Camera; Olympus Imaging Europa GmbH). Digital images were processed and analysed using an image analysis software package (ANALYSIS; Soft Imaging Systems GmbH). Measurements of total goblet cell number (full and empty) and villi height were based on the analysis of eight to ten randomly chosen fields from the intestinal mucosa. Goblet cell counts in intestinal villi were expressed over a contour length of 100 μm, whereas villi height and width were calculated according to Escaffre *et al.*[22]. Size (S) of hepatic fat deposits (unstained vacuoles within hepatocytes that corresponded to lipids dissolved during the embedding process of the tissue in paraffin) was estimated at ×400 magnification according to the formula: $S\ (\mu m^2) = 1/4 \times \pi \times a \times b$, where a and b were the minimum and maximum diameters of the vacuole[23].

Quantitative PCR and gene expression analyses of intestinal markers

Total RNA was extracted from the mid-posterior intestine of fish using the TRIzol reagent (Invitrogen®) as specified by the manufacturer. The quantity of RNA isolated was determined using a Gene-Quant spectrophotometer (Amersham Biosciences), measuring optical density at 260 nm, its purity was established by the absorbance ratio 260:280 nm, and quality of the RNA was evaluated using 1·2 % agarose gel electrophoresis. A reverse transcription reaction was carried out using equal quantities of total RNA (1 μg) from each sample and Quanti Tect Reverse Transcription Kit (Qiagen®). Electrophoresis using a 1·2 % agarose gel was run to assess the quality of the RT-PCR product used for real-time quantitative PCR analyses of gene expression.

Differences in dietary-induced gene expression patterns in the intestine among groups were only compared between the control group (Diet A) and the experimental group displaying a greater increase in body weight and overall condition (Diet C). In this sense, we used a previously validated specific PCR-array of eighty-eight target genes[24], this PCR-array was designed to cover different key biological functions: (i) cell differentiation and proliferation, n 14; (ii) intestinal architecture and permeability, n 20; (iii) enterocyte mass and epithelial damage, n 9; (iv) IL and cytokines, n 22; (v) pathogen recognition receptors, n 14; and (vi) mitochondria function and biogenesis, n 11 (Table 2). Real-time quantitative PCR analysis of target genes was performed in eight specimens from Diets A and C using an iCycler IQ Real-time Detection System (Bio-Rad) as described in Pérez-Sánchez *et al.*[24]. In brief, each PCR-well contained a SYBR Green Master Mix (Bio-Rad) and specific primers were used at a final concentration of 0·9 μM. DNA polymerase was activated and complementary DNA denatured by pre-incubation for 3 min at 95°C, the template was amplified for forty cycles of denaturation for 15 s at 95°C and annealing/extension at 60°C for 60 s. All pipetting operations were made with a handling robot (Eppendorf epMotion 5070) to minimise technical variability. β-Actin was chosen as a house keeping gene and no differences in gene expression were found between Diet A (control) and Diet C (experimental group) (cycle threshold (C_t) values of β-actin: 19·72 *v.* 19·83). The efficiency of PCR reactions for all target and reference genes varied between 90 and 98 %. The dynamic range of standard curves (serial dilutions of RT-PCR reactions) spanned five orders of magnitude, and the amount of product in a particular sample was determined by interpolation of the C_t value. The specificity of each reaction was verified by analysis of melting curves. Fluorescence data acquired during the extension phase were ultimately normalised to β-actin by the $\Delta\Delta C_t$ method[25].

Statistical analyses

The mean values of BW_f, SL_f and K were expressed as means and standard deviations. The calculation was based on the values of the individual BW_f, SL and K of all the fish belonging to the same treatment (fish from the four tanks/replicate per dietary treatment analysed together, since there were no statistical differences between replicates), and consequently, the SD describes the dispersion of the individual values. In contrast, the mean values of survival, BW_f size classes, SGR, FCR, SFI, intestinal goblet cell number, villi width and height, and size of hepatic lipid vacuoles were calculated from each replicate tank (n 4) and expressed as means with their standard errors. Data expressed as percentage were arcsine square root transformed before being analysed. Data were compared by means of one-way ANOVA (data normally distributed, Kolmogorov–Smirnov test) and comparisons between experimental groups after finding statistical significances were performed by a Bonferroni test. C_t values between samples obtained from fish fed Diet A and C were compared by means of a t test. Statistically significant differences were indicated by different letters. Data sets were analysed using the SigmaStat 3® software package (Systat Software Inc.).

Results

Survival, somatic growth performance and feed utilisation parameters

Data on survival, somatic growth performance in terms of BW_f, SL_f and K, and feed utilisation parameters of gilthead sea bream fingerlings fed experimental diets are shown in Table 3. No differences in survival were found among experimental groups with mean values ranging between 96·0 and 97·5 %

Table 2. Full list of genes (abbreviation, gene name and accession number) analysed by real-time PCR in intestinal samples of gilthead sea bream (*Sparus aurata*) fed experimental diets containing different levels of a bioactive extract of olive oil

Symbols	Gene names	Accession numbers	Symbols	Gene names	Accession numbers
Cell differentiation and proliferation pathways			IL and cytokines		
Pcna	Proliferating cell nuclear antigen	KF857335	*Il1β*	IL 1 beta	CAD11603
Bmpr1a	Bone morphogenetic protein receptor type-1A	KF857333	*Il1r1*	IL 1 receptor type 1	JX976615
Ihh	Indian hedgehog protein	KF857334	*Il6*	IL 6	B6CKP4
Gli1	Zinc finger protein GLI1	KF857336	*Il6rb*	IL 6 receptor subunit beta	JX976617
Hhip	Hedgehog-interacting protein	KF857338	*Il7*	IL 7	JX976618
Wls	Protein wntless homolog	KF857339	*Il8*	IL 8	JX976619
Myc	Transcriptional regulator Myc	KF857340	*Il8ra*	High affinity IL-8 receptor A	JX976620
Ctnnb1	Catenin β-1	KF857341	*Il10*	IL 10	JX976621
Tcf4	Transcription factor 4	KF857342	*Il10ra*	IL 10 receptor subunit alpha	JX976621
Nle1	Notcheless protein homolog 1	KF857343	*Il12b*	IL 12 B	JX976629
Hes1-b	Transcription factor HES-1-B	KF857344	*Tnfa*	TNFα	AJ413189
Gfi-1	Zinc finger protein GFI-1	KF857345	*Csf1r1*	Macrophage colony-stimulating factor 1 receptor 1	AM050293
Klf4	Krueppel-like factor 4	KF857346	*Cxcl9*	C–X–C motif chemokine 9	KF857315
Intestinal architecture and permeability			*Ccl21*	C–C motif chemokine 21	KF857316
Itgb1bp1	Integrin β-1-binding protein 1	KF861987	*Ccr3*	C–C chemokine receptor type 3	KF857317
Itgb6	Integrin β-6	KF861988	*Ccr9*	C–C chemokine receptor type 9	KF857318
Ilk	Integrin-linked protein kinase	KF861989	*Ccr11*	C–C chemokine receptor type 11	KF857319
Ocln	Occludin	KF861990	*Ccl20*	C–C chemokine CK8	GU181393
Cldn3	Claudin 3	KF861991	*Cd48*	CD48 antigen	KF857320
Cldn12	Claudin 12	KF861992	*Cd276*	CD276 antigen	KF857321
Cldn15	Claudin 15	KF861993	Pathogen recognition receptors		
Tjp1	Tight junction protein ZO-1	KF861994	*Tlr1*	Toll-like receptor 1	KF857322
Cdh1	Cadherin 1	KF861995	*Tlr2*	Toll-like receptor 2	KF857323
Cdh17	Cadherin 17	KF861996	*Tlr5*	Toll-like receptor 5	KF857324
F11r	Junctional adhesion molecule A	KF861997	*Tlr9*	Toll-like receptor 9	AY751797
Cxadr	Coxsackievirus and adenovirus receptor homolog	KF861998	*Nod1*	Nucleotide-binding protein oligomerisation domain-containing protein 1	KF857325
Dsp	Desmoplakin	KF861999	*Mrc1*	Macrophage mannose receptor 1	KF857326
Cx32·2	Gap junction Cx32·2 protein	KF862000	*Cd209*	CD209 antigen	KF857327
Gjb4	Gap junction β-4 protein	KF862002	*Cd302*	CD302 antigen	KF857328
Muc2	Mucin 2	JQ27710	*Clec10a*	C-type lectin domain family 10 member A	KF857329
MUC2-like	Mucin 2-like	JQ27711	*Lgals1*	Galectin-1	KF862003
Muc13	Mucin 13	JQ27713	*Lgals8*	Galectin-8	KF862004
I-muc	Intestinal mucin	JQ27712	*Csl2*	L-Rhamnose-binding lectin CSL2	KF857330
Enterocyte function and epithelial damage			*Fcl*	Fucolectin	KF857331
Alpi	Intestinal-type alkaline phosphatase	KF857309	*Vim*	Vimentin	KF857332
Fabp1	Liver type fatty acid-binding protein	KF857311	Mitochondria function and biogenesis		
Fabp2	Intestinal fatty acid-binding protein	KF857310	*Mthsp10*	Mitochondrial 10 kDa heat shock protein	JX975224
Fabp6	Ileal fatty acid-binding protein	KF857312	*Mthsp60*	Mitochondrial 60 kDa heat shock protein	JX975227
Calr	Calreticulin	KF857313	*Mthsp70*	Mitochondrial 70 kDa heat shock protein	DQ524993
Canx	Calnexin	KF857314	*Ech*	Enoyl-CoA hydratase	JQ308826
Gr	Glutathione reductase	AJ937873	*Hadh*	Hydroxyacyl-CoA dehydrogenase	JQ308829
Gst3	Glutathione *S*-transferase 3	JQ308828	*Cs*	Citrate synthase	JX975229
Sod1	Superoxide dismutase (Cu–Zn), cytoplasmatic	JQ308833	*Tim44*	Mitochondrial import inner membrane translocase subunit 44	JX975239
Prdx1	Peroxiredoxin 1	GQ252679	*Tom22*	Mitochondrial import receptor subunit Tom 22	JX975236
Prdx2	Peroxiredoxin 2	GQ252680	*Mttfa*	Mitochondrial transcription factor A	JX975262
House keeping gene			*Nrf1*	Nuclear respiratory factor 1	JX975263
Actb	β-actin	X89920	*Pgc1α*	Proliferator-activated receptor γ coactivator 1 α	JX975264

(ANOVA, $P > 0{\cdot}05$). At the end of the trial, BW_f values were significantly different depending on the tested diet (ANOVA, $P < 0{\cdot}05$). In this sense, fish fed Diets C and D displayed the best values in BW_f, those fed Diet A the lowest growth performance, whereas fish fed Diets B and E showed intermediate values. On average, fish fed Diets C and D were 5 % heavier than those fed the other diets. Results in BW_f were supported by those from the distribution of BW_f size classes (Fig. 1). In brief, the greatest proportion of larger animals in terms of BW_f (61–80 g) was observed among fish fed Diets C and D (51·7 (SE 0·9) and

Table 3. Survival, growth performance in body weight (BW_f), standard length (SL_f), specific growth rate (SGR) and Fulton's condition factor (*K*), feed efficiency parameters (feed conversion ratio (FCR) and specific feed intake (SFI)) and carcass chemical composition (in DM) of gilthead sea bream (*Sparus aurata*) fingerlings fed experimental diets containing different levels of a bioactive extract of olive-oil (Mean values and standard deviations)

	Diets									
	A		B		C		D		E	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Survival (%)*	96·0	0·6	97·3	0·4	97·0	0·3	97·5	0·5	96·7	0·4
$BW_{(30\,d)}$	20·2	0·3	21·2	0·1	21·8	0·2	22·0	0·4	20·9	0·3
$BW_{(60\,d)}$	39·2	0·3	41·0	0·3	42·2	0·6	42·7	0·4	40·1	0·6
$BW_{f(90\,d)}$†	57·0^{b}	0·7	58·1^{a,b}	1·5	61·1^{a}	1·6	60·3^{a}	1·1	58·8^{a,b}	1·4
SL_f†	11·8	0·2	11·8	0·2	12·0	0·2	12·1	0·1	11·8	0·1
K†	3·5	0·08	3·6	0·02	3·5	0·05	3·4	0·03	3·6	0·15
$SGR_{(0–90\,d)}$ (%/d)*	2·60	0·02	2·62	0·06	2·67	0·03	2·67	0·03	2·63	0·02
FCR*	1·06	0·01	1·07	0·01	1·05	0·01	1·06	0·01	1·06	0·01
SFI*	2·76	0·02	2·77	0·02	2·83	0·02	2·78	0·02	2·78	0·02
Carcass composition‡										
Protein (%)	66·9	2·5	68·7	3·3	69·3	1·0	70·0	2·7	66·8	1·0
Lipid (%)	12·3	1·0	13·6	0·8	13·6	0·9	14·7	0·4	12·6	0·8
Ash (%)	2·1	0·2	2·1	0·2	2·1	0·2	2·0	0·1	2·0	0·1
Lipid liver content‡	17·1	0·7	17·9	0·7	18·7	1·5	18·8	1·9	18·9	1·5

^{a,b} Mean values within rows with unlike superscript letters denote significant differences between dietary groups (ANOVA, $P < 0·05$).

* Based on four replicate tanks per diet with seventy-two to seventy-four fish per tank.

† Mean BW_f, SL_f and *K* factor values were calculated using the individual values from all fish within the same dietary treatment (four replicate tanks per diet with seventy-two to seventy-four fish per tank).

‡ Based on twenty fish per diet (four replicate tanks per diet with five randomly selected fish per tank).

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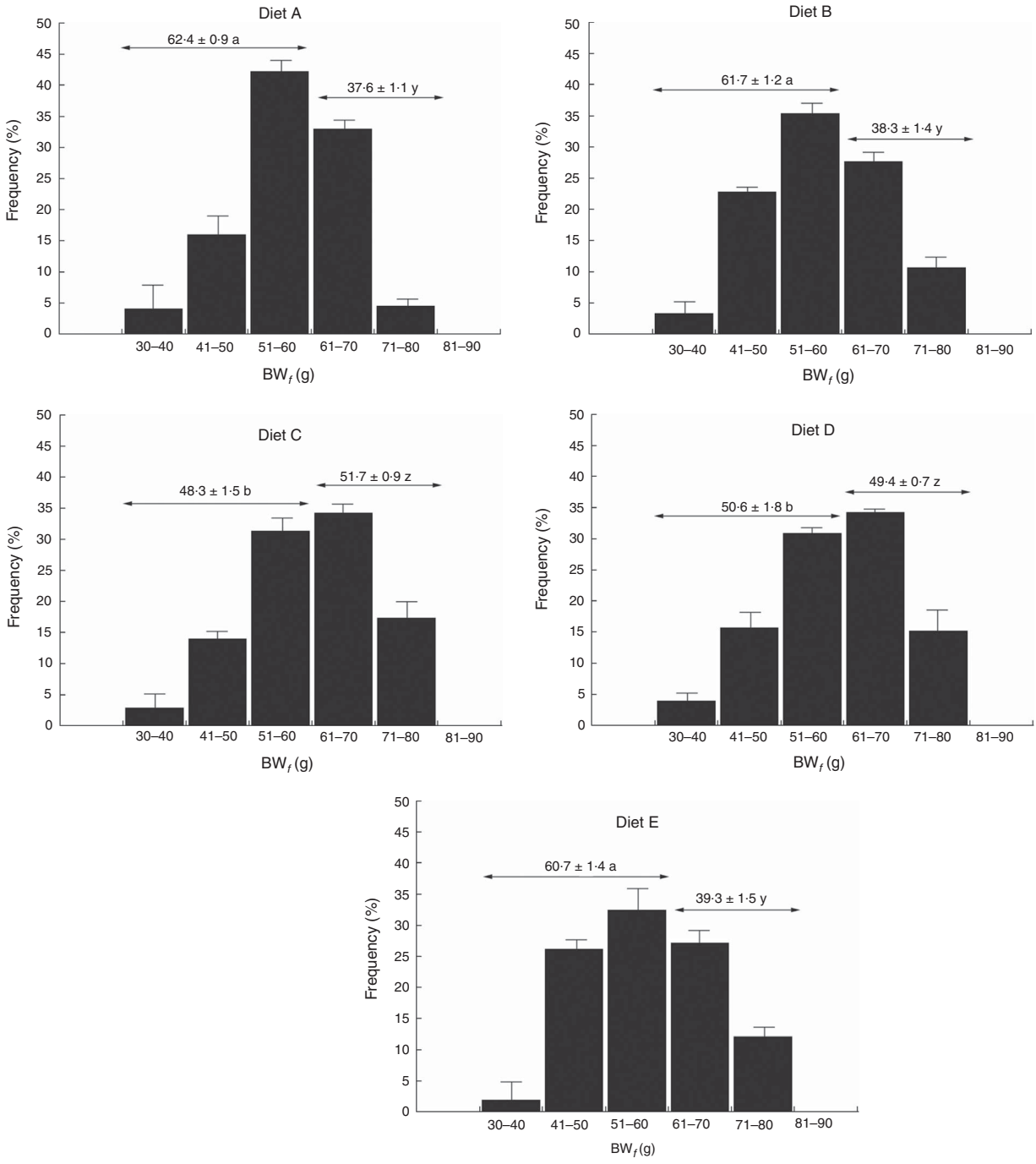

Fig. 1. Distribution of final body weight (BW$_f$) of gilthead sea bream (*Sparus aurata*) fed experimental diets containing different levels of a bioactive extract of olive oil. Values are mean frequencies for each size category calculated, with their standard errors, from all fish from each replicate. [a,b,y,z] Mean values with unlike letters denote differences among dietary groups ($P < 0{\cdot}001$).

49·4 (SE 0·7) %, respectively), whereas it was only 37·6 (SE 1·1), 38·3 (SE 1·4) and 39·3 (SE 1·5) % in fish fed Diets A, B and E, respectively (ANOVA, $P < 0{\cdot}05$). However, there were no statistically significant differences in SGR values among groups (ANOVA, $P > 0{\cdot}05$). In addition, SL$_f$ and K values were similar among different experimental groups (ANOVA, $P > 0{\cdot}05$), and no differences in FCR and SFI were found among groups fed different diets (ANOVA, $P > 0{\cdot}05$).

Carcass proximate composition, hepatic fat content, and lipid peroxidation and antioxidative stress enzymes in liver and intestine

There were no differences in the carcass composition and hepatic fat content among fish fed experimental diets (ANOVA, $P > 0{\cdot}05$; Table 3). There were no statistically significant differences in LPO levels and activity of CAT, GR and

GST in the intestine and liver of gilthead sea bream fed different diets (ANOVA, $P > 0{\cdot}05$; Table 4).

Histological organisation of the intestinal mucosa and liver

In all experimental groups, the intestine was lined by a simple columnar epithelium with basal nuclei, basophilic cytoplasm and prominent microvilli. The organisation of the lamina propria, submucosa and *tunica muscularis* was normal. No lipid deposits were found either within enterocytes or in the vascular system, indicating that the lipid content in tested diets did not imbalance the absorptive and transporting lipid capacities of the intestine (online Supplementary Fig. S2). No differences in villus size, width and height were found among treatments (Table 4), whereas the number of intestinal goblet cells was significantly affected by the presence of OBE in the diet (ANOVA, $P < 0{\cdot}001$; Table 5). In this sense, goblet cell density in fish fed Diet A (1·8 (SE 0·05) goblet cells in 100 μm of intestinal epithelium) was on average 14·3 % lower than in fish fed diets containing OBE (treatment mean = 2·1 (SE 0·05) goblet cells in 100 μm of intestinal epithelium).

Under present experimental conditions, the histological organisation of the liver was normal, with hepatocytes arranged along sinusoids. In any case, hepatic steatosis (severe fat accumulation in hepatocytes affecting their integrity and functionality) was not observed in any of the examined fish. Data on the semi-quantitative evaluation of lipid accumulation in hepatocytes of gilthead sea bream fed experimental diets are shown in Table 5. In general terms, fish showed similar levels of hepatic fat accumulation within hepatocytes (online Supplementary Fig. S2); however, the size of lipidic vacuoles and the level of peripheral displacement of nuclei within hepatocytes was slightly lower in fish fed Diets C and D in comparison to the control group (Diet A), whereas the rest of groups showed intermediate values.

Intestinal gene expression profiling

The C_t values for all the target genes analysed in this study with regard to the effect of supplementing the diet C with OBE on the integrity and health of the intestine are shown in the online Supplementary Table S1. Among the eighty-eight genes evaluated, twenty-nine were differentially expressed in response to the presence of OBE in the diet (Table 6; *t* test, $P < 0{\cdot}05$). Among the thirteen studied genes related to cell differentiation and proliferation, four of them were significantly affected by the diet. In particular, proliferating cell nuclear antigen (*Pcna*) was the only one down-regulated, whereas bone morphogenetic protein receptor type-1A (*Bmpr1a*), transcription factor 4 (*Tcf4*) and Krueppel-like factor 4 (*Klf4*) were up-regulated in fish fed the OBE diet. Six of the sixteen genes analysed related to intestinal architecture and permeability were up-regulated (occludin (*Ocln*), *Cldm3*, claudin 12 (*Cldn12*), junctional adhesion molecule A (*F11r*), coxsackievirus and adenovirus receptor homolog (*Cxadr*) and desmoplakin (*Dsp*)), whereas the rest of them were not affected by the diet. The expression of calreticulin (*Calr*) decreased, whereas that of three of the nine genes studied involved in enterocyte function and epithelial damage were up-regulated (intestinal-type alkaline phosphatase (*Alpi*), liver type fatty acid-binding protein (*Fabp1*) and *Gr*). Almost half of the genes coding for IL and cytokines were differentially expressed (10/22); in particular, *Il1r1*, *Il6rb*, *Il8*, *Il10ra*, *Il12b*, *Tnfα*, *Csf1r1*, *Ccr9* and *Ccr11* were up-regulated, whereas *Il7* was down-regulated. Among the fourteen pathogen recognition receptor genes analysed, four of them (Toll-like receptor 2 (*Tlr2*), nucleotide-binding protein oligomerisation domain-containing protein 1 (*Nod1*), CD302 antigen (*Cd302*) and fucolectin (*Fcl*)) were up-regulated in the intestinal mucosa of fish fed the OBE diet. Regarding the expression of genes related to mitochondria function and biogenesis, molecular chaperones (mitochondrial 10 kDa heat shock protein (*mtHsp10*), mitochondrial 60 kDa heat shock protein (*mtHsp60*)) and membrane protein translocases (mitochondrial import inner membrane translocase subunit 44 (*Tim44*), mitochondrial import receptor subunit Tom 22 (*Tom22*)) were down-regulated in fish fed the OBE diet, whereas an opposite pattern was found for the transcription factor *Pgc1*.

Discussion

Previous studies dealing with the inclusion of bioactive compounds derived from olive oil in aqua feeds have been mostly limited to maslinic acid. In particular, it has been shown that a diet supplemented with maslinic acid (250 mg/kg feed) promoted growth in *Oncorhynchus mykiss*[9], whereas similar or lower levels (100 mg/kg feed) of this triterpenic compound did not affect growth performance in gilthead sea bream[10,11], even though it enhanced hepatic protein-turnover rates[10] and induced hypertrophy in muscle fibres were found[11]. In addition, no effect on growth performance in *Dentex dentex* was observed when using higher doses of maslinic acid (20, 40, 80 g/kg feed)[26]. Under present experimental conditions, the inclusion of OBE slightly enhanced growth independently of feed intake without affecting neither feed efficiency parameters nor the proximate composition of the fillet. The effect of OBE on growth in body weight was not-linear, as the highest mean values of BW_f were observed in fish fed 0·17 and 0·42 % of OBE and not in those fed the highest inclusion of OBE in diets (0·73 %), which indicated a quadratic growth response to OBE inclusion in diets for gilthead sea bream. In addition, the inclusion of OBE in diets affected the final fish size distribution in BW, resulting in a more homogeneous fish size distribution in those groups fed the OBE diets, although the rationale of these findings with regard to the administered diet needs to be further investigated. However, these findings are of practical importance since the use of OBE in diets might reduce the effort required for size selection during processing of production lots, and also in a reduction of hierarchical dominance situations[27,28]. Although these results might be partially attributed to the potential growth-promoting effect of polyphenols[29], our data suggest that they were most likely related to the enhancement of the health condition of the intestinal mucosa as described below.

The intestine is involved not only in digestion and feed absorption but also in water and electrolyte balance, nutrient sensing and immunity[30]. This functional diversity is now

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Table 4. Levels of lipid peroxidation (LPO) and catalase (CAT), glutathione reductase (GR) and glutathione *S*-transferase (GST) from the intestine and liver of gilthead sea bream (*Sparus aurata*) fed experimental diets containing different levels of a bioactive extract of olive oil
(Mean values with their standard errors; *n* 4)

	Diets									
	A		B		C		D		E	
Intestine	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
LPO (nmol MDA mg/protein)	1·44	0·14	1·30	0·01	1·20	0·02	1·23	0·19	1·03	0·05
CAT (nmol/mg protein)	2694	350·4	2490·1	441·4	2135·1	223·1	1981·6	252·2	2376·7	219·6
GR (nmol/mg protein)	12·14	0·54	13·26	1·62	13·12	0·23	13·63	1·05	11·54	0·38
GST (nmol/mg protein)	28·41	4·30	27·90	4·20	27·56	4·95	26·57	2·37	28·23	2·94
Liver										
LPO (nmol MDA mg/protein)	1·44	0·15	1·30	0·01	1·20	0·02	1·23	0·19	1·28	0·07
CAT (nmol/mg protein)	923·5	125·9	857·3	235·3	865·3	148·2	927·6	65·2	868·2	322·1
GR (nmol/mg protein)	17·72	0·58	12·75	1·60	11·34	2·09	12·74	1·27	12·64	1·98
GST (nmol/mg protein)	16·09	2·17	12·07	0·87	13·09	1·95	13·47	1·86	13·89	0·97

MDA, malondialdehyde.

Table 5. Size of hepatic lipid deposits (μm^2), villi size in height and width (μm) and goblet cell density (number of goblet cells in 100 μm of epithelium) in the intestine of gilthead sea bream (*Sparus aurata*) fed experimental diets with different levels of an olive oil bioactive extract
(Mean values with their standard errors; *n* 4)

	Diets									
	A		B		C		D		E	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Hepatic lipid vacuoles' size	133·1^{b}	7·5	115·9^{a,b}	10·9	86·6^{a}	9·8	89·9^{a}	3·5	104·9^{a,b}	5·4
Villus height	1273·8	68·3	1330·6	50·5	1452	36·6	1457	49·5	1390	48·5
Villus width	287·5	45·6	297·4	65·4	291·9	41·9	311·4	51·1	294·5	34·8
Goblet cell density	1·80^{b}	0·06	2·17^{a}	0·14	2·17^{a}	0·13	2·20^{a}	0·11	2·26^{a}	0·10

^{a,b} Mean values within rows with unlike superscript letters denote significant differences between dietary treatments (ANOVA, $P < 0·05$).

Table 6. Differentially expressed genes (Student *t* test, $P < 0{\cdot}05$) in the intestine of fish fed the control (Diet A) and Diet C containing 1·7 g olive oil bioactive extract per kg feed*
(Mean values with their standard errors; *n* 8)

	Fold change	
Genes	Mean	SE
Cell differentiation and proliferation (4)		
Pcna	0·54	0·11
Bmpr1a	1·50	0·11
Tcf4	1·68	0·16
Klf4	1·55	0·15
Intestinal architecture and permeability (6)		
Ocln	2·47	0·26
Cldn3	1·49	0·18
Cldn12	1·36	0·13
F11r	1·27	0·08
Cxadr	1·38	0·11
Dsp	1·87	0·19
Enterocyte mass and epithelia damage (4)		
Alpi	1·68	0·20
Fabp1	1·26	0·08
Calr	0·64	0·08
Gr	1·38	0·14
IL and cytokines (10)		
Il1r1	2·18	0·33
Il6rb	1·39	0·14
Il7	0·81	0·05
Il8	1·81	0·25
Il10ra	1·48	0·15
Il12b	1·71	0·20
Tnfa	1·60	0·19
Csf1r1	1·42	0·10
Ccr9	1·57	0·12
Ccr11	3·77	0·65
Pathogen recognition receptors (4)		
Tlr2	1·34	0·10
Nod1	1·61	0·11
Cd302	1·53	0·15
Fcl	3·14	0·52
Mitochondria function and biogenesis (5)		
mtHsp10	0·49	0·05
mtHsp60	0·48	0·04
Tim44	0·71	0·04
Tom22	0·78	0·06
Pgc1α	1·74	0·19

Pcna, proliferating cell nuclear antigen; *Bmpr1a*, bone morphogenetic protein receptor type 1A; *Tcf4*, transcription factor 4; *Klf4*, Krueppel-like factor 4; *Ocln*, occluding; *Cldn3*, claudin 3; *Cldn12*, claudin 12; *F11r*, junctional adhesion molecule A; *Cxadr*, coxsackievirus and adenovirus receptor homolog; *Dsp*, desmoplakin; *Il1r1*, IL 1 receptor type 1; *Il6rb*, IL 6 receptor subunit *β*; *Il10ra*, IL 10 receptor subunit *α*; *Csf1r1*, macrophage colony-stimulating factor 1 receptor 1; *Ccr9*, C–C chemokine receptor type 9; *Ccr11*, C–C chemokine receptor type 11; *Tlr2*, toll-like receptor 2; *Nod1*, nucleotide-binding protein oligomerisation domain-containing protein 1; *Cd302*, CD302 antigen; *Fcl*, fucolectin; *Mthsp10*, mitochondrial 10 kDa heat shock protein; *Mthsp60*, mitochondrial 60 kDa heat shock protein; *Tim44*, mitochondrial import inner membrane translocase subunit 44; *Tom22*, mitochondrial import receptor subunit Tom 22.

* Values >1 are up-regulated genes in fish fed Diet C. Values <1 are down-regulated genes in fish fed Diet C.

starting to be elucidated in fish and different histological and molecular approaches are helping to understand the many vital functions conducted along the gastrointestinal tract[31,32]. The inclusion of OBE in the diet resulted in an increase of the goblet cell population in the intestinal epithelium. This histological observation was supported by the up-regulated expression of the zinc-finger transcription factor *Klf4*, a goblet-cell specific intestinal differentiation factor[33]. The increase in goblet cell number would benefit fish by providing an effective immune barrier against potentially pathogenic gut bacteria[34], which also agrees with an enhanced expression of several pathogen recognition receptors (*Tlr2*, *Nod1*, *Cd302*, *Fcl*). In higher vertebrates, it has been demonstrated that TLR4- and TLR2-dependent pathways can stimulate *β*-defensin-2 expression by intestinal epithelial cells that are able to respond to pathogen-associated molecular patterns (PAMP) by secreting antimicrobial peptides, as well as protecting the epithelium from injuries[35] and reducing intestinal permeability by protecting tight junctions[36]. In addition, NOD1-mediated innate immune responses are critically involved in the intestinal homoeostasis of higher vertebrates[35], where epithelial cells remain responsive to invasive bacteria via ligand binding to NOD1 or NOD2[36]. Similarly, the *CD302* gene encodes for a C-type lectin receptor involved in cell adhesion and migration, as well as in endocytosis and phagocytosis processes[37]. It is noteworthy that the highest fold change in expression among pathogen recognition receptors was found in *fcl*, a gene coding for fucolectin that has been described to enhance phagocytosis in *in vitro* studies with peritoneal macrophages in *Dicentrarchus labrax*[38].

The inclusion of the OBE in the diet affected gene expression of approximately half of the IL and cytokines analysed in this study. The up-regulation of a vast array of pathogen recognition receptors and pro-inflammatory IL, IL receptors and cytokines (*Il1r1*, *Il6rβ*, *Il12*, *Il8*, *Tnfα*, *Csf1r1*, *Ccr9* and *Ccr11*) highly supports an immunostimulatory action[39] of OBE when included at 0·17 % of the diet. Early elimination of potential pathogens has clear implications for improved health, but also for the bioenergenetic balance of the whole animal; much less energy is expended when pathogens are removed before they have opportunities for proliferation. Further, the enhanced mucin production due to increased goblet cell populations provides a physical displacement of potential pathogenic organisms; a more diverse microbiota leads to a thickening of the mucus layer and this improves the microniches of the gut inhabited by these beneficial bacteria[40]. In this sense, further research is needed to evaluate the potential effects of OBE in modulating the microbiota.

The homoeostasis of the constantly renewing intestinal epithelium relies on an integrated control of proliferation, differentiation and apoptosis, as well as on the functional architecture of the epithelial cells. Thus, the down-regulation of *pcna* in fish fed OBE at 0·17 % of the diet in comparison to the control group might be attributed to lower epithelial turnover rate associated with a better health condition of enterocytes, which was in agreement with the higher transcription of the *bmpr1a* that codes for the bone morphogenetic protein receptor, type 1 A that plays a major role in cell differentiation. This hypothesis is supported by the up-regulation of *Tcf4* in this group of fish, as this transcription factor is a key player in the Wnt pathway signalling and maintaining the homoeostasis of the intestinal epithelium[41]. However, BMPR1A inhibits WNT signalling. Thus, although both inhibitory (*Bmpr1a*) and stimulatory (*Tcf4*) signals of stem cell proliferation were up-regulated by the OBE diet, the net result would be prone to promote cell differentiation rather than cell proliferation.

Enhanced terminal cell differentiation would have a variety of potentially beneficial consequences, but one possible is the observed increase in goblet cells. Prevention of the entrance of toxic molecules or infectious agents, such as solutes, antigens and micro-organisms, is ensured by the gastrointestinal mucosa. A key structure of the intercellular space is the tight junction, which plays a major role in regulating the paracellular passage of luminal elements. Therefore, proper functioning and regulation of tight junctions is crucial. These junctions are under the influence of intestinal microflora, inflammation and even alimentary components, which can compromise tight junctions[42]. Three of the main protein families found in tight junctions are occludins, claudins and junction-associated membrane proteins[43]. In particular, OBE may reduce intestinal permeability and consequently, the risk of intestinal disorders by increasing the expression of genes coding for mucosal tight junction proteins like occludin, claudin 3 and 12, and F11 receptor, the so-called junctional adhesion molecule A, as well as desmoplakin that is involved in maintaining the desmosome structure.

In addition, the inclusion of OBE at 0·17 % in the diet improved the condition of enterocytes by reducing the expression of several gene markers related to mitochondrial function (*mtHsp10*, *mtHsp60*, *Tim44*, *Tom22*). In particular, TOM22 and TIM44 proteins are involved in the translocation of transit peptide-containing proteins from the outer and inner mitochondrial membranes[44,45], whereas PGC1α is a transcriptional co-activator that is involved in controlling global oxidative metabolism by regulating mitochondrial biogenesis and function[46]. Furthermore, OBE induced the down-regulation of *mtHsp60 and mtHsp10*, two genes encoding for UPR^{mt}-responsive proteins that are involved in protein homoeostasis in mitochondria[47]. Thus, the down-regulation of the above-mentioned genes coupled with the increase in expression of *pgc1α* may correlate with a lower metabolic rate of mitochondria, protein turnover and potentially lower oxidative stress in the intestinal mucosa of fish fed the OBE diet[48]. Indeed, this suggestion is in line with the tendency found for fish fed the OBE diets to show lower LPO values ($P=0·091$) in comparison with the control group.

Regarding the expression of genes involved in enterocytes' function and epithelial damage, 45 % of the analysed genes were differentially expressed when fish were fed the diet containing OBE at 0·17 % of the diet. In particular, *Alpi* regulates lipid absorption across the apical membrane of enterocytes, participates in the regulation of bicarbonate secretion and duodenal surface pH, limits bacterial transepithelial passage and finally protects against bacterial endotoxin-induced inflammation by dephosphorylating lipopolysaccharides[30]. Thus, the up-regulation of *Alpi* may indicate a more mature enterocyte in fish fed the OBE diet[49], as well as reinforce the idea of an enhanced intestinal immune function[28]. In addition, the higher expression of *Alpi* and *Fabp1* might be related to their role in fatty acid uptake from the lumen of the intestine and trafficking within the enterocyte[50]. CALR is reported to play a role in many cellular functions including lectin-like chaperoning, Ca^{2+} storage and signalling, regulation of gene expression, cell adhesion, wound healing and autoimmunity[51,52]; thus, the down-regulation of *Calr* in fish fed the OBE diet is in agreement with the proposed decrease in intestinal epithelial permeability and overall improvement of intestinal health, as it has been recently reported in *Ictalurus punctatus*[53].

Regarding the impact of the feed additive on the liver, present results revealed that the inclusion of OBE reduced the size of hepatic deposits within hepatocytes, which may be in agreement with the tendency for lower LPO values in the liver of fish fed diets containing OBE. These results were in agreement with *in vitro*[54] and *in vivo*[55] models that have shown that polyphenols reduce hepatocellular lipid accumulation by means of the regulation of the AMP-activated protein kinase signalling and hepatocyte lipid metabolism. In addition, triterpenic acids have been also reported to be involved in regulating hepatic lipid accumulation and reducing hepatic steatosis[56], stimulating liver protein-synthesis rates[9], as well as regulating proteins involved in liver metabolism and detoxification processes[57–59]. However, further research is needed in fish model species in order to properly characterise by means of transcriptomic and physiological approaches the impact of OBE in the health condition and metabolism of the liver, especially in fish fed diets with a high level of fish oil substituted by vegetal oil sources.

Conclusions

The inclusion of OBE in the diet had a positive effect on growth performance in gilthead sea bream juveniles. This effect depended on the dose, becoming significant when OBE was included at 0·17 and 0·42 % of the diet. The inclusion of OBE reduced the size of hepatic deposits within hepatocytes, which may be in agreement with the tendency for lower LPO values in the liver of fish fed diets containing OBE, suggesting that OBE may positively affect lipid metabolism in the liver and potentially prevent hepatic steatosis. Results from the transcriptomic profiling of the intestine suggested that OBE enhanced fish growth primarily by improving the condition and defensive role of the intestine. When OBE was fed at 0·17 % of the diet such a response appeared to involve augmented enterocytes' maturation, increased integrity of the intestinal epithelium and goblet cell density, and improved intestinal innate immune function, as gene-expression data indicated. These results showed that OBE is a promising feed additive for the aquaculture industry with multiple beneficial properties.

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The authors declare that there are no conflicts of interest.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114517000228

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