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Effects of dietary NEXT ENHANCE ®150 on growth performance and expression of immune and intestinal integrity related genes in gilthead sea bream (*Sparus aurata* L.).

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ABSTRACT

Gilthead sea bream juveniles were fed different doses (0, 50, 100, 200, 300 ppm) of NEXT ENHANCE ®150 (NE) for 9 weeks. Feed gain ratio (FGR) was improved by a 10% with all the doses, but feed intake decreased in a dose dependent manner. The optimum inclusion level to achieve maximum growth was set at 100 ppm. The hepatosomatic index did not vary and only at the highest dose, viscerosomatic and splenosomatic indexes were significantly decreased. No significant changes were found in haematological parameters, plasma biochemistry, total antioxidant capacity and respiratory burst. In a second trial, NE was given at 100 ppm alone (D1) or in combination with the prebiotic PREVIDA® (0.5%) (PRE) (D2) for 17 weeks. There were no differences in the growth rates, and FGR was equally improved for D1 and D2. No significant changes in haematology and plasma antioxidant capacity were detected. The histological examination of the liver and the intestine showed no outstanding differences in the liver, but the number of mucosal foldings appeared to be higher in D1 and D2 *vs* CTRL diet and the density of enterocytes and goblet cells also appeared higher, particularly in the anterior intestine. A 87-gene PCR-array was constructed based on our transcriptomic database (www.nutrigroup-iats.org/seabreamdb) and applied to samples of anterior (AI) and posterior (PI) intestine. It included 54 new gene sequences and other sequences as markers of cell differentiation and proliferation, intestinal architecture and permeability, enterocyte mass and epithelial damage, interleukins and cytokines, pattern recognition receptors (PRR), and mitochondrial function and biogenesis. More than half of the studied genes had significantly different expression between AI and PI segments. The functional significance of this differential tissue expression is discussed. The experimental diets induced significant changes in the

expression of 26 genes. The intensity of these changes and the number of genes that were significantly regulated were higher at PI than at AI. At PI, both diets invoked a clear down-regulation of genes involved in cell differentiation and proliferation, some involved in cell to cell communication, cytokines and several PRR. By contrast, up-regulation was mostly found for genes related to enterocyte mass, cell epithelial damage and mitochondrial activity at AI. The changes were of the same order for D1 and D2, except for fatty acid-binding proteins 2 and 6 and the PRR fucolectin, which were higher in D2 and D1 fed fish, respectively. Thus, NE alone or in combination with PRE seems to induce an anti-inflammatory and anti-proliferative transcriptomic profile with probable improvement in the absorptive capacity of the intestine that would explain the improved FGR.

Key words: Teleostei, prebiotics, carvacrol, thymol, aquafeeds, intestine, transcriptomics, immunology

1. Introduction

The role of dietary nutrients and additives on immune system function in fish has been investigated for several decades as a means of reducing the presence of opportunistic pathogens and simultaneously stimulating the host immunological responses [1, 2]. Among the different nutraceutical products, prebiotics, probiotics and antioxidants are most studied. Prebiotics are non-digestible selectively fermented feed ingredients that allow specific changes in the composition and/or activity in the gut microbiota that confers benefits upon host wealth and health, which mediate immunological development and functionality, particularly at the mucosal interface within the gastrointestinal (GI) tract [3-5]. The use of dietary prebiotics in farmed fish has been approached, sometimes with contradictory effects [6-10].

Phytoadditives obtained from different herbs have also been tested in fish feeds for effects on fish immune response and disease susceptibility [11-15]. They represent a promising alternative to traditional drugs as they have low costs and some of them are legally registered as food additives for human consumption [16]. Phytogenic extracts containing phenolic and flavonoid chemical compounds are known to exert an array of positive effects that include improvement of gut microbiota composition, enhanced immune function and resistance to pathogens, and improved gut barrier structure and function in humans and animals [17, 18]. Among these additives, carvacrol and thymol are essential oils from oregano (*Origanum vulgare*) that have been shown to improve growth performance in different animal production systems [19, 20]. They are also known for their antibacterial [21] and antioxidant [22] properties, for synergizing effects of antibiotics [23], and for their potential applications in foods [24], including fish fillets [25, 26]. Carvacrol in

particular exhibits a range of biological activities: antimicrobial, antitumoral, antimutagenic, antigenotoxic, analgesic, antispasmodic, antiinflammatory, angiogenic, antiparasitic, antiplatelet, AChe inhibitory, antielastase, insecticidal, antihepatotoxic and hepatoprotective (reviewed by Baser [27]). However, its role as an immunostimulant is rather controversial, with contradictory results in swine [28-30]. Carvacrol administration in fish species is starting to be documented. Thus far, it has been tested alone in tilapia [31] and European sea bass [32], or in combination with thymol in channel catfish [33] and rainbow trout [34, 35]. In these previous experiments, these phytoadditives had a beneficial effect on growth and disease resistance to bacterial challenges. However, the pathways involved in their immunostimulant and/or antioxidant roles in fish are still poorly understand.

Thus, in the present study we tested the effect of Next Enhance NE®150 (NE), an encapsulated combination of carvacrol and thymol, as a feed additive for gilthead sea bream, alone or in combination with the prebiotic Previda® (PRE). In a first short-term trial, the best dose of NE was established in terms of growth performance. Subsequently, a longer trial was set up to assess the effect on gut and liver histomorphology, antioxidant status, and the expression of immune and intestinal integrity related genes, using an 87-gene PCR-array derived from the updated transcriptomic database of gilthead sea bream [36] hosted at www.nutrigroup-iats.org/seabreamdb. The development of the array involved the molecular definition of 54 new gilthead sea bream sequences, selected together with other known sequences as markers of intestine function, immunity and integrity, based on the intestine gene expression profiling in fish challenged with different diets and the parasite *Enteromyxum leei* [37-40].

2. Materials and Methods

2.1. Animal care, feeding trials design and sampling procedure

Clinically healthy juvenile gilthead sea bream (GSB) were obtained from a local commercial hatchery and acclimatized to the IATS rearing facilities for four weeks. A first feeding trial (trial 1) was set up to establish the best NE dose. Fish with an initial weight of 26-27 g (1st May) were randomly distributed in triplicated 90L tanks (20 animals/tank) and fed a basal diet (CTRL, see below) or the experimental diets with four inclusion levels of the active compound NE: 50 ppm (D50), 100 ppm (D100), 200 ppm (D200) and 300 ppm (D300). Fish were fed twice a day by visual satiety during 9 weeks and were sampled at the end of the experiment for total biomass biometry. At this time, 24 fish per diet were sacrificed to obtain organosomatic indexes, blood, plasma and tissues samples.

In a second feeding trial (trial 2), the best NE dose established in trial 1 (D100) was used alone (D1) or in combination with the prebiotic PRE at 0.5 % (D2), using the same basal diet (CTRL). Fish were distributed in June in three replicated 500L tanks for each diet with an initial number of 40 fish per tank. Fish were fed *ad libitum* by hand twice a day. Daily feed intake was recorded. A biometric sampling was performed on all fish after 17 weeks of feeding. A subsample of 30 fish per diet (10 per tank) was randomly sacrificed to obtain blood, plasma and tissue for gene expression and histology.

In both trials, day length followed natural changes at IATS latitude (40º5' N, 0º10'E). Natural sea water (37.5‰ salinity) was 5 µm-filtered and UV irradiated for supply to the fish in a flow through system. Flow rate and oxygen content were checked daily and kept uniform in all tanks. Water temperature ranged from 19 to 25 ºC. In lethal samplings, blood was quickly drawn from caudal vessels with heparinized syringes, one blood aliquot

was immediately used to measure the respiratory burst activity, another aliquot was used to measure haemoglobin and the remaining blood was centrifuged at 3000 *g* for 20 min at 4 ◦C, and plasma aliquots were stored at −80 ◦C until used in anti-oxidant and metabolite analyses. Fish were killed by an overdose of the anaesthetic aminobenzoic acid ethyl ester (MS-222; Sigma, Saint Louis, MO, USA) prior to organ weight and tissue collection for histology and gene expression analysis. Anterior and posterior intestine portions were rapidly excised, frozen in liquid nitrogen and stored at -80ºC until RNA isolation.

All procedures were carried out in accordance with the principles published in the European animal directive (86/609/EEC) for the protection of experimental animals, and was approved by the Consejo Superior de Investigaciones Científicas (CSIC) ethics committee and IATS Review Board.

2.2. Diet composition and preparation

The concentration of NE in the different diets was calculated according to the amount of the active compound and both NE and PRE were incorporated into the basal diet before extrusion by SPAROS Lda (Faro, Portugal). The composition of the basal diet (CTRL) can be found in Table 1. NE has a 50% of the active compound and to obtain the target doses it was added to the basal diet from 100 to 600 mg/kg. The active ingredients in NE include thymol and carvacrol at a 1:1 ratio. PRE consists of broad range of oligosaccharides, from all-natural hemicellulose extract. Mannose represents more than 51% and xylose, glucose and galactose are the other main oligosaccharide components. Small quantities of arabinose, rhamnose and fucose are the remaining constituents. The broad degree of polymerization distribution in the oligosaccharides contributes to the broad spectrum prebiotic activity found in the gut [6, 41].

158

159 *2.3. Biometrical data*

160 Body weight, whole viscera, liver and spleen weights were measured and the
161 viscerosomatic (VSI), the hepatosomatic (HSI) and splenosomatic (SSI) indexes were
162 calculated as the ratio between the organ weight and body weight. Specific growth rates
163 (SGR) were calculated as follows: SGR (%) = 100 × (lnWt – lnW0)/t, where W0 represents
164 weight at the beginning of the period, Wt the weight at the end of the trial and t the number
165 of growth days. Feed gain ratio (FGR) was calculated as the ratio between feed intake and
166 weight gain.

167

168 *2.4. Blood haematology and biochemistry*

169 Haemoglobin concentration (Hb) was determined with a HemoCue B-Haemoglobin
170 Analyser® (AB, Leo Diagnostic, Sweden), which uses a modified azide methaemoglobin
171 reaction for haemoglobin quantification. Blood was drawn into disposable microcuvettes
172 which contain reagents in dried form that produce the red blood cell lysis and the
173 conversion of haemoglobin to methaemoglobin by sodium nitrate, which is then combined
174 with azide. The absorbance of the azide methaemoglobin is then photometrically measured
175 at 565 nm and 880 nm.

176 Plasma glucose was measured by the glucose oxidase method (ThermoFisher
177 Scientific, Waltham, Massachusetts, USA). Total plasma cholesterol was determined using
178 cholesterol esterase/cholesterol dehydrogenase reagent (ThermoFisher Scientific). Plasma
179 triglycerides (TG) were determined using lipase/glycerol kinase/glycerol-3phosphate
180 oxidase reagent (ThermoFisher Scientific). Total plasma proteins were measured with the

Bio-Rad protein reagent (Hercules, California, USA) with bovine serum albumin as standard.

The total antioxidant capacity (TAC) was measured in serum samples with a commercial kit (Cayman Chemical), as previously described [42]. Induction of the respiratory burst (RB) activity in blood leucocytes was measured directly from heparinised blood [42]. Briefly, blood was incubated with a luminol suspension containing PMA for 1 h and the resulting integral chemiluminescence in relative light units (RLU) was calculated.

2.5. Histology

For histological examination, pieces of liver, anterior and posterior intestine were fixed in 10% buffered formalin, embedded in Technovit resin (Kulzer, Germany), 1.5 µm-sectioned and stained with haematoxylin and eosin (H&E) and toluidine blue.

2.6. Gene sequence analysis

The nutrigroup-iats.org/seabreamdb database contains more than 60,000 assembled contigs with 17,797 non-redundant sequences and more than 14,500 Swissprot descriptions. This set of sequences is specially enriched on intestinal reads and this allowed the unequivocal identification (E-value > 3e-42) of 54 new intestinal-related genes, uploaded to GenBank with accession numbers KF857309-KF857334/KF857336-KF857346/KF861987-KF862004 (Table 2). The list included 14 markers of cell differentiation and proliferation (BMPR1A, IHH, GLI1, GLIS3, HHIP, WLs, Myc, CTNNB1, Tcf4, NLE1, HES1-B, GFI-1, KLF4, VIM), 3 markers of cell adhesion (ITGB1BP1, ITGB6, ILK), 8 markers of tight-junctions (OCLN, CLDN12, CLDN15, TJP1, CDH1, CDH17, F11R, CXADR), 1 marker of desmosomes (DSP), 3 markers of gap junctions (CX32.2, CX32.7, GJB4), 4 markers of

differentiated enterocytes (ALPI, FABP1, PABP2, FABP6), 2 markers of oxidative stress (CALR, CANX), 7 chemokine receptors (CXC, CCL25, CCR3, CCR9, CR11, CCL20, CD48, CD276) and 12 pattern recognition receptors (PRRs) (TLR1, TLR2, TLR5, NOD1, MRC1, CD209, CD302, CLEC10A, LGALS1, LGALS8, CSL2, FCL). Thirty three among them comprised complete codifying sequences with open reading frames of 332-2708 nucleotides in length and a variable number of reads (2-4026) composing the assembled sequences.

2.7. Gene expression analysis

Total RNA of 6 fish from each dietary treatment of trial 2 was extracted from anterior and posterior intestine samples with the MagMAX TM-96 total RNA isolation kit (Life Technologies). The RNA yield was 50-100 μg with absorbance measures (A260/280) of 1.9–2.1 and RIN (RNA integrity number) values of 8-10 with the Agilent 2100 Bioanalyzer, which is indicative of clean and intact RNA. Reverse transcription (RT) of 500 ng total RNA was performed with random decamers, using the High-Capacity cDNA Archive Kit (Applied Biosystems) following manufacturer's instructions. Negative control reactions were run without reverse transcriptase and real-time quantitative PCR was carried out with a CFX96 Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), using a 96-well PCR array layout designed for simultaneously profiling a panel of 87 genes under uniform cycling conditions (Table 3). The GenBank accession numbers for all the genes included in the assay and the PCR-array layout are shown in Supplementary Table 1. This set of genes included markers of cell differentiation and proliferation (14), intestinal architecture and permeability (19), enterocyte function and epithelia damage (9), immune-surveillance (interleukins, cytokines and chemokines receptors, 21; PRRs, 13) and

mitochondria function and biogenesis (11). Housekeeping genes and controls of general PCR performance are included on each array, and all the liquid manipulations required to perform the PCR array were made by means of the EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany). Briefly, for each RT reaction, 660 pg of total input RNA was diluted to a 25 ml volume for each PCR reaction. PCR-wells contained a 2x SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA), and specific primers at a final concentration of 0.9 μM were used to obtain amplicons of 50–150 bp in length (Supplementary Table 2).

The program used for PCR amplification included an initial denaturation step at 95ºC for 3 min, followed by 40 cycles of denaturation for 15 s at 95ºC and annealing/extension for 60 s at 60ºC. The efficiency of PCR reactions was always higher than 90% (amplification factor > 1.90 and similar for all genes), and negative controls without sample templates were routinely used for each primer set. The specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5ºC/10 s over a temperature range of 55–95ºC), linearity of serial dilutions of RT reactions, and electrophoresis and sequencing of PCR amplified products. Fluorescence data acquired during the PCR extension phase were normalised using the delta-delta Ct method [43]. β-actin, elongation factor 1, α-tubulin and 18S rRNA were initially tested for gene expression stability using GeNorm software, and the most stable gene was found to be β-actin (M score = 0.17); therefore, this gene was used as a housekeeping gene in the normalisation procedure for routine assays. Technical replicates of the samples were run initially to test the reproducibility of the method, but the obtained data had a very high reproducibility score and technical replicates were finally omitted. For multi-gene analysis comparisons,

all data values were referenced to the expression level of IL-1β at the anterior intestine of CTRL fish with an arbitrarily assigned value of 1.

2.8. Statistical analysis

Data on fish performance, biochemistry and gene expression were analyzed using one-way analysis of variance (ANOVA-I), followed by a Student-Newman-Keuls post hoc test. When the test of normality or equal variance failed, a Mann-Whitney Rank Sum test or a Kruskal-Wallis ANOVA on ranks followed by Dunn's method was applied instead, respectively. The significance level was set at $P < 0.05$. All analyses were conducted using SPSS package version 19.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Trial 1: dose-effect of NE

Table 4 shows the dose-dependent effects of NE on the growth performance and organosomatic indexes. Feed intake was significantly and progressively reduced in fish fed with the four levels of NE. There was also a progressive, but not statistically significant decrease in the final body weight, which did not negatively affect specific growth rate (SGR). Consequently, feed gain ratio (FGR) was progressively improved with increasing inclusion of NE. The weight of whole viscera and liver, as well as the viscerosomatic (VSI) and splenic (SI) indexes were significantly decreased only in D300 fish.

No statistically significant changes were detected in Hb and plasma biochemistry, TAC and respiratory burst for any of the experimental diets (Table 4). Nevertheless, a decreasing trend in plasmatic cholesterol and glucose levels and an increasing trend in the

276 respiratory burst (RB) of circulating leucocytes were observed with increasing doses of
277 NE®150. In fact, the mean RB of D300 fish almost doubled that of CTRL fish, but
278 differences were not significant due to the high individual variability.
279
280 *3.2. Trial 2: long term effects of NE*
281 *Growth performance.* Table 5 shows the growth performance of fish after 17 weeks
282 of experimental feeding. There were no differences in the final body weight or in the SGR
283 between the three diets, but feed intake was significantly lower in D2 than CTRL fish, and
284 FGR was significantly better for D1 and D2 than for CTRL. There were no significant
285 changes in Hb, though a certain decrease was found in both experimental diets, and a slight
286 increase in plasma TAC was also found.
287 *Histological observations.* The histological examination of the liver and the two
288 segments of the intestine of CTRL and D1 and D2 fed fish showed no outstanding
289 differences in the liver, with a high level of fat in hepatocytes in general (Fig. 1A-B). Initial
290 steatosis was observed only in one D2 fish and one D1 fish. The number of mucosal
291 foldings appeared to be higher in D1 and D2 *vs* CTRL diet and the density of enterocytes
292 and goblet cells also appeared higher, particularly at the anterior segment (Fig. 1C-F).
293 *Transcriptomic profile*. The relative expression of the 87 studied genes at the
294 anterior (AI) and posterior (PI) intestine are shown in supplementary Table 3. To simplify
295 the interpretation of the results, only the fold changes of the significantly different genes
296 are represented in Figs. 2 and 3. The comparison of the constitutive expression at PI *vs* AI
297 in CTRL fish revealed that 52.8 % (46) of the studied genes were significantly different (P
298 < 0.05) (Fig. 2). In particular, genes related to cell differentiation and proliferation,
299 intestinal architecture and permeability, cytokines and PRRs were more expressed at PI,

whereas those related to enterocyte mass and epithelial damage and mitochondrial function and biogenesis were more expressed at AI. It was especially remarkable the higher expression of I-MUC at PI and IHH at AI. FABP6 transcripts were only consistently detected at PI, whereas FABP2 only at AI.

Fig. 3 shows the 26 differentially expressed genes for each diet and intestinal segment. The trend of the changes (red for up-regulation, green for down-regulation) induced by experimental diets was mainly down-regulation and the intensity of the changes and the number of significantly regulated genes were higher at PI than AI. In general, the magnitude of the changes was similar for D1 and D2 fish, and for some genes (TLR5 and TLR9) the magnitude was even higher than constitutional differences between the two intestinal segments in CTRL fish. Among the 14 studied genes related to cell differentiation and proliferation, a pattern of down-regulation was observed for six genes only at PI, and particularly more in D1 fish. Bone morphogenetic protein receptor type IA (BMPR1A) was the only gene that showed exclusive down-regulation in D2 fish. Vimentin (VIM) was down-regulated both in D1 and D2 fish at PI. The transcriptomic profile of the 19 studied genes involved in intestinal architecture and permeability (cell adhesion, tight junction, desmosome and gap junction proteins and mucins) was also decreased for four genes only at PI and very similarly with both experimental diets.

More than a fourth (26.5%) of the 34 measured genes related to immunosurveillance was also significantly down-regulated. The dis-regulation affected cytokines, including interleukins (IL), and PRRs. IL-6 was the only gene decreased in both intestinal segments by both diets, whereas TNF-α was the only gene decreased exclusively by D2. The only significantly up-regulated PRR, which was also the gene with the highest fold change, was fucolectin (FCL), but only by D1 at both intestinal segments. By contrast, up-regulation

was mostly found for genes related to enterocyte mass, cell epithelial damage and mitochondrial activity (4 out of 20 measured genes), and none of them was differentially expressed at both intestinal segments. Interestingly, the gene expression of fatty acid-binding proteins 2 and 6 (FABP2, FABP6) was significantly increased at AI and PI by D2, respectively. The only down-regulated genes in this gene category were CANX and ALPI, both by D2 at PI and AI, respectively.

4. Discussion

This is the first integrated study of the effects of combining essential oils and a prebiotic in the diet of a Mediterranean farmed fish. The threshold dose of NE was clearly established at 100 ppm in the first trial, as higher doses slowed growth due to a reduced feed intake, and possibly a lower lymphohaematopoietic function (low SI). When using the 100 ppm dose alone (D1) or combined with PRE (D2) in the second trial, the decrease in feed intake was confirmed and FGR was equally improved. Previously, carvacrol has been shown to be a growth enhancer in poultry and pig studies [27] and feed conversion was also improved with carvacrol or thymol in some fish trials with tilapia [31], channel catfish [33] and rainbow trout [35]. However, no improvement was obtained when using a combination of carvacrol and thymol in rainbow trout [33] or carvacrol alone in European sea bass [32] for 8 or 9 weeks, respectively. The use of some prebiotics also improved feed conversion in several fish species [6, 44-47], but we did not find significant differences between not adding (D1) or adding (D2) the prebiotic with the assayed dose. In the current study, organosomatic indexes (HSI, VSI and SI) were only altered with the highest dose (D300). Similarly, HSI and VSI were not altered in channel catfish fed thymol and/or carvacrol

[33], in European sea bass fed with carvacrol [32] or in starry flounder fed a dairy-yeast prebiotic [45]. By contrast, these indexes were decreased when Orego-Stim® (OS) (contains natural oregano oil) was added [33].

No significant changes in plasma metabolites, Hb, respiratory burst and TAC were detected in the feeding trials, though a slight increase in TAC was detected with D100 and D2. Similarly, in channel catfish fed carvacrol + thymol no significant changes were found in catalase and superoxide dismutase activity. These activities were only increased with OS diet [33]. By contrast, serum catalase was significantly increased in rainbow trout fed either carvacrol or thymol [35] and key enzymes of antioxidant defences were increased in white sea bream fed high levels of non-starch polysaccharides [48].

In the current work, the transcriptomic study focused on the gut because the intestine not only digests food and absorbs nutrients, but also provides a defence barrier against pathogens and noxious agents ingested [49]. The transcriptomic profile was based on the molecular definition of 87 (including 54 new) gilthead sea bream sequences, selected as markers of intestine function, integrity and immunity. For some of these genes, limited previous information has been published for salmonids and model fish species, while others have no data available on their functional regulation in fish. Thus, the new data published here will open the door to new studies focused on gut immunity and function. This approach is aligned with current strategies in human health to study nutritional aspects of metabolic inflammation, in which transcriptomic biomarkers have been shown to have a potential in profiling pro- and anti-inflammatory mechanisms [50].

First of all, it is interesting to note the differential constitutive profile of more than half of the studied genes between the two intestinal segments. Structurally, it is known that the AI of fish is primarily involved in the absorption of lipids and proteins, while the PI can

372 take up macromolecular proteins. The PI also appears to be the primary site where antigen
373 uptake occurs and an immune response is initiated [51]. This is in accordance with the
374 observed higher expression of some cytokines and many pattern recognition receptors
375 (PRR), including TLRs, NODs and lectins at the PI than at the AI of GSB. PRRs are
376 expressed by innate immune cells and activated by specific pathogen-associated molecular
377 patterns (PAMPs) present in microbial molecules or by damage-associated molecular
378 patterns (DAMPs) exposed on the surface of, or released by, damaged cells. Binding of
379 PAMPs or DAMPs to PRRs promotes the synthesis of cytokines and the subsequent
380 triggering of the innate and specific immune response. Information on fish TLRs has
381 increased considerably in the last years [52, 53] and TLRs in fish are known to be
382 expressed especially in immune-related organs (spleen, head-kidney) and mucosal–
383 epithelial barriers (gills, gut, skin), but there is no information on its differential expression
384 along the GI tract in fish.

385 Another group of genes more represented at the PI of GSB included several cell to
386 cell communication genes, such as some claudins, occludin and other tight junction (TJ)
387 and gap junctions (GJ) proteins and mucins. TJs are the most apical component of the
388 junctional complex, providing one form of cell-cell adhesion in enterocytes and playing a
389 critical role in regulating paracellular barrier permeability. Claudins are the major structural
390 and functional components of TJs that largely determine TJ permeability, with at least 24
391 members in mammals [54]. In fish, about 63 genes encoding for claudins have been
392 reported in 16 teleost species [55]. The structure and function of the TJ complex in teleosts
393 appears to be fundamentally similar to those of other vertebrate groups [56], though some
394 claudins have no orthologous in mammals. Even though at least 30 claudins have been
395 reported in the GI tract of teleosts, only a small fraction of them have been examined to

396 date and almost no functional studies have been conducted [57]. The GI tract of teleosts has
397 been reported to progressively "tighten," from the anterior to posterior part, thus preventing
398 leakage of water back into the gut lumen [58], this would agree with the observed higher
399 expression of genes related to TJ complexes at the PI, especially those that are considered
400 as tightening, such as TJP1, occludin and CXADR, and would mean a decreased
401 paracellular permeability. Claudins 12 and 15 have previously been found in the intestine of
402 fish, however their functions throughout the animals groups are unclear, as they vary
403 depending on the system studied. Claudin 15 is considered in humans a pore-forming type,
404 but it has been found to increase in Atlantic salmon intestine in response to seawater
405 acclimation [59] and claudin 12 expression decreases from unfertilized oocytes to
406 segmented embryo in Atlantic cod [57]. The different mucin types have a tissue-specific
407 distribution in the GI of GSB, and the current results confirmed the higher expression of
408 intestinal mucin (I-MUC) at the PI [40].

409 Direct communication between adjacent cells mainly occurs through GJ, which are
410 intercellular channels formed by members of the connexin family. GJ allow the direct cell-
411 to-cell passage of electrical signals, ions, and small molecules up to approximately 1,000
412 daltons in mass [59] and are also master regulators of cell growth and cell death [60].
413 Numerous factors are known to drive physiological GJ production and activity and
414 connexin gene transcription is ruled by specific sets of transcription factors in mammals
415 [61]. The connexin family comprises at least twenty isoforms in mammals, and there seems
416 to be a higher number in fish, and some of them, such as CX32.2 and CX32.7 are fish-
417 specific [62]. This is the first report on the expression of these two connexins in fish
418 intestine, and the first report of GJB4 (also named connexin 30.3) regulation in fish. The
419 previous scarce information on CX32.2 and CX32.7 in fish is mainly related to their

presence and regulation in gonads and gamets [58, 63, 64], and GJB4 in mammals is mainly found in skin and kidney and its mutations provoke skin diseases in humans [65]. Therefore we can only speculate about their differential expression in the two intestinal segments of GSB. What we know about gastrointestinal GJ is that they play a specific role in pacemaking and neurotransmission and thus the regulation of motility in mammals, and some enteric bacteria use these channels for cell invasion [66].

The differential function of both intestinal segments also explains the differential expression of enterocyte mass and epithelial damage related genes, such as FABPs. Although numerous experiments have provided evidence for several biological functions of FABPs, the precise role of FABPs in cell physiology remains unresolved. These studies suggest several functions: (1) the uptake and transportation of fatty acids and other hydrophobic ligands; (2) cell growth and differentiation; (3) regulation of specific genes; (4) sequestering of fatty acids to protect the cell from the detergent effects of excess fatty acids; and (5) targeting of fatty acids to various transport and signalling pathways. FABP2 transcripts were mainly found at AI, in agreement with the previous studies that showed a proximal to distal decrease in the intestine of several fish species [67-69], which is related to the major absorption of nutrients at the anterior segment. By contrast, FABP6 transcripts were detected exclusively at PI, the tissue homologous to the mammalian ileum, as well as in the distal region of zebrafish intestine [70], which suggests that FABP6 may have the same role in the uptake of bile salts, which are absorbed in the ileal epithelium of mammals [71].

Concerning the effects of the experimental diets on the gut transcriptomic profile, the results showed that several cell to cell communication genes, such as some claudins and other TJ proteins, were down-regulated, particularly at PI. Alteration of TJs in higher

vertebrates leads to a number of pathophysiological diseases causing malabsorption of nutrients and intestinal structure disruption, which may even contribute to systemic organ failure. Among the eight genes related to TJ proteins (ocludins, claudins, cadherins, etc.) described for the first time in GSB in the current study, three of them were affected by the experimental diets besides the gap junction protein GJB4. There is no information of the effect of diets or nutrients in fish TJs, though in mammals, several studies have shown that intestinal bacteria and various dietary components can regulate epithelial permeability by modifying expression and localization of TJ proteins [72]. The effects of the detected down-regulation on the intestinal permeability of GSB are unknown, but in any case it did not affect any of the measured plasma metabolites. Most probably, it would contribute to a higher permeability and therefore to an enhanced intestinal absorption, and would explain the observed higher FGR. This would be in line with the effects of chitosan, a polysaccharide widely used in the food industry, known for its absorption-enhancing properties due to the demonstrated increased paracellular permeability, by altering the distribution of TJP1 [73]. Considering the importance and complexity of the teleostean GI tract, the role of all these proteins in this tissue will be an exciting area for future study.

Most of the differentially expressed genes related to cell differentiation and proliferation were down-regulated at PI, in D1 fish, with vimentin (VIM) being the only one affected by both diets. VIM is one of the large intermediate filaments of eukaryotic cells' cytoskeleton. It is the major protein in mesenchymal cells and is frequently used as a developmental marker of epithelial to mesenchymal transitions that take place during embryogenesis and metastasis [74], yet the functional implications of the expression of this protein are poorly understood in mammals [75] and almost unknown in fish. In salmon, VIM-like transcripts were co-localized with the presence of intermediate filaments in

chordocytes, indicating that the transcript is translated and directly used in the cytoskeleton [76]. It is also considered a mesenchymal marker in GSB hepatocytes [77] and VIM+ cells were demonstrated by immunocytochemistry in the gill filament epithelium of *Oreochromis niloticus* [78]. In the current study, VIM was down regulated in all the experimental diet groups and intestinal segments, though the statistical differences were only found at PI. As VIM is considered a marker of undifferentiated cells, this down regulation could indicate a higher degree of cell maturation in experimentally fed fish. Interestingly, VIM has also been described as an endogenous, activating ligand for Dectin-1 (a PRR), and has been found in an extracellular form within areas of inflammation and necrosis in human atherosclerotic lesions [79]. Therefore, its down-regulation in experimentally fed GSB could also have anti-inflammatory effects.

Regarding immune-related genes, the down-regulation of three typically pro-inflammatory cytokines, TNFα, IL-1β and IL-8, concurrent with the down-regulation of IL-6 indicates the induction of a clear anti-inflammatory profile. In fact, IL-6 is a multifunctional, pleiotropic interleukin, with both pro- and anti-inflammatory functions [80]. In previous studies, it has been shown that in *E. leei*-infected GSB, IL-6, IL-8 and its receptor, IL-12 and TNFα were up-regulated short after exposure to the parasite during the inflammatory reaction [39]. Another group of genes related with immunity, PRRs, was also mainly down-regulated, especially at PI by D1 and only TLR5 and CLEC10A were equally decreased by both experimental diets. Although very few data are available on their functional regulation at the intestine or by nutrients in fish, it has been shown that TLR2, TLR4 and TLR5 can be up-regulated by feeding with probiotics [81, 82]. Some lectins induce the synthesis of pro-inflammatory cytokines, including IL1-β1, IL1-β2, TNF-α1, TNF-α2 and IL8 in rainbow trout macrophages and fibroblast-like cells [83]. The low

492 expression of most PRRs would be therefore in line with the observed low expression of
493 pro-inflammatory cytokines. The only significantly up-regulated PRR, which was also the
494 gene with the highest fold change, was fucolectin (FCL). In fact, it was significantly over-
495 expressed by D1 at both intestinal segments. Fucolectins — lectins that bind fucose— have
496 been described as immune-recognition molecules in both invertebrates and vertebrates.
497 They have been isolated and characterized from the sera of several fish species such as
498 European and Japanese eels, striped bass, European sea bass and even GSB [84]. Lectins
499 are typically multivalent proteins that recognize and bind specific carbohydrate moieties
500 through carbohydrate recognition domains (CRD). The presence of multiple CRDs in
501 combination with other protein domains, enable not only the recognition of carbohydrates
502 on the surface of potential pathogens, but also on the surface of immunocompetent cells.
503 Thus, vertebrate lectins play an active role in innate immunity, particularly in PAMP
504 recognition, opsonisation, phagocytosis, and complement activation [85, 86]. Thus, it is
505 tempting to suggest that those pathogens that express fucose antigens would easily be
506 recognized by fish with increased levels of FCL, activating the corresponding cascade of
507 immune events. In fact, *Anguilla japonica* fucolectin is induced by the presence of bacterial
508 liposaccharides [87], however in *E. leei*-infected GSB, FCL was down-regulated [88]. As
509 D1 fish expressed higher levels of FCL than D2 fish, we discard the possible effect of the
510 prebiotic (rich in oligosaccharides, but with a small percentage of fucose) as the triggering
511 agent. Further studies are needed to identify the cell types that express these high levels of
512 FCL at AI and the possible consequences. The global down-regulation of
513 immunosurveillance genes would be in agreement with the reported decrease of some
514 immune factors (lysozyme, complement, immunoglobulins) by carvacrol or thymol in
515 several fish species [32, 35].

Most of the genes of enterocyte mass and cell epithelial activity were over-expressed in experimentally fed fish. Some of them, such as FABP2, FABP6, were significantly increased at AI and PI of D2 fish, respectively, which could indicate a synergizing effect of the prebiotic. Recently, it has been shown in Atlantic salmon a progressive reduction in FABP2 staining of intestinal folds and a significant decrease of both transcriptional and protein levels of FABP2 during progression of intestinal inflammation associated to a diet induced enteritis [67]. This enteritis is characterized by increasing cell proliferation and infiltration in the lamina propria and submucosa. In addition, it has been shown that FABPs genes can be modulated by dietary fatty acids and peroxisome proliferators in zebrafish [89], as previously found in mammals [90]. Interestingly, FABP2 and FABP6 were significantly down-regulated in GSB with *E. leei*-induced enteritis (and clinical cachexia) [88] and in blue catfish after a short-term fasting [91]. The increase in FABPs in our experimental fish was coincident with an increase in the transcripts of Enoyl-CoA hydratase (ECH) and hydroxyacyl-CoA dehydrogenase (HADH), which are enzymes essential for metabolizing fatty acids to produce both acetyl CoA and energy, as they catalyse the second and third steps, respectively, of β-oxidation in fatty acid metabolism in the mitochondrial matrix. The increase in the expression of this group of genes would be in accordance with the observed increased FGR, and probably also with the increased number of intestinal villi, enterocyte density and goblets cells in the intestine, which probably involved a higher uptake and transport of fatty acids and nutrients in general. These changes on the intestinal morphology have also been observed in red drum fed PRE [6], as well as other species of fish fed other prebiotics [92-95].

In conclusion, it appears that the combination of NE and PRE induces an anti-inflammatory and anti-proliferative transcriptomic status in the intestine of GSB, mainly at

the posterior segment, and possibly changes in the absorptive capacity which would explain the observed improvement of feed conversion. These results are in agreement with those obtained for PRE in sea bass in reducing diet-induced enteritis (Peggs et al., personal communication), and with field results of the current additive combination (D2) in GSB in cage farms, in which cumulative mortality due to idiopathic enteritis was decreased (Andromeda S.A., unpublished results). Therefore, this dietary combination could have a potential use for overcoming some types of nutritionally or pathologically induced gut inflammation. In any case, the possible uses of these additives have to be further evaluated with pathogen challenges, to check if the induction of an anti-inflammatory/anti-proliferative transcriptomic profile can help to alleviate the immunopathological consequences of high inflammation levels. The involvement of changes in the microbiota composition are also to be studied in the future.

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858 **Figure legends**

859

860 **Fig. 1.** Microphotographs of representative liver (A, B), anterior intestine (C, D) and
861 posterior intestine (E, F) of gilthead sea bream fed with control diet (A,C, E) or the D2
862 (NE + PRE) (B, D, F). Arrowheads point to some goblet cells. Staining: Toluidine blue (A,
863 B), H&E (C-F). Scale bars: 50 µm (A, B), 100 µm (C-F).

864 **Fig. 2.** Representation of the statistically significant changed genes ($P < 0.05$) in the
865 posterior (PI) and anterior (AI) intestine of gilthead sea bream fed the control diet (without
866 additives). Bars above 0 stand for those genes with higher constitutive expression levels at
867 the PI, and those below 0 for those more expressed at the AI.

868 **Fig. 3.** Collorary of the gene expression changes found at anterior and posterior intestine of
869 gilthead sea bream fed with diets D1 (NE) and D2 (NE + PRE). Fold changes are relative to
870 the control diet. Red tones correspond to up-regulated genes and green tones correspond to
871 down-regulation. The intensity of the colour represents the degree of change. Statistically
872 significant differences between CTRL and diets groups are indicated (*$P < 0.05$). For each
873 gene, the symbol and the category in which they are classified are included: 1 = cell
874 differentiation and proliferation; 2 = Tight junctions; 3 = Cytokines; 4 = PRR; 5 =
875 Enterocyte absorption and epithelial damage; 6 = mitochondrial activity.

876

Table 3. Full list of gilthead sea bream genes included in real-time PCR (in alphabetical order). The symbols of new sequences uploaded to GenBank are labelled in italics.

Gene name	Symbol	Gene name	Symbol
Bone morphogenetic protein receptor type-1A	*BMPR1A*	Interleukin 8	IL-8
Cadherin-1	*CDH1*	Interleukin-8 receptor A	IL-8RA
Cadherin-17	*CDH17*	Interleukin 10	IL-10
Calnexin	*CANX*	Interleukin10 receptor subunit alpha	IL-10RA
Calreticulin	*CALR*	Interleukin 15	IL-15
Catenin beta-1	*CTNNB1*	Interleukin 34	IL-34
C-C chemokine receptor type 3	*CCR3*	Intestinal fatty acid-binding protein	*FABP2*
C-C chemokine receptor type 9	*CCR9*	Intestinal mucin	I-MUC
C-C chemokine receptor type 11	*CCR11*	Intestinal-type alkaline phosphatase	*ALPI*
C-C motif chemokine 20	*CCL20*	Junctional adhesion molecule A	*F11R*
C-C motif chemokine 25	*CCL25*	Krueppel-like factor 4	*KLF4*
CD48 antigen	*CD48*	Liver type fatty acid-binding protein	*FABP1*
CD209 antigen	*CD209*	L-rhamnose-binding lectin CSL2	*CSL2*
CD276 antigen	*CD276*	Macrophage colony-stimulating factor 1 receptor 1	CSF1R1
CD302 antigen	*CD302*	Macrophage mannose receptor 1	*MRC1*
Citrate synthase	CS	Mitochondrial 10 kDa heat shock protein	mtHsp10
Claudin-12	*CLDN12*	Mitochondrial 60 kDa heat shock protein	mtHsp60
Claudin-15	*CLDN15*	Mitochondrial 70 kDa heat shock preotin	mtHsp70
Coxsackievirus and adenovirus receptor homolog	*CXADR*	Mitochondrial import inner membrane translocase subunit 44	Tim44
C-type lectin domain family 10 member A	*CLEC10A*	Mitochondrial import receptor subunit Tom22	Tom22
C-X-C chemokine	*CXC*	Mitochondrial Transcription factor A	mtTFA
Desmoplakin	*DSP*	Mucin 2	MUC2
Enoyl-CoA hydratase	ECH	Mucin 2-like	MUC2-like
Fucolectin	*FCL*	Mucin 13	MUC13
Galectin-1	*LGALS1*	Notcheless protein homolog 1	*NLE1*
Galectin-8	*LGALS8*	Nuclear respiratory factor 1	NRF1
Gap junction beta-4 protein	*GJB4*	Nucleotide-binding protein oligomerization domain-containing protein 1	*NOD1*
Gap junction Cx32.2 protein	*CX32.2*	Occludin	*OCLN*
Gap junction Cx32.7 protein	*CX32.7*	Proliferator-activated receptor gamma coactivator 1 alpha	PGC1α
Glutathione reductase	GR	Protein wntless homolog	*WLs*
Glutathione S-transferase 3	GST3	Superoxide dismutase [Cu-Zn]	SOD1
Hedgehog-interacting protein	*HHIP*	Tight junction protein ZO-1	*TJP1*
Hydroxyacyl-CoA dehydrogenase	HADH	Toll-like receptor 1	*TLR1*
Ileal fatty acid-binding protein	*FABP6*	Toll-like receptor 2	*TLR2*
Indian hedgehog protein	*IHH*	Toll-like receptor 5	*TLR5*
Integrin beta-1-binding protein 1	*ITGB1BP1*	Toll-like receptor 9	*TLR9*
Integrin beta-6	*ITGB6*	Transcription factor 4	*Tcf4*
Integrin-linked protein kinase	*ILK*	Transcription factor HES-1-B	*HES1-B*
Interleukin 1 beta	IL1-β	Transcriptional regulator Myc	*Myc*
Interleukin 1 receptor type 1	IL-1R1	Tumor necrosis factor alpha	TNF-α
Interleukin 6	IL-6	Vimentin	*VIM*
Interleukin 6 receptor subunit beta	IL-6RB	Zinc finger protein GFI-1	*GFI-1*
Interleukin 7	IL-7	Zinc finger protein GLI1	*GLI1*
		Zinc finger protein GLIS3	*GLIS3*

Table 1. Ingredients of the basal diet (CTRL), to which PRE (5 g/kg diet) (D2) and/or NE was added at 100 (D50), 200 (D100, D1), 400 (D200) or 600 (D300) mg/kg diet.

Ingredient	Amount (g/kg)
Fish meal (70) [a]	190
Fish meal (65) [b]	180
Corn gluten meal [c]	160
Soybean meal [d]	180
Wheat meal	142.5
Fish oil [e]	140
Monocalcium phosphate [f]	5
Mineral & vitamim premix [g]	2.5

[a] Fish meal (Peruvian): 68% crude protein (CP), 9% crude fat (CF), EXALMAR, Peru.

[b] Fair Average Quality (FAQ) fish meal: 63% CP, 11 % CF, COFACO, Portugal.

[c] GLUTALYS: 61% CP, 8% CF, ROQETTE, France.

[d] Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL SA, Portugal

[e] Marine oil omega 3: Henry Lamotte Oils GmbH, Germany.

[f] Monocalcium phosphate: 22% phosphorous, 16% calcium, Fosfitalia, Italy.

[g] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (mg/kg diet, except as noted): DL-α tocopherol acetate, 250; sodium menadione bisulphite, 10; retinyl acetate, 10,000 IU/kg; DL-cholecalciferol, 2,000 IU/kg; thiamine, 20; riboflavin, 20; pyridoxine, 20; cobalamine, 0.2; nicotinic acid, 150; folic acid, 4; ascorbic acid, 300; inositol, 150; biotin, 0.8; calcium pantothenate, 60. Minerals (mg/kg diet): potassium iodide, 4; iron sulphate, 45; copper sulphate, 7; manganese oxide, 35; sodium selenite, 150; zinc oxide, 60.

Table 2. Characteristics of assembled new sequences of gilthead sea bream according to BLAST searches.

Contigs	F[a]	Size (nt)	Annotation[b]	Best match[c]	E[d]	CDS[e]	GenBank Accession No.
C2_68236	2	669	BMPR1A	CBH32481	3e-42	<1-233	KF857333
C2_43802	6	611	IHH	XP_003963214	7e-122	<1->611	KF857334
C2_16781	22	837	GLI1	CBN80831	1e-163	<1->837	KF857336
C2_61777	2	536	GLIS3	XP_003453957	2e-102	<1->536	KF857337
C2_24684	19	1778	HHIP	XP_003972462	2e-173	<1-822	KF857338
C2_3167	109	2394	WLs	XP_003975755	0.0	126-1766	KF857339
C2_66983	6	595	Myc	CBN82098	3e-102	<1-570	KF857340
C2_25912	22	1809	CTNNB1	XP_003965981	0.0	<1-978	KF857341
C2_17080	17	986	Tcf4	XP_003440245	3e-118	175->986	KF857342
C2_32386	8	1084	NLE1	XP_003446779	0.0	83->1084	KF857343
C2_15474	33	2081	HES1-B	XP_003974264	2e-156	234-1067	KF857344
C2_8854	7	2090	GFI-1	XP_003978426	3e-170	196-1182	KF857345
C2_64245	2	431	KLF4	XP_003965249	6e-78	<1-353	KF857346
C2_3381	180	1925	VIM	XP_003438114	0.0	<1-973	KF857332
C2_1720	145	1987	ITGB1BP1	XP_004082307	3e-95	98-694	KF861987
C2_26407	14	1397	ITGB6	XP_008283045	5e-107	<1-669	KF861988
C2_2296	148	2884	ILK	XP_003968610	0	183-1541	KF861989
C2_15809	32	1077	OCLN	XP_003445179	0	<1->1077	KF861990
C2_10139	87	2435	CLDN12	AAT64072	5e-153	210-1241	KF861992
C2_218	1275	1485	CLDN15	XP_004079873	2e-137	176-844	KF861993
C2_57261	6	708	TJP1	XP_008288986	3e-104	<3->708	KF861994
C2_2045	4026	230	CDH1	CAF91005	0	193-2901	KF861995
C2_3038	169	3092	CDH17	XP_004078163	0	75-2666	KF861996
C2_1814	296	2334	F11R	XP_003971293	4e-126	198-1100	KF861997
C2_12148	39	1210	CXADR	XP_003978491	4e-163	<1-894	KF861998
C2_20476	32	2019	DSP	CAG07577	0	<1-1917	KF861999
C2_501	648	1391	CX32.2	P51915	0	131-991	KF862000
C2_7763	52	1557	CX32.7	XP_005737265	3e-164	326-1222	KF862001
C2_79555	888	4	GJB4	XP_003962600	8e-98	50-556	KF862002
C2_2049	370	1912	ALPI	XP_003974329	0.0	88-1656	KF857309
C2_23355	21	1187	FABP1	Q8JJ04	7e-68	169-552	KF857311
C2_1175	972	1474	FABP2	ACI68448	6e-79	64-462	KF857310
C2_750	460	696	FABP6	XP_003975579	2e-78	205-588	KF857312
C2_1023	720	1920	CALR	ABG00263	0.0	101-1350	KF857313
C2_19770	27	1558	CANX	ADX97134	0.0	<1-1266	KF857314
C2_11437	63	842	CXC	ACQ59055	4e-45	36-452	KF857315
C2_6505	202	1043	CCL25	XP_003448528	3e-36	111-443	KF857316
C2_1992	154	1295	CCR3	BAC87713	0.0	119-1219	KF857317
C2_3514	136	1901	CCR9	CBJ23501	0.0	252-1358	KF857318
C2_18315	44	1454	CCR11	CBN82022	0.0	121-1233	KF857319
C2_6437	83	1665	CD48	ACQ58805	3e-48	125-949	KF857320
C2_43130	10	1136	CD276	NP_001177294	7e-141	77-973	KF857321
C2_13781	16	1188	TLR1	ADX01348	0.0	<1->1188	KF857322
C2_12348	52	2293	TLR2	AFZ81806	0.0	<1-2115	KF857323
C2_20508	30	2076	TLR5	AEN71825	0.0	<1-1695	KF857324
C2_14384	25	1303	NOD1	AFV53357	0.0	125->1303	KF857325
C2_1940	246	3462	MRC1	XP_008284986	0.0	<1-3045	KF857326
C2_1976	149	1531	CD209	XP_003966073	4e-94	71-844	KF857327
C2_4689	179	3364	CD302	XP_003962014	7e-81	102-857	KF857328
C2_27915	9	1079	CLEC10A	XP_003446031	5e-126	57-953	KF857329
C2_111	990	1324	LGALS1	ADV35589	8e-74	312-719	KF862003
C2_12854	25	1564	LGALS8	AFJ79965	4e-142	56-997	KF862004
C2_2032	263	1244	CSL2	XP_003455367	4e-103	40-708	KF857330
C2_1599	588	1822	FCL	XP_003450311	2e-154	110-1042	KF857331

[a]Number of reads composing the assembled sequences.
[b]Gene identity determined through BLAST searches; full name of the genes can be found in table 3.
[c]Best BLAST-X protein sequence match (lowest E value).
[d]Expectation value.
[e]Codifying sequence.

Table 4. Dose-dependent effects of NE (0-300 ppm) on the growth performance, haematological parameters, plasma biochemistry, total antioxidant capacity (TAC) and respiratory burst (RB) of gilthead sea bream juveniles fed to visual satiety for 9 weeks (Trial 1). Data on body weight, feed intake, and growth indices are the mean (S.E.M) of triplicate tanks. Data on viscera and liver weight are the mean (S.E.M) of 20-25 fish and the remaining values are the mean (S.E.M) of 8-10 fish. Different superscript letters in each row indicate significant differences among dietary treatments (ANOVA-I followed by Student Newman-Keuls test, $P < 0.05$).

	CTRL	D50	D100	D200	D300	*P*
Initial body weight(g)	27.2(0.019)	27.0(0.45)	27.1(0.25)	27.0(0.55)	27.1(0.12)	*0.97*
Final body weight (g)	96.7(2.53)	92.5(0.28)	91.6(0.31)	90.8(0.86)	88.4(1.31)	*0.098*
Feed intake (g DM/fish)	75.2(3.96)^{a}	63.9(0.67)^{b}	62.9(2.24)^{b}	62.4(0.06)^{b}	58.6(1.99)^{b}	*0.033*
Viscera (g)	8.16(0.29)^{a}	7.70(0.25)^{ab}	7.15(0.25)^{ab}	7.25(0.28)^{ab}	6.65(0.23)^{b}	*0.003*
Liver (g)	1.30(0.06)^{a}	1.22(0.06)^{ab}	1.12(0.03)^{ab}	1.15(0.05)^{ab}	1.13(0.01)^{b}	*0.045*
Spleen (g)	0.19(0.02)	0.19(0.02)	0.17(0.04)	0.15(0.02)	0.13(0.01)	*0.057*
VSI (%)[1]	8.21(0.16)^{a}	8.14(0.15)^{ab}	7.99(0.13)^{ab}	7.82(0.18)^{ab}	7.57(0.12)^{b}	*0.030*
HI (%)[2]	1.32(0.04)	1.29(0.05)	1.25(0.02)	1.26(0.05)	1.29(0.04)	*0.76*
SI (%)[3]	0.19(0.03)^{a}	0.20(0.02)^{a}	0.19(0.04)^{ab}	0.17(0.01)^{ab}	0.14(0.004)^{b}	*0.005*
SGR (%)[4]	2.04(0.03)	2.02(0.02)	2.00(0.02)	1.99(0.02)	1.94(0.03)	*0.16*
FGR (%)[5]	1.08(0.038)^{a}	0.97(0.01)^{b}	0.97(0.04)^{b}	0.97(0.005)^{b}	0.96(0.05)^{b}	*0.047*
Haemoglobin (g/dl)	8.90(0.44)	8.97(0.28)	8.6(0.15)	8.57(0.25)	8.63(0.18)	*0.249*
Proteins (g/dl)	3.59(0.13)	3.70(0.14)	3.76(0.09)	3.62(0.12)	3.67(0.11)	*0.790*
TG (mM/l)	0.38(0.04)	0.32(0.04)	0.35(0.03)	0.32(0.05)	0.33(0.04)	*0.877*
Cholesterol (mg/dl)	176.3(12.9)	157.7(14.6)	140.5(19.4)	136.5(11.2)	159.7(11.5)	*0.156*
Glucose (mg/dl)	50.1(4.01)	50.6(3.06)	49.2(2.21)	53.1(1.31)	49.5(1.91)	*0.207*
TAC (mM Trolox)	1.99(0.07)	1.92(0.04)	2.00(0.08)	1.86(0.06)	1.85(0.07)	*0.411*
RB (RLU)[6]	14431.9 (2353.8)	9737.5 (3048.6)	12877.5(4004.3)	17920(4287.8)	26282.5(12080.6)	*0.297*

[1] Viscerosomatix index = (100 x viscera weight) / fish weight
[2] Hepatosomatic index = (100 x liver weight) / fish weight
[3] Splenosomatic index = (100 x spleen weight) / fish weight
[4] Specific growth rate = 100 x (ln final body weight - ln initial body weight / days)
[5] Feed gain ratio = dry feed intake / wet weight gain
[6] Of circulating leukocytes after PMA stimulation

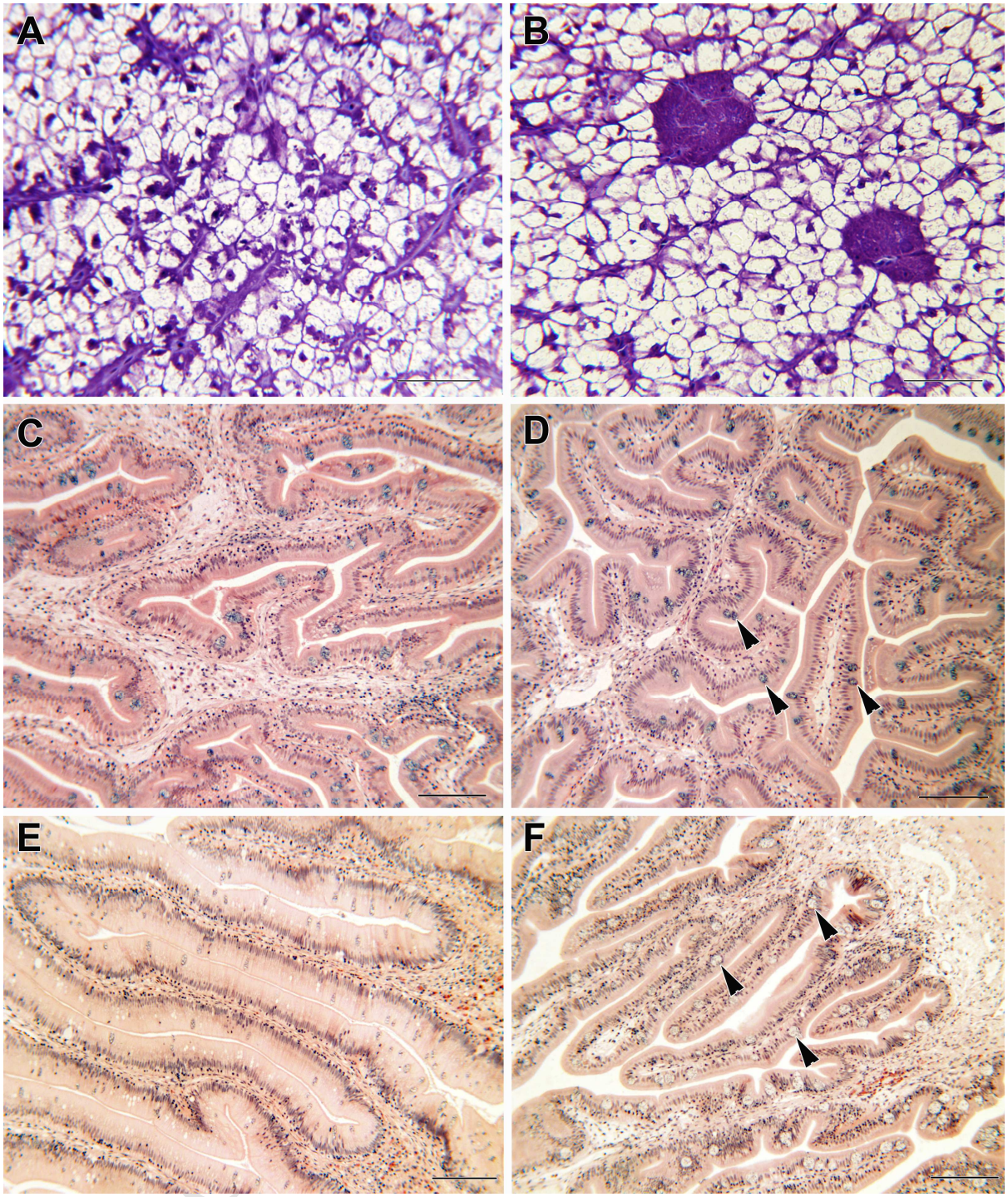
Table 5. Effects of NE (100 ppm) alone (D1) or in combination with PRE (0.5%) (D2), in comparison to the control diet (CTRL) on performance of gilthead sea bream juveniles (Trial 2). Data are the mean (S.E.M) of triplicated tanks. Different superscript letters in each row indicate significant differences among dietary treatments ANOVA-I followed by Student Newman-Keuls test ($P < 0.05$).

	CTRL	**D1**	**D2**	***P***
Initial body weight (g)	24.2 (0.54)	24.4 (0.13)	24.9 (0.01)	0.39
Final body weight (g)	102.7 (2.86)	103.08 (1.18)	100.5 (3.95)	0.811
Feed intake (g/fish)	87.9 (1.7)^{a}	75.5 (3.35)^{ab}	71.3 (0.99)^{b}	0.028
SGR (%)[1]	1.97 (0.06)	1.97 (0.008)	1.91 (0.05)	0.613
FGR (%)[2]	1.12 (0.027)^{b}	0.96 (0.029)^{a}	0.94 (0.036)^{a}	0.048
Haemoglobin (g/dl)	9.52(0.24)	8.92(0.45)	8.91(0.72)	0.632
TAC (mM Trolox)[3]	1.18(0.08)	1.14(0.09)	1.24(0.05)	0.652

[1]Specific growth rate = 100 x (ln final body weight - ln initial body weight / days)

[2]Feed gain ratio = dry feed intake / wet weight gain

[3] Total antioxidant capacity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B
C
D
E
F

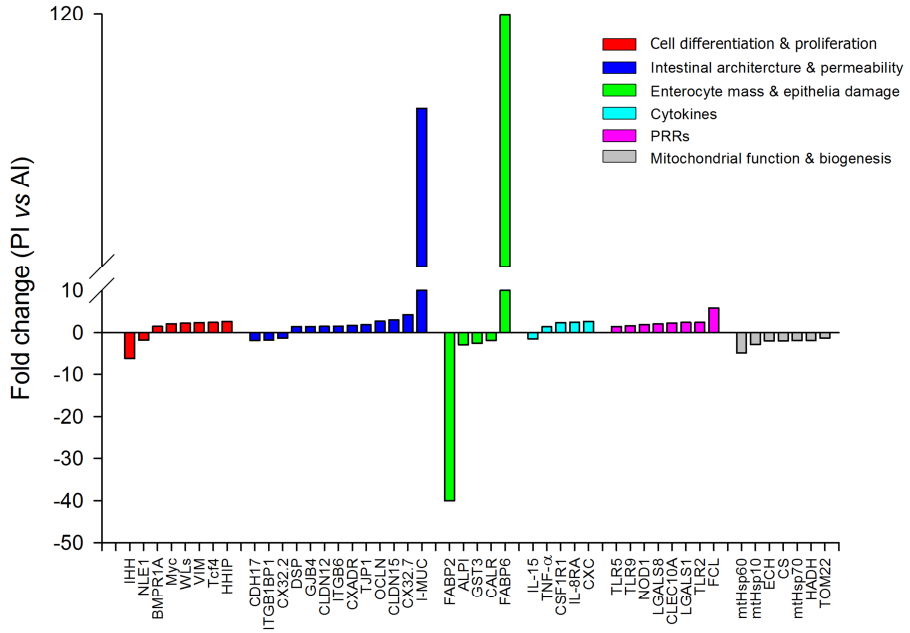

Figure 3.

GENE		ANTERIOR INTESTINE		POSTERIOR INTESTINE	
Category	Symbol	D1	D2	D1	D2
1	BMPR1A	1.08	0.96	0.85	0.75*
	GLI1	0.90	0.81	0.73*	0.82
	WLs	0.80	0.93	0.65*	0.86
	Myc	0.92	0.92	0.64*	0.78
	Tcf4	0.77	0.79	0.57*	0.72
	VIM	0.89	0.99	0.65*	0.62*
2	CLDN12	0.91	0.80	0.76*	0.75*
	CLDN15	0.90	0.98	0.78	0.73*
	TJP1	0.84	0.85	0.68*	0.69*
	GJB4	0.87	0.86	0.65*	0.71*
3	IL-1β	0.47*	0.64*	0.64	0.68
	IL-6	0.23*	0.39*	0.12*	0.25*
	IL-8	0.74	0.38*	0.95	0.67
	TNFα	0.92	0.81	0.76	0.64*
4	TLR1	0.69	0.92	0.72*	0.95
	TLR5	0.53	0.67	0.55*	0.60*
	TLR9	1.14	0.80	0.57*	0.91
	CLEC10A	0.68	0.79	0.53*	0.60*
	LGALS1	0.94	1.14	0.69*	0.93
	FCL	11.7*	3.34	6.12*	3.33
5	ALPI	1.09	0.45*	0.91	0.72
	FABP2	1.57	3.33*	-	-
	FABP6	-	-	1.52*	2.45*
	CANX	1.02	0.81	0.85	0.77*
6	ECH	1.98*	1.55*	1.21	1.28
	HADH	1.51*	1.16	1.30	1.01

Highlights

- ✓ 54 new intestinal-related gene sequences were identified
- ✓ More than half of the studied genes was more expressed at the posterior intestine
- ✓ The additives improved feed gain ratio
- ✓ The additives induced more changes in the transcriptome of posterior intestine
- ✓ The additives induced an anti-inflammatory and anti-proliferative transcriptomic profile

Supplementary Table 2. Forward and reverse primers of gilthead sea bream genes for real-time PCR. The symbols of new sequences uploaded to GenBank are labelled in italics

Gene name	Symbol		Primer sequence
Bone morphogenetic protein receptor type-1A	*BMPR1A*	F	AGT GCT GGG CTC ATA ACC
		R	CAT CTT GGC GAG TGT CTT CT
Indian hedgehog protein	*IHH*	F	ACA GGT TGG CTA TCG CAG TG
		R	CCT CCG TCA CAC GCA AGT
Zinc finger protein GLI1	*GLI1*	F	AGA ACC AGC GAG GAA TGC CGT ATT
		R	TTG AAG TGG GTC GGT GTC TGT TGA TT
Zinc finger protein GLIS3	*GLIS3*	F	CGA CAG TTG CGG AAG AAG ATG
		R	AGG GTG GAT GGT TAA ACA GTC T
Hedgehog-interacting protein	*HHIP*	F	CTG TGT AAG AGC GGC TAC T
		R	CCT GGT CGT TGG GCA TAC
Protein wntless homolog	*WLs*	F	GAG GTC GGC AGC GTG GCT CAT AAG TA
		R	GTT GAC AGG CAG ACG GAT GTT GAG AAG GT
Transcriptional regulator Myc	*Myc*	F	CAG CAG CAA CCG CAA GTG T
		R	TGT CGT AGT CCT CCG TGT CAG A
Catenin beta-1	*CTNNB1*	F	ACA CAG AGA CGC ACC AGC AT
		R	CTC CAT ACG AAC TCC CTC CAC AAA
Transcription factor 4	*Tcf4*	F	CAG AGA GCC CAA CCC ACA CT
		R	CCC AAC TCG CCA CCC AGT AT
Notcheless protein homolog 1	*NLE1*	F	GGA CTT GAC GAC GGA GAC
		R	ACC AGG CGA TGC TCA GTA
Transcription factor HES-1-B	*HES1-B*	F	GCC TGC CGA TAT GAT GGA A
		R	GGA GTT GTG TTC ATG CTT GC
Zinc finger protein GFI-1	*GFI-1*	F	CTC AGC AGC CTC TGG ACT
		R	GCA GTG GTA GGT GTT GGA G
Krueppel-like factor 4	*KLF4*	F	ACA TCA CCG CAC GCA CAC
		R	AAC CAC AGC CCT CCC AGT C
Vimentin	*VIM*	F	GCT TCA GAC AGG ATG TGG ACA AC
		R	AGT GAT TCT ACC TTC CGC TCC AG
Integrin beta-1-binding protein 1	*ITGB1BP1*	F	GCC ACC CTC TCT ACC TGA TAG T
		R	TTG AGA GCC AGG AGG TTC TTC
Integrin beta-6	*ITGB6*	F	AGC CTC CCA ACA TCC CTA TGA TTA TTC
		R	CTT CCA CAC ACC CAG CAG AA
Integrin-linked protein kinase	*ILK*	F	GCC AAT GAA CAC GGG AAC AC
		R	ACG AGA TCC TCA GCC ACT TG
Occludin	*OCLN*	F	GTG TCA GAA CCT CTA CCA GAC CAG CTA CTC
		R	GAA AGC CTC CCA CTC CTC CCA TCT
Claudin-12	*CLDN12*	F	CTC TCA GGG CTA CAC ATC TAC CTA TGC
		R	ACA TTC GTG AGC GGC TGG AG
Claudin-15	*CLDN15*	F	CCG ATT GTG GAA GTA GTG GCT CTG GT
		R	CAG CAT CAC CCA ACC GAC GAA CC
Tight junction protein ZO-1	*TJP1*	F	AAG CAG TAT TAC GGT GAC TCA
		R	TGC ATC CCT GGC TTG TAG
Cadherin-1	*CDH1*	F	TGC TCC ATA CAG CGT CAC CTT ACA
		R	CTC GTT CAT CCT AGC CGT CCA GTT
Cadherin-17	*CDH17*	F	GAT GCC CGC AAC CCA GAG
		R	CCG TTG ATT CAC TGC CGT AGA C

Supplementary Table 2. Continued-I

Gene name	Symbol		Primer sequence
Junctional adhesion molecule A	*F11R*	F	GAC TGG TTT CGG TGG CTT TGT TC
		R	TGG CTT GGG AGG TAG TGA CTG TA
Coxsackievirus and adenovirus receptor homolog	*CXADR*	F	CAT CAG AGG ACT ACG AGA GG
		R	CAT CTT GGC AGC ATT TGG T
Desmoplakin	*DSP*	F	GCA GAA GGA GCA CGA GAC CATC
		R	GGG TGT TCT TGT CGC AGG TGA A
Gap junction Cx32.2 protein	*CX32.2*	F	CGA GGT GTT CTA TCT GCT CTG TA
		R	CTT GTG GGT GCG AGT CCT
Gap junction Cx32.7 protein	*CX32.7*	F	CGC TCA CCT TGC CCT CAC AA
		R	AAC CAG ATG ATG ACC GAC TTC TCT
Gap junction beta-4 protein	*GJB4*	F	TGA AAT CCT CTA CCT GGT CGG CAA AC
		R	TGG CGA GAA TTA TGG AAC GAG GTG AAG
Mucin 2	MUC2	F	ACG CTT CAG CAA TCG CAC CAT
		R	CCA CAA CCA CAC TCC TCC ACA T
Mucin 2-like	MUC2-like	F	GTG TGT GGC TGT GTT CCT TGC TTT GT
		R	GCG AAC CAG TCT GGC TTG GAC ATC A
Mucin 13	MUC13	F	TTC AAA CCC GTG TGG TCC AG
		R	GCA CAA GCA GAC ATA GTT CGG ATA T
Intestinal mucin	I-MUC	F	GTG TGA CCT CTT CCG TTA
		R	GCA ATG ACA GCA ATG ACA
Intestinal-type alkaline phosphatase	*ALPI*	F	CCG CTA TGA GTT GGA CCG TGA T
		R	GCT TTC TCC ACC ATC TCA GTA AGG G
Liver type fatty acid-binding protein	*FABP1*	F	GTC CTC GTC AAC ACC TTC ACC AT
		R	CGC CTT CAT CTT CTC GCC AGT
Intestinal fatty acid-binding protein	*FABP2*	F	CGA GCA CAT TCC GCA CCA AAG
		R	CCC ACG CAC CCG AGA CTT C
Ileal fatty acid-binding protein	*FABP6*	F	ACC CAG GAC GGC AAT ACC
		R	CGA CGG TGA AGT TGT TGG T
Calreticulin	*CALR*	F	GGC GGC GGC TAT GTG AAG
		R	GCA TCG CAG TCT GAT CCA AGT C
Calnexin	*CANX*	F	CCC GAG GGT TGG CTA GAT GA
		R	GGC GTC TGG GTC TCC GAT AT
Glutathione reductase	GR	F	TGT TCA GCC ACC CAC CCA TCG G
		R	GCG TGA TAC ATC GGA GTG AAT GAA GTC TTG
Glutathione S-transferase 3	GST3	F	CCA GAT GAT CAG TAC GTG AAG ACC GTC
		R	CTG CTG ATG TGA GGA ATG TAC CGT AAC
Superoxide dismutase [Cu-Zn]	SOD1	F	TCA CGG ACA AGA TGC TCA CTC TC
		R	GGT TCT GCC AAT GAT GGA CAA GG
Interleukin 1 beta	IL1-β	F	GCGACCTACCTGCCACCTACACC
		R	TCG TCC ACC GCC TCC AGA TGC
Interleukin 1 receptor type 1	IL-1R1	F	GAA GCT GTA CGA CGC CTA C
		R	CTC CAC TGC CTT ACT GTA TCC
Interleukin 6	IL-6	F	TCT TGA AGG TGG TGC TGG AAG TG
		R	AAG GAC AAT CTG CTG GAA GTG AGG

Supplementary Table 2. Continued-II

Gene name	Symbol		Primer sequence
Interleukin 6 receptor subunit beta	IL-6RB	F	CAG TGT CGG AGT ATG TGG TTG AGT
		R	CCC TCT GCC AGT CTG TCC AA
Interleukin 7	IL-7	F	CTA TCT CTG TCC CTG TCC TGT GA
			TGC GGA TGG TTG CCT TGT AAT
Interleukin 15	IL-15	F	GAG ACC AGC GAG CGA AAG GCA TCC
		R	GCC AGA ACA GGT TAC AGG TTG ACA GGA A
Interleukin 8	IL-8	F	CAG CAG AGT CTT CAT CGT CAC TAT TG
		R	AGG CTC GCT TCA CTG ATG G
High affinity interleukin-8 receptor A	IL-8RA	F	CTT GTT TCA TCT GAC GAT AG
		R	AAG AGG ATG CTT GTG TAG
Interleukin 10	IL-10	F	AAC ATC CTG GGC TTC TAT CTG
		R	GTG TCC TCC GTC TCA TCT G
Interleukin10 receptor subunit alpha	IL-10RA	F	GAG GAC AAT GAA GAG GAA GAC AGG AG
		R	TGT TCG TAG CGG AGT TGG ACT
Interleukin 34	IL-34	F	TCT GTC TGC CTG CTG GTA G
		R	ATG CTG GCT GGT GTC TGG
Tumor necrosis factor alpha	TNF-α	F	CAGGCGTCGTTCAGAGTCTC
		R	CTGTGGCTGAGAGGTGTGAG
Macrophage colony-stimulating factor 1 receptor 1	CSF1R1	F	TTG CGT GTG GTG AGG AAG GAA GGT
		R	AGC AGG CAG GGC AGC AGG TA
C-X-C chemokine	*CXC*	F	CTG AGG AGT AAC GAG ACA GTG TG
		R	CCT GTT CCA GCA GCG TAT CA
C-C motif chemokine 25	*CCL25*	F	GCA ACA TCC CTG CCA CAA TCT TCA
		R	TCC TTC AGT TCT ATG ACC CAC ATC TCT C
C-C chemokine receptor type 3	*CCR3*	F	CTA CAT CAG CAT CAC CAT ACG CAT CCT
		R	TGG CAC GGC ACT TCT CCT TCA
C-C chemokine receptor type 9	*CCR9*	F	TCC CTG AGT TAA TCT TCG CCC AAG TG
		R	TGT TGT ATT CGT TGT TCC AGT AGA CCA GAG
C-C chemokine receptor type 11	*CCR11*	F	GCT ACG ATT ACA GTT ATG AA
		R	TAG ATG ATT GGG AGG AAG
C-C motif chemokine 20	*CCL20*	F	CCG TCC TCA TCT GCT TCA TAC T
		R	GCT CTG CCG TTG ATG GAA C
CD48 antigen	*CD48*	F	GAC ATA CTT CGA GGT TGG CGG TAA ACT
		R	GAT GTT GTC GAT AGT CTC CGT CAC TGT AGG
CD276 antigen	*CD276*	F	GTC ACA CTC AAC TGC TCC TTC A
		R	CGC CAG AAG ACG GTC AGA T
Toll-like receptor 1	*TLR1*	F	GGG ACC TGC CAG TGT GTA AC
		R	GCG TGG ATA GAG TTG GAC TTG AG
Toll-like receptor 2	*TLR2*	F	CAT CTG CGA CTC TCC TCT CTT CCT
		R	ATT CAA CAA TGG AGC GGT GGA CTT
Toll-like receptor 5	*TLR5*	F	TCG CCA ATC TGA CGG ACC TGA G
		R	CAG AAC GCC GAT GTG GTT GTA AGA C
Toll-like receptor 9	*TLR9*	F	GCC TTC CTT GTC TGC TCT TTC T
		R	GCC GTA GAG GTG CTT CAG TAG

Supplementary Table 2. Continued-III

Gene name	Symbol		Primer sequence
Nucleotide-binding protein oligomerization domain-containing protein 1	*NOD1*	F	GTC CAG GTT GAG GAG CAT CCA GTG
		R	TGA AGC CAC AAG CCG ACA GGT T
Macrophage mannose receptor 1	*MRC1*	F	CTT CCG ACC GTA CCT GTA CCT ACT CA
		R	CGA TTC CAG CCT TCC GCA CAC TTA
CD209 antigen	*CD209*	F	CGC CAC GAG CAT GAG GAC AA
		R	TCT TGC CAG AAT CCA TCA CCA TCC A
CD302 antigen	*CD302*	F	GGA CCA GAG GAA GAG CAC ATC
		R	GAC CAG GGC GGA CAT CAG
C-type lectin domain family 10 member A	*CLEC10A*	F	CGA CTC TGG ACT CCC TCA
		R	CGT TGT TGA TGG TGC GTT C
Galectin-1	*LGALS1*	F	GTG TGA GGA GGT CCG TGA TG
		R	ACT GTA GAG CCG TCC GAT AGG
Galectin-8	*LGALS8*	F	GGC GGT GAA CGG CGG TCA
		R	GCT CCA GCT CCA GTC TGT GTT GAT AC
L-rhamnose-binding lectin CSL2	*CSL2*	F	GCT CAC CAA TAC AAA GTG CTC TCA G
		R	CTT GCC ATC ACA CCT CCT CCT
Fucolectin	*FCL*	F	CCA TAC TGC TGA ACA GAC CAA CC
		R	TGA TGG AGG TGA CGA TGT AGG A
Mitochondrial 10 kDa heat shock protein	mtHsp10	F	CAT GCT GCC AGA GAA GTC TCA AGG
		R	AGG TCC CAC TGC CAC TAC TGT
Mitochondrial 60 kDa heat shock protein	mtHsp60	F	TGT GGC TGA GGA TGT GGA TGG AGA G
		R	GCC TGT TGA GAA CCA AGG TGC TGA G
Mitochondrial 70 kDa heat shock preotin	mtHsp70	F	TCC GGT GTG GAT CTG ACC AAA GAC
		R	TGT TTA GGC CCA GAA GCA TCC ATG
Enoyl-CoA hydratase	ECH	F	GCC CAA GAA GCC AAG CAA TCA G
		R	CTT TAG CCA TAG CAG AGA CCA GTT TG
Hydroxyacyl-CoA dehydrogenase	HADH	F	GAA CCT CAG CAA CAA GCC AAG AG
		R	CTA AGA GGC GGT TGA CAA TGA ATC C
Citrate synthase	CS	F	TCC AGG AGG TGA CGA GCC
		R	GTG ACC AGC AGC CAG AAG AG
Mitochondrial import inner membrane translocase subunit 44	Tim44	F	GAT GAC CTG GGA CAC ACT GG
		R	TCA CTC CTC TTC CTG AGT CTG G
Mitochondrial import receptor subunit Tom22	Tom22	F	CGC TCT GGG TGG GTA CTA CCT CCT T
		R	CGA ACA CAA CAG GCA GCA CCA GGA T
Mitochondrial Transcription factor A	mtTFA	F	GAG CCC GCA ACA GAA ACA GCC ATT
		R	ACT GCT CCC TGT CCC GCT GAT AG
Nuclear respiratory factor 1	NRF1	F	CAG ATA GTC CTG GCA GAG A
		R	GAC CTG TGG CAT CTT GAA
Proliferator-activated receptor gamma coactivator 1 alpha	PGC1α	F	CGT GGG ACA GGT GTA ACC AGG ACT C
		R	ACC AAC CAA GGC AGC ACA CTC TAA TTC T
β-actin	ACTB	F	TCC TGC GGA ATC CAT GAG A
		R	GAC GTC GCA CTT CAT GAT GCT

Supplementary Table 1. PCR-array layout of 87 genes of gilthead sea bream with extra-wells for housekeeping genes and general controls of PCR performance.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BMPR1A	Tcf4	ILK	CXADR	I-MUC	GST3	IL8	CCL25	TLR2	LGALS1	HADH	PPC1
B	IHH	NLE1	OCLN	DSP	ALPI	SOD1	IL8RA	CCR3	TLR5	LGALS8	CS	PPC2
C	GLI1	HES1-B	CLDN12	CX32.2	FABP1	IL1β	IL10	CCR9	TLR9	CSL2	Tim44	PPC3
D	GLIS3	GFI-1	CLDN15	CX32.7	FABP2	IL1R1	IL10RA	CCR11	NOD1	FCL	Tom22	PPC4
E	HHIP	KLF4	TJP1	GJB4	FABP6	IL6	IL34	CCL20	MRC1	mtHsp10	mtTFA	NPC
F	WLs	VIM	CDH1	MUC2	CALR	IL6RB	TNFα	CD48	CD209	mtHsp60	NRF1	NPC
G	Myc	ITGB1BP1	CDH17	MUC2-like	CANX	IL7	CSF1R1	CD276	CD302	mtHsp70	PGC1α	ACTB
H	CTNNB1	ITGB6	F11R	MUC13	GR	IL15	CXC	TLR1	CLEC10A	ECH		ACTB

Position	Symbol	Description	Accession No.
A1	BMPR1A	Bone morphogenetic protein receptor type-1A	KF857333
B1	IHH	Indian hedgehog protein	KF857334
C1	GLI1	Zinc finger protein GLI1	KF857336
D1	GLIS3	Zinc finger protein GLIS3	KF857337
E1	HHIP	Hedgehog-interacting protein	KF857338
F1	WLs	Protein wntless homolog	KF857339
G1	Myc	Transcriptional regulator Myc	KF857340
H1	CTNNB1	Catenin beta-1	KF857341
A2	Tcf4	Transcription factor 4	KF857342
B2	NLE1	Notcheless protein homolog 1	KF857343
C2	HES1B	Transcription factor HES-1-B	KF857344
D2	GFI-1	Zinc finger protein GFI-1	KF857345
E2	KLF4	Krueppel-like factor 4	KF857346
F2	VIM	Vimentin	KF857332
G2	ITGB1BP1	Integrin beta-1-binding protein 1	KF861987
H2	ITGB6	Integrin beta-6	KF861988
A3	ILK	Integrin-linked protein kinase	KF861989
B3	OCLN	Occludin	KF861990
C3	CLDN12	Claudin 12	KF861992
D3	CLDN15	Claudin 15	KF861993
E3	TJP1	Tight junction protein ZO-1	KF861994
F3	CDH1	Cadherin 1	KF861995
G3	CDH17	Cadherin 17	KF861996
H3	F11R	Junctional adhesion molecule A	KF861997
A4	CXADR	Coxsackievirus and adenovirus receptor homolog	KF861998
B4	DSP	Desmoplakin	KF861999
C4	CX32.2	Gap junction Cx32.2 protein	KF862000
D4	CX32.7	Gap junction Cx32.7 protein	KF862001
E4	GJB4	Gap junction beta-4 protein	KF862002
F4	MUC2	Mucin 2	JQ27710
G4	MUC2-like	Mucin 2-like	JQ27711
H4	MUC13	Mucin 13	JQ27713
A5	I-MUC	Intestinal mucin	JQ27712

Supplementary Table 1. Continued-I.

Position	Symbol	Description	Accession No.
B5	ALPI	Intestinal-type alkaline phosphatase	KF857309
C5	FABP1	Liver type fatty acid-binding protein	KF857311
D5	FABP2	Intestinal fatty acid-binding protein	KF857310
E5	FABP6	Ileal fatty acid-binding protein	KF857312
F5	CALR	Calreticulin	KF857313
G5	CANX	Calnexin	KF857314
H5	GR	Glutathione reductase	AJ937873
A6	GST3	Glutathione S-transferase 3	JQ308828
B6	SOD1	Superoxide dismutase [Cu-Zn], cytoplasmatic	JQ308833
C6	IL-1β	Interleukin 1 beta	AJ419178
D6	IL-1R1	Interleukin 1 receptor type 1	JX976615
E6	IL-6	Interleukin 6	EU244588
F6	IL-6RB	Interleukin 6 receptor subunit beta	JX976617
G6	IL-7	Interleukin 7	JX976618
H6	IL-15	Interleukin 15	JX976625
A7	IL-8	Interleukin 8	JX976619
B7	IL-8RA	High affinity interleukin-8 receptor A	JX976620
C7	IL-10	Interleukin 10	JX976621
D7	IL-10RA	Interleukin10 receptor subunit alpha	JX976621
E7	IL-34	Interleukin 34	JX976629
F7	TNF-α	Tumor necrosis factor alpha	AJ413189
G7	CSF1R1	Macrophage colony-stimulating factor 1 receptor 1	AM050293
H7	CXX	C-X-C chemokine	KF857315
A8	CCL25	C-C motif chemokine 25	KF857316
B8	CCR3	C-C chemokine receptor type 3	KF857317
C8	CCR9	C-C chemokine receptor type 9	KF857318
D8	CCR11	C-C chemokine receptor type 11	KF857319
E8	CCL20	C-C chemokine CK8	GU181393
F8	CD48	CD48 antigen	KF857320
G8	CD276	CD276 antigen	KF857321
H8	TLR1	Toll-like receptor 1	KF857322
A9	TLR2	Toll-like receptor 2	KF857323
B9	TLR5	Toll-like receptor 5	KF857324
C9	TLR9	Toll-like receptor 9	AY751797
D9	NOD1	Nucleotide-binding protein oligomerization domain-containing protein 1	KF857325
E9	MRC1	Macrophage mannose receptor 1	KF857326
F9	CD209	CD209 antigen	KF857327
G9	CD302	CD302 antigen	KF857328
H9	CLEC10A	C-type lectin domain family 10 member A	KF857329
A10	LGALS1	Galectin-1	KF862003
B10	LGALS8	Galectin-8	KF862004
C10	CSL2	L-rhamnose-binding lectin CSL2	KF857330
D10	FCL	Fucolectin	KF857331
E10	mtHsp10	Mitochondrial 10 kDa heat shock protein	JX975224

Supplementary Table 1. Continued-II.

Position	Symbol	Description	Accession No.
F10	mtHsp60	Mitochondrial 60 kDa heat shock protein	JX975227
G10	mtHsp70	Mitochondrial 70 kDa heat shock protein	DQ524993
H10	ECH	Enoyl-CoA hydratase	JQ308826
A11	HADH	Hydroxyacyl-CoA dehydrogenase	JQ308829
B11	CS	Citrate synthase	JX975229
C11	Tim44	Mitochondrial import inner membrane translocase subunit 44	JX975239
D11	Tom22	Mitochondrial import receptor subunit Tom22	JX975236
E11	mtTFA	Mitochondrial transcription factor A	JX975262
F11	NRF1	Nuclear respiratory factor 1	JX975263
G11	PGC1α	Proliferator-activated receptor gamma coactivator 1 alpha	JX975264
A12-D12	PPC	Positive PCR control (serial dilutions of standard gene)	AY590304
E12/F12	NPC	Negative PCR control	
G12/H12	ACTB	ß-Actin	X89920

Cell differentiation and proliferation (14): BMP pathway (BMPR1A), Hh pathway (IHH, GLI1, GLIS3), Wnt pathway (HHIP, WLs, Myc, CTNNB1, Tcf4), Notch pathway (NLE1, HES1-B, GFI-1, KLF4), VIM.
Intestinal architecture and permeability (19): ITGB1BP1, ITGB6, ILK, OCLN, CLDN12, CLDN15, TJP1, CDH1, CDH17, F11R, CXADR, DSP, CX32.2, CX32.7, GJB4, MUC2, MUC2-like, MUC13, I-MUC.
Enterocyte mass and epithelia damage (9): ALPI, FABP1, FABP2, FABP6, CALR, CANX, GR, GST3, SOD1.
Interleukines, cytokines and chemokine receptors (21): IL-1β, IL-1R1, IL-6, IL-6RB, IL-7, IL-15, IL-8, IL-8RA, IL-10, IL-10RA, IL-34, TNF-α, CSF1R1, CXC, CCL25, CCR3, CCR9, CR11, CCL20, CD48, CD276.
Pattern recognition receptors (13): TLR1, TLR2, TLR5, TLR9, NOD1, MRC1, CD209, CD302, CLEC10A, LGALS1, LGALS8, CSL2, FCL.
Mitochondria function and biogenesis (11): mtHsp10, mtHsp60, mtHsp70, ECH, HADH, CS, Tim44, Tim22, mtTFA, NRF1, PGC1α.

Supplementary Table 3. Gene expression profile of interior and posterior intestine sections in gilthead sea bream fed CTRL and experimental diets D1 (NE150) and D2 (NE150 + Previda). Values are the mean ± S.E.M. (n = 6). Rows with unlike superscript letters were significantly different ($P < 0.05$; Student-Newman-Keuls). β-actin was used as a housekeeping gene and all data values are referred to the expression level of IL-1β in CTRL fish (arbitrary value of 1).

	Anterior intestine			Posterior intestine			ANOVA
Gene name	CTRL	D1	D2	CTRL	D1	D2	*P*-value
BMPR1A	28.8±1.2^{b}	31.1±2.2^{b}	27.8±3.1^{b}	42.8±3.7^{a}	36.4±5.2^{ab}	32.2±2.1^{b}	0.031
IHH	2.4±0.62^{a}	2.2±0.28^{a}	2.3±0.63^{a}	0.39±0.12^{b}	0.2±0.03^{b}	0.14±0.01^{b}	<0.001
GLI1	5.0±0.43^{ab}	4.5±0.25^{b}	4.0±0.42^{b}	6.2±0.55^{a}	4.5±0.4^{b}	5.1±0.43^{ab}	0.021
GLIS3	0.62±0.15	0.56±0.20	0.56±0.13	0.3±0.16	0.3±0.09	0.23±0.08	0.256
HHIP	16.4±1.6^{b}	17.4±1.5^{b}	18.2±3.0^{b}	43.3±10.5^{a}	37.5±5.2^{ab}	30.3±2.8^{ab}	0.003
WLs	7.7±0.63^{c}	6.1±0.30^{c}	7.1±0.85^{c}	17.3±2.1^{a}	11.3±0.97^{b}	14.9±0.94^{a}	<0.001
Myc	3.3±0.43^{b}	3.1±0.29^{b}	3.1±0.53^{b}	7.0±1.1^{a}	4.4±0.65^{b}	5.4±0.53^{ab}	0.001
CTNNB1	127.4±10.3	107.7±4.8	100.0±3.3	135.8±11.5	111.8±9.7	117.9±9.5	0.075
Tcf4	1.3±0.10^{cd}	1.0±0.09^{d}	1.1±0.21^{d}	3.2±0.28^{a}	1.8±0.23^{bc}	2.3±0.19^{b}	<0.001
NLE1	6.6±0.71^{a}	6.4±0.63^{ab}	6.1±1.0^{ab}	3.7±0.28^{b}	4.1±0.63^{ab}	4.3±0.44^{ab}	0.011
HES1	80.2±10.3	78.1±10.6	64.4±7.7	112.9±13.6	110.8±19.0	88.8±10.4	0.064
GFI-1	0.49±0.05	0.70±0.04	0.59±0.07	0.62±0.08	0.5±0.07	0.6±0.07	0.378
KLF4	20.9±2.0	21.1±1.9	19.7±1.6	19.4±3.9	17.5±4.1	18.0±2.4	0.936
VIM	5.1±0.67^{b}	4.6±0.57^{b}	5.1±1.1^{b}	12.1±0.66^{a}	7.9±0.98^{b}	7.6±1.2^{b}	<0.001
ITGB1BP1	12.4±1.1^{a}	10.6±1.3^{ab}	9.2±1.1^{abc}	7.0±0.37^{bc}	6.3±0.8^{c}	6.9±0.97^{bc}	0.001
ITGB6	45.5±4.1^{b}	50.5±2.7^{b}	53.9±2.9^{b}	69.6±2.9^{a}	77.2±7.9^{a}	75.4±6.1^{a}	<0.001
ILK	51.7±2.4	47.3±1.5	45.9±2.3	58.2±2.2	54.8±7.1	57.0±4.6	0.168
OCLN	81.7±10.0^{b}	79.4±6.6^{b}	92.2±11.8^{b}	221.8±26.2^{a}	185.1±29.3^{a}	218.3±30.8^{a}	<0.001
CLDN12	15.9±2.1^{b}	14.5±1.5^{b}	12.7±1.0^{b}	23.8±1.9^{a}	18.1±1.8^{b}	17.8±1.25^{b}	0.001
CLDN15	598.2±62.7^{c}	540.1±63.3^{c}	588.9±56.5^{c}	1797±182^{a}	1405.0±273	1319.4±139	<0.001
TJP1	7.4±0.85^{c}	6.2±0.45^{c}	6.3±0.53^{c}	14.0±1.2^{a}	9.6±1.7^{b}	9.7±0.89^{b}	<0.001
CDH1	372.7±30.8	381.2±46.6	309.1±39.6	305.6±28.2	288.9±39.6	274.6±31.9	0.231
CDH17	995 ± 77.5^{ab}	1090 ± 102.1^{a}	970.0 ± 74.8^{ab}	548.7±69.6^{c}	722.3±118.	587.1±95.9^{c}	<0.001
F11R	166.1 ± 19.1^{ab}	172 ± 14.3^{ab}	148.4 ± 13.1^{b}	227.1±16.1^{a}	210.3±22.3^{a}	222.4±24.0^{a}	0.021
CXADR	64.1±7.7^{b}	60.2±4.6^{b}	69.1±9.7^{b}	112.6±8.0^{a}	101.8±8.7^{a}	105.7±17.0^{a}	0.001
DSP	160.6±5.4^{bc}	131.7±11.6^{c}	121.7±7.0^{c}	229.6±17.9^{a}	212.2±24.2^{a}	211.2±24.3^{a}	<0.001
CX32.2	1608±163^{b}	1862±193^{b}	1537±146^{b}	1276±212^{a}	1290±238^{a}	1350±98^{a}	0.194
CX32.7	0.96±0.19^{b}	0.81±0.18^{b}	0.89±0.13^{b}	4.0±1.1^{a}	3.4±0.64^{a}	4.9±0.68^{a}	<0.001
GJB4	3.8±0.30^{b}	3.3±0.29^{b}	3.3±0.30^{b}	5.5±0.5^{a}	3.6±0.39^{b}	3.9±0.17^{b}	0.001
MUC2	799±90.1	923±85	838±98	727±212	704±259	611±123	0.801
MUC2-like	691.2±162.5	842.3±132.5	724.3±94.5	634.6±224	608.1±167	519.4±35.2	0.743
MUC13	1214± 171	1217 ± 72	1073 ± 169	1200±225	713.9±43	1036±209	0.334
I-MUC	7.4 ± 2.9^{b}	5.6 ± 0.83^{b}	6.3 ± 1.8^{b}	836.8±161^{a}	976.1±329^{a}	637.1±134^{a}	<0.001

Supplementary Table 3. Continued-II.

	Anterior intestine			Posterior intestine			ANOVA
Gene	CTRL	D1	D2	CTRL	D1	D2	*P*-value
ALPI	1858±170^{a}	2024±302^{a}	845.2±172^{b}	661.1±124.7^{b}	600.9±146.4^{b}	637.1±134.7^{b}	<0.001
FABP1	1698±307	1499±204	1478±205	796.6±492	654.2±365	701.1±272	0.107
FABP2	2873± 597^{b}	4523± 825^{b}	9568 ± 914^{a}	-	-	-	0.001
FABP6	-	-	-	44725±14285	67844±19029	109784±24786	0.091
CALR	692±126^{a}	665.6±78^{a}	512 ± 42^{ab}	379.1±65.1^{b}	330.9±50.8^{b}	305.6±47.0^{b}	0.002
CANX	359.1±31.5^{ab}	364± 27^{ab}	289± 21^{ab}	330±19^{a}	280±39^{ab}	255±22^{b}	0.048
GR	59±12	54±6.6	44.4±3.9	34.5±3.8	36.3±4.2	38.1±5.7	0.073
GST3	704± 79^{a}	745±60^{a}	677±106^{a}	280±45^{b}	305±18^{b}	280±39^{b}	<0.001
SOD1	248.0±32.0	300.9±16.9	302.4±18.5	179.6±37.4	214.5±43.3	211.6±38.0	0.062
IL-1β	1.1±0.28^{a}	0.54±0.07^{b}	0.73±0.06^{b}	1.8±0.52^{a}	1.1±0.19^{ab}	1.2±0.13^{ab}	0.023
IL-1R1	31.4±2.1	32.1±2.2	28.9±1.5	28.1±2.8	28.3±3.7	31.6±4.0	0.854
IL-6	0.84±0.29^{a}	0.19±0.04^{b}	0.33±0.20^{b}	1.28±0.48^{a}	0.15±0.03^{b}	0.33±0.15^{b}	0.019
IL-6R	17.3±3.8	13.3±1.8	12.9±2.0	17.2±1.9	14.8±1.7	16.5±2.7	0.656
IL-7	23.4±2.7^{a}	23.2±2.0^{a}	17.8±3.4^{a}	14.1±2.9^{ab}	9.9±0.97^{b}	9.3±0.79^{b}	<0.001
IL-15	12.2±1.9^{a}	11.2±1.8^{ab}	9.4±0.52^{ab}	8.3±0.35^{b}	7.1±1.1^{b}	7.4±0.94^{b}	0.037
IL-8	11.8± 2.7^{a}	8.8±2.4^{ab}	4.5±0.56^{b}	7.6±2.1^{ab}	7.2±3.7^{ab}	5.1±0.84^{ab}	0.04
IL-8RA	0.90±0.17^{b}	0.64±0.07^{b}	0.81±0.19^{b}	2.2±0.34^{a}	1.5±0.28^{ab}	1.3±0.38^{b}	0.004
IL-10	4.3±1.0	3.4±0.73	4.1±0.80	7.2±0.74	5.5±1.9	6.4±1.4	0.251
IL-10RA	15.6±2.9	10.3±1.0	9.8±0.96	12.2±1.4	10.1±1.3	14.8±3.2	0.200
IL-34	21.6 ± 2.8	18.4 ± 2.4	17.8±1.5	24.1±0.52	19.5±2.9	19.9±2.8	0.443
TNF-α	2.1±0.08 ^{b}	1.9±0.28^{b}	1.7± 0.18^{b}	3.0±0.38^{a}	2.2±0.39^{ab}	1.9±0.09^{b}	0.030
CSF1R1	7.2±1.4^{b}	5.4±0.82^{b}	6.0±1.9^{b}	17.3±1.89^{a}	12.7±1.7^{a}	13.7±1.7^{a}	<0.001
CXC	57.7±10.3^{b}	42.7±8.7^{b}	57.0±13.9^{b}	153.4±17.0^{a}	129.0±16.0^{a}	134.2±13.6^{a}	<0.001
CCL25	583.4±69.3	529.8±60.8	510.1±65.1	420.4±43.6	469.1±80.9	378.3±48.0	0.241
CCR3	8.1±1.57^{ab}	6.8±0.66^{b}	7.1 ± 0.56^{b}	12.1±1.7^{ab}	10.6±2.2^{ab}	11.7±1.1^{a}	0.038
CCR9	21.0±4.0^{b}	18.2±2.1^{b}	19.0 ± 2.5^{b}	31.7±5.7^{ab}	28.6±3.8^{ab}	42.1±6.1^{a}	0.003
CCR11	64.9 ± 11.5	82.4±7.8	75.3±10.0	90.2±10.1	95.7±10.9	102.7±7.0	0.101
CCL20	137.0±38.0	109.1±11.3	81.4±24.2	126.3±24.3	134.9±21.1	115.1±14.3	0.588
CD48	55.6±4.3	49.1±2.4	47.9±3.8	46.4±2.3	42.4±3.26	49.0±5.0	0.253
CD276	13.2±1.2	12.0±1.3	12.3±2.2	16.1±0.7	12.2±2.0	12.6±1.1	0.406
TLR1	11.7±2.0^{ab}	8.1±0.90^{b}	8.3±0.67^{b}	14.3±1.2^{a}	10.3±1.1^{b}	13.6±1.3^{a}	0.006
TLR2	5.2±0.82^{b}	4.6±0.73^{b}	4.8±0.88^{b}	12.6±0.94^{a}	9.9±1.1^{a}	11.8±1.3^{a}	<0.001
TLR5	1.1±0.27^{b}	0.61±0.11^{b}	0.77 ± 0.14^{b}	1.6±0.21^{a}	0.93±0.17^{b}	1.0±0.11^{b}	0.004
TLR9	0.69±0.03^{bc}	0.78±0.12^{bc}	0.5±0.05^{c}	1.1±0.1^{a}	0.65±0.09^{bc}	1.0±0.16^{ab}	0.001
NOD1	19.0±1.2^{b}	16.4±1.6^{b}	16.7±1.2^{b}	36.7±5.7^{a}	27.4±3.5^{ab}	26.2±2.2^{ab}	<0.001
MRC1	17.2± 2.8	13.5±1.1	13.3±1.6	18.0±1.7	13.9±1.8	17.7±1.4	0.207

Supplementary Table 3. Continued-III.

	Anterior intestine			Posterior intestine			ANOVA
Gene	CTRL	D1	D2	CTRL	D1	D2	*P*-value
CD209	3.7±0.33	3.5±0.31	3.7±0.47	3.4±0.5	2.4±0.41	2.4±0.57	0.146
CD302	127.9±10.81	133.4±15.1	123.7±10.6	143.5±4.1	126.5±13.4	138.3±11.9	0.821
CLEC10A	0.77±0.11^{b}	0.52±0.07^{b}	0.61±0.10^{b}	1.7±0.28^{a}	0.94±0.16^{b}	1.0±0.09^{b}	<0.001
LGALS1	123.6±11.2^{b}	116.7±12.0^{b}	141.1±33.9^{b}	301.1±31.6^{a}	206.6±26.7^{b}	279.0±39.3^{a}	<0.001
LGALS8	49.8±4.1^{b}	45.6±5.5^{b}	43.0±3.6^{b}	102.9±10.3^{a}	87.8±15.0^{a}	91.9±10.9^{a}	<0.001
CSL2	117.15±45.1	58.0±21.6	28.2±5.5	183.5±96.1	90.7±35.5	92.2±22.1	0.358
FCL	62±28^{c}	764±193^{b}	217±129^{bc}	381±68^{b}	2334.7±963^{a}	1271±600^{ab}	0.033
mtHsp10	94.1±10.5^{a}	129.1±14.6^{a}	105.5±15.2^{a}	33.9±3.7^{b}	38.2±8.1^{b}	43.5±8.7^{b}	<0.001
mtHsp60	51.6±8.6^{a}	58.0±9.2^{a}	46.8± 11.3^{a}	10.4±1.2^{b}	13.0±4.2^{b}	12.7±2.5^{b}	<0.001
mtHsp70	63.9±9.6^{ab}	74.0±4.1^{a}	71.5±14.5^{a}	34.7±4.2^{b}	34.9±4.2^{b}	37.2±4.5^{b}	0.001
ECH	216.4±27.5^{c}	427.6±43.0^{a}	335.8±43.2^{b}	111.4±21.5^{d}	134.7±18^{d}	143.0±13^{d}	<0.001
HADH	303.0±57.2^{b}	456.6±43.9^{a}	351.5±42.4^{ab}	169.0±19.1^{c}	219.7±18.0^{c}	171.4±16.4^{c}	<0.001
CS	524.7±33.4^{a}	630.0±56.6^{a}	563.3±75.8^{a}	277.4±44.9^{b}	303.4±63.8^{b}	314.9±57.2^{b}	<0.001
Tim44	8.4±0.72^{a}	8.0±0.51^{a}	6.0±0.65^{ab}	6.6±0.85^{ab}	4.8±0.6^{b}	4.5±0.45^{b}	0.001
Tom22	28.6±2.0^{a}	27.2±2.1^{ab}	25.7±1.1^{ab}	22.6±1.3^{b}	21.8±1.5^{b}	21.4±1.6^{b}	0.018
mtTFA	25.2±2.7	23.9±3.3	23.0±1.7	23.0±2.3	21.8±3.2	21.8±3.4	0.956
NRF1	5.2±1.2	5.1±1.1	4.0±0.82	6.9±0.42	5.7±0.68	5.6±0.54	0.268
PGC1α	43.6±4.2	43.8±5.0	40.5±7.0	40.8±4.0	33.2±5.6	35.6±6.2	0.696