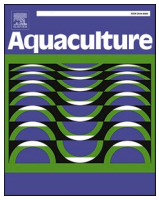
Aquaculture 586 (2024) 740770



Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture





Can the genetic background modulate the effects of feed additives? Answers from gut microbiome and transcriptome interactions in farmed gilthead sea bream (*Sparus aurata*) fed with a mix of phytogenics, organic acids or probiotics

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ARTICLE INFO

Keywords:
Feed additives
Gut microbiota
Host transcriptomics
Phytobiotics
Probiotics
Organic acids

ABSTRACT

The synergies between selective breeding and feed additives remain under-explored in farmed fish, despite their sustainability. Reference (REF) and selected gilthead sea bream for growth (GS) were fed with the control (CTRL) diet during 14 days. CTRL diet was oil-coated with three functional additives (PHY: phytogenic based on garlic and medium chain fatty acid; OA: organic acid mixture with a 70% of butyric acid sodium salt; PROB: probiotic based on *Bacillus subtilis, pumillus* and *licheniformes* species). These experimental diets were then sequentially administered at high (PHY/OA = 7.5 g/kg, PROB = 2×10^{11} CFU/kg; 2 weeks) and low (PHY = 5 g/kg, OA = 3 g/kg, PROB = 4×10^{10} CFU/kg; 10 weeks) additive doses. The capacity of a given genotype and additive to modify the fish growth performance, gut health and the host interaction with its anterior intestine (AI) microbiota was evaluated as a whole population or individually (9 fish/diet/genetics). GS fish showed a better growth and feed conversion ratio, linked to a reduced individual variability of gut microbial composition. The PHY additive had a major impact upon the intestinal transcriptome of GS-PHY fish, with the up-regulation of markers of epithelial integrity, sphingolipid and cholesterol/bile salt metabolism. With the OA additive, impaired growth performance, reduced AI goblet cell area and enhanced AI granulocyte infiltration were concomitant with a down-regulation of neutrophil degranulation markers associated with a decrease of pathogenic genera (*Staphylococcus/Streptococcus/Neisseria*), and an over-representation of acetone/butanol/ethanol fermentation and vitamin K biosynthesis inferred pathways. *Bacillus* establishment and lack of AI inflammation were parallel in PROB fish of both genetic backgrounds. However, GS fish grew and utilized feed better with the additive, whereas a worsening appeared in REF fish. This amelioration was related with a higher abundance of the nitrate-reducer *Kocuria*, an up-regulation of markers of epithelial cell maintenance and proliferation, and a down-regulation of microbiota-correlated protein synthesis and ubiquitination markers, supporting a reduced epithelial turnover and improved intestinal barrier function. Overall, the success of nutritional innovations in gilthead sea bream is largely dependent on the host genome predisposition, but also on the intestinal microbiota according to the hologenome theory.

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https://doi.org/10.1016/j.aquaculture.2024.740770
Received 7 November 2023; Received in revised form 7 February 2024; Accepted 4 March 2024
Available online 5 March 2024
0044-8486/© 2024 Published by Elsevier B.V.

1. Introduction

The sustainable growth of modern aquaculture must rely on the production of healthy and robust fish overcoming the dependence on limited marine feedstuffs resources (Glencross et al., 2023; Hua et al., 2019; Perera et al., 2019; Ytrestøyl et al., 2015). Hence, a number of studies gather the benefits of alternative and locally sourced feedstuffs according to the principles of circular economy (Aragão et al., 2022; Chakraborty et al., 2019; Eroldoğan et al., 2022; Hodar et al., 2020). However, the utilization of new fish feed formulations often discloses drawback effects on fish health and welfare (Montero et al., 2010; Estensoro et al., 2016; Piazzon et al., 2017), fish quality, as well as a reduced fillet content in long-chain n-3 PUFAs (Benedito-Palos et al., 2008; Izquierdo et al., 2005). Some of these negative effects can be reversed, at least in part, with the use of feed additives, which are commercially available after authorisation and scientific evaluation demonstrating that the additive has no harmful effects on human and animal health, nor on the environment (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2022). The nature and characteristics of feed additives is quite diverse, but according to their specific application they are classified as technological (*e.g.*, food preservatives), sensory (*e.g.*, flavourings and colourings), nutritional (*e.g.*, vitamins and minerals), and zootechnical (*e.g.* enzymes and micro-organisms used to favourably affect the performance of animals in good health) (Bampidis et al., 2021). This last category encompasses the most heterogeneous group of compounds, including, among others, feeding attractants, prebiotics, probiotics, acidifiers and essential oils (Encarnação, 2016; Marimuthu et al., 2022), which have been used for an overall improvement of growth (Balbuena-Pecino et al., 2022; Jang et al., 2023; Ng and Koh, 2017; Ruiz et al., 2023a, 2023b; Simó-Mirabet et al., 2017), nutrient digestion and absorption (Liang et al., 2022; Murashita et al., 2021; Stejskal et al., 2023), as well as antibacterial, antifungal, and anti-inflammatory immune responses (Abd-elaziz et al., 2023; Cabello-Gómez et al., 2022; Firmino et al., 2021a; Rimoldi et al., 2020; Salomón et al., 2022, Valenzuela-Gutiérrez et al., 2021), thus reducing the need for the use of antibiotics (Bharati et al., 2019; Dawood et al., 2018; Hernandez Dios et al., 2022; López-Pedrouso et al., 2020; Ramos-Pinto et al., 2019). In this regard, the gut is becoming a main target tissue of feed additives (Butt and Volkoff, 2019), and phytobiotics, organic acids and probiotics are able to modulate the microbial intestinal population contributing to improve fish disease resistance in different infective models (Abdel-latif and Khalil, 2014; El-Saadony et al., 2021; Hoseinifar et al., 2019; Lückstädt, 2008; Vazirzadeh et al., 2020). Regarding transcriptional responses, zootechnical compounds also fuel the expression and activity of digestive enzymes (Castillo et al., 2014), protective antioxidant responses (Reverter et al., 2021), and markers involved in different essential biological processes, such as immune response and lipid or vitamin metabolism (Firmino et al., 2021b; Moroni et al., 2021; Núñez-Acuña et al., 2015), which contribute to balance the cost-benefit of animal production systems with a reduced carbon footprint.

Substantial progress on the sustainability of aquaculture production systems has also been achieved by selective breeding for different productive traits (*e.g.* growth, feed conversion, fillet yield, disease resistance) (Boudry et al., 2021; Calduch-Giner et al., 2023; Kause et al., 2022; Song et al., 2023; Vandeputte et al., 2022) that co-select for a number of physiological attributes with an impact on gut microbiome and host transcriptome (Ferrocino et al., 2022; Hoseinifar et al., 2019; Lin et al., 2021; Nichols and Davenport, 2021). Indeed, gilthead sea bream selected for accelerated growth within a Spanish National Breeding Program, PROGENSA® selection program (García-Celdrán et al., 2015; Lorenzo-Felipe et al., 2021) displayed a more plastic microbiota (Piazzon et al., 2020; Naya-Català et al., 2021) in association with an easily changing intestinal and hepatic transcriptome for dealing with changes in diet composition (Naya-Català et al., 2022a; Naya-Català et al., 2023). There is also experimental evidence linking selective breeding with the effectiveness of feed additives in aquaculture practice (Landsman et al., 2019; Rimoldi et al., 2023), though we are still far from establishing a specific mode of action for a given additive, farmed fish species and genetic background. Hence, the aim of this study is to contribute to cover this gap of knowledge with focus on a representative set of feed additives, including a phytobiotic (PHY), a mix of organic acids (OA) and a *Bacillus*-based probiotic (PROB), for their testing in a farmed fish model, using reference (REF) fish and fish genetically selected (GS) for improved growth performance. Such approach paid special attention on fish performance, intestinal morphology and the association of intestinal microbiota (metataxonomics) and wide-transcriptomics (RNA-seq) to discern additive-driven specific pathways and mode of action upon gut health and function in a context of different fish growth potentiality.

2. Materials and methods

2.1. Ethics statement

Fish manipulation and tissue collection were carried out according to the Spanish (Royal Decree RD53/2013) and the current EU (2010/63/EU) legislations on the handling of experimental fish. The Bioethical Committee of the University of las Palmas de Gran Canaria approved all the protocols used in the present study (approval no. OE-BA_ULPGC_10/2020).

2.2. Broodstock crosses

A population of 6122 adult fish from the Canary Islands at the 3rd generation of National Breeding Program (PROGENSA®) (García-Celdrán et al., 2015; Perera et al., 2019) were evaluated for growth. The estimated breeding values (EBV, expressed as g of whole body) ranged between −159.14 for reference (REF) and + 223.18 for selected fish for growth (GS) with an average value of 8.59 and a standard deviation value of 52.84. A subset of 196 fish (98 fish per broodstock) was then selected as breeders with values for the EBV varying from −25.95 in the group of REF fish to +39.68 in the group of GS fish, comprising almost the 47% of the evaluated population.

2.3. Diets and feed additives

Control and experimental diets were formulated and delivered by Skretting Aquaculture Research Centre (Stavanger, Norway). The control diet (CTRL, without feed additive supplementation) was a low fish meal diet with a high inclusion level of vegetable proteins and poultry meal, completely devoid of fish oil (FO) with the use of poultry oil (2.1%) and DHA-rich algae oil (2.5%) as alternative dietary oils. The proximate composition of the feed (Table 1) and its nutritional values (Supplementary Table 1) were analysed according to standard procedures (AOAC, 2000). Ash content was determined by combustion in a muffle furnace (600 °C, 12 h) and moisture content was determined after drying in an oven (110 °C) to constant weight. The crude lipids were extracted as described by Folch et al. (1957) and crude protein content (Nx6.25) by following the Kjeldahl method. Fatty acid methyl esters were obtained by transmethylation as described by Christie (1998) and separated by gas chromatography (GC-14 A, Shimadzu, Japan). A GC Supercolovax-10-fused silica capillary column (Supelco, Bellefonte, USA) was used for the separation applying the conditions described by Izquierdo et al. (1992). Fatty acid methyl esters were quantified by flame ionizator detector and identified by comparing them with external and well-characterized fish oils standards (EPA 28, Nippai, Ltd. Tokyo, Japan).

Three proprietary experimental feed additives, including a phytobiotic (PHY), a mixture of organic acids (OA), and a probiotic (PROB) were provided by INVE (Dendermonde, Belgium) and added to the CTRL diet by oil-coating. Oil is the preferential option for performing this

Table 1
Ingredients and chemical composition of the CTRL basal experimental diet. Ingredients are expressed in as-fed (%) basis.

Ingredients	(%)
Corn gluten	5
Hi Pro Soy bean meal	5.08
Wheat gluten	14.44
Faba bean dehulled	8
Soy protein concentrate	17
Fish oil	0
Fish meal	7.5
Rapeseed oil	6.52
Phosphate	0.44
Vitamin and mineral premix*	0.3
Wheat	19.09
Poultry meal	10
Poultry oil	2.1
DHA-rich Algae oil	2.53
Lecithin	2
Proximal composition	
Crude Protein	48.6
Crude Lipid	15.9
Crude Ash	4.6
EPA + DHA	9.79

* Premix and vitamin composition are according fish requirements from NRC (2011).

coating, as this typically leads to a reduction of leaching in the water during feeding (and thus less leaching), and also allows for a longer in-cold storage of the coated pellets. More in detail, PHY booster contained as active ingredient for 16% (*w*/w) natural extracts from herbaceous plants of the Alliaceae family (mainly garlic) in combination with medium chain fatty acid sources (Aquagarlic P Protec, Domca, Spain), in addition to product fillers. The OA supplement consisted for 70% of butyric acid sodium salt (GBM CMR, Sanluc, Belgium) as the active ingredient. PROB was an experimental probiotic with as active ingredient a mixture three *Bacillus* species (*B. subtilis*, *B. licheniformis*, and *B. pumilus*) at a total bacterial concentration of 2×10^{10} CFU/g of product, with each of the species in an equal ratio.

2.4. Experimental setup and sample collection

The progeny of either GS and REF fish were grown up until 16 g. Fish were then fed to visual satiety with the CTRL diet during two weeks. After that, fish were distributed in triplicate 500 L tanks (34 fish/tank; 102 fish per diet) and CTRL diet was oil-coated with the three functional additives. The different supplemented diets were used with a high additive dose (HD; PHY and OA = 7.5 g/kg, PROB = 2×10^{11} CFU/kg) during 2 weeks, decreasing thereafter to a low additive dose (LD; PHY = 5 g/kg, OA = 3 g/kg, PROB = 4×10^{10} CFU/kg) until the end of the trial (10 extra weeks) (Fig. 1). Such procedure was based on previous INVE experience with these additives at the farm level. The water was provided by a flow-through system, tanks were provided with aeration, and temperature and dissolved oxygen was recorded in continuous, with a natural photoperiod (12 L:12D). The temperature ranged from19.1 to 20.3 °C and the dissolved oxygen from 6.1 to 6.8 ppm. The renovation rate was 1 tank per hour, to keep tanks clean in terms of nitrogenous compounds and floating particles. Once per week, cleaning of the tanks was carried out. Fish were fed at apparent satiety, three times per day. At the end of the trial and following overnight fasting, fish were euthanized by immersion in an overdose of 5 mL of natural clove oil /L (Guinama S. L; Spain, Ref. Mg83168).

Several transverse sections of anterior intestine (AI; immediately after the pyloric caeca) and rectum (PI; rectum section, immediately after the ileorectal valve) (72 fish, 9 fish per diet and genetics) were taken and fixed with 4% paraformaldehyde at 4 °C for subsequent morphological analyses. An additional section (~0.4 cm; 72 fish, 9 fish per diet and genetics) of the AI was excised, submerged in RNAlater and stored at −80 °C until RNA extraction. The remaining AI of the same fish was opened, washed with sterile phosphate-buffered saline (PBS) and transferred to a Petri dish, where the internal mucus was scraped with the blunt end of a sterile scalpel for DNA extraction immediately after sampling.

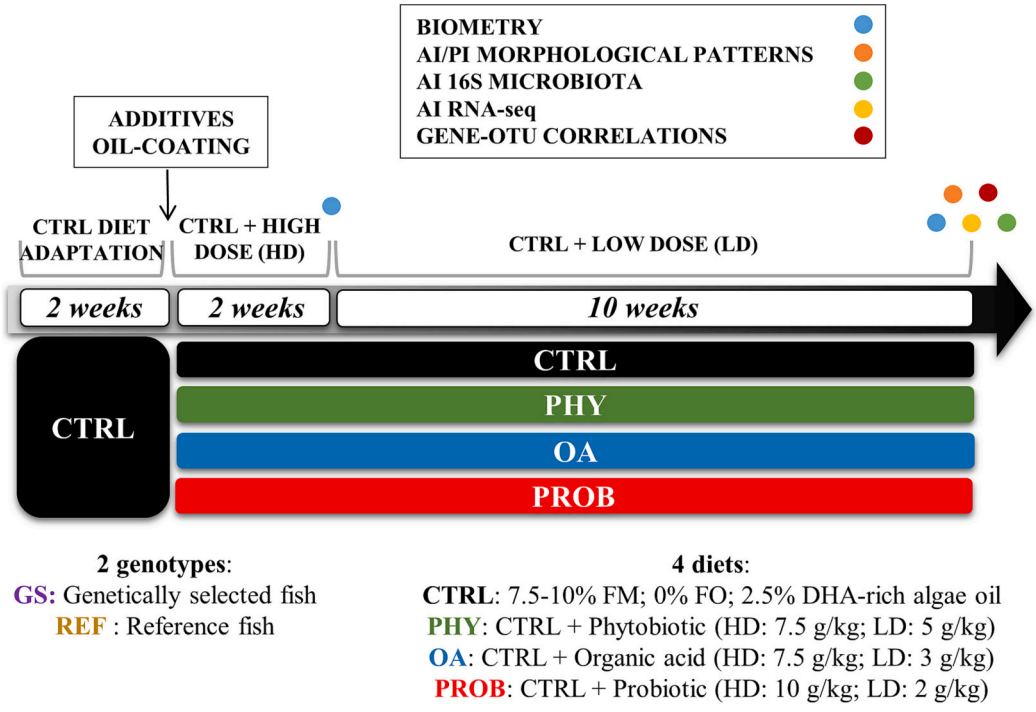


Fig. 1. Experimental design and timing of the gilthead sea bream feeding trial with the basal diet and the functional additives, as well as the type of data recorded at the end-sampling point. AI: anterior intestine, PI: posterior intestine. Diets: CTRL (control diet), PHY (phytogenic), OA (organic acid), PROB (probiotic).

2.5. Histological evaluation

After 48–72 h in 4% paraformaldehyde at 4 °C, intestinal samples were dehydrated and embedded in paraffin. The regions sampled for histological evaluation were anterior intestine (AI; 1.5 cm immediately after the pyloric caeca) and posterior intestine (PI; 1 cm sections after the ileorectal valve). Sections of 4 μm-thickness of both regions were stained with Alcian Blue (pH = 2.5) in order to differentiate goblet cell secreting acid mucins, and with May-Grünwald/Giemsa (MGG) to study the distribution of leukocyte populations (Martoja and Martoja-Pierson, 1970). Slides were scanned using an Olympus VS120 digital scanner (Optic system BX61VS, Tokyo, Japan) equipped with VC50 and *VS*-XM10 camera and images were acquired using Olympus *VS* software (*VS*-NIS-SQL-V2.6, Tokyo, Japan). Digitalized images of intestinal transverse sections (Alcian Blue pH = 2.5) were used to determine the intestinal goblet cells area (μm^2) for each intestinal region evaluated using CellSens Dimension Desktop 1.16 (Olympus Iberia, Spain), as previously described for a similar fish species (Torrecillas et al., 2019). Three ranges of goblet cell area were defined (30–100 μm^2; 100–200 μm^2; 200–500 μm^2), as well as the percentage of cells detected within each range with respect to the total number of goblet cells detected. The general intestinal inflammatory pattern was evaluated for the mucosa, *lamina propria* and submucosa of the anterior and posterior intestines, based on the width, leukocyte infiltration and/or connective tissue hyperplasia as described for *Salmo salar* by Penn et al., 2011, and based on a previously stablished scale (ND: not detected, 1: low, 2: moderate and 3: high for each area/cell evaluated) (Torrecillas et al., 2023). The abundance of infiltrated leukocytes subpopulations (lymphocytes and granulocytes) was evaluated based on a similar scale (Torrecillas et al., 2023).

2.6. DNA/RNA extraction

The extraction of mucosal DNA for 16S rRNA microbiota analysis was performed using the High Pure PCR Template Preparation Kit (Sigma-Aldrich, St. Louis, MO, United States), with an initial lysozyme lysis step (Piazzon et al., 2019). Total RNA from the AI of sampled fish was extracted with the MagMAXTM-96 Total RNA Isolation Kit (Applied Biosystems, Foster City, CA, USA). The DNA and RNA concentration and purity were determined using a Nanodrop 2000c (Thermo Scientific, Wilmington, DE, USA). Quality and integrity of the isolated RNA was checked on an Agilent Bioanalyzer 2100 total RNA Nano series II chip (Agilent, Amstelveen, The Netherlands), yielding RNA integrity numbers (RINs) between 8 and 10. Extracted DNA and RNA were stored at −80 °C until sequencing.

2.7. Sequencing and bioinformatics protocols

Up to 72 samples (9 per diet and genetic condition) were processed for microbiota analysis. The hypervariable V3-V4 region of the 16S rRNA gene (341–805 nucleotide region) was amplified and sequenced using the Illumina MiSeq System (Illumina, San Diego, CA, USA) under a 2 × 300 paired-end (PE) read format at the Genomics Unit from the Madrid Science Park Foundation (FPCM). Details of PCR amplification and amplicon sequencing were described elsewhere (Piazzon et al., 2019). Bioinformatic analyses included a quality assessment with FastQC and Prinseq (Schmieder and Edwards, 2011), a 97% identity clustering, and a stringent (≥90% sequence identity, ≥90% query coverage) taxonomic assignment using VSEARCH and BLAST (Altschul et al., 1990; Rognes et al., 2016) against the Ribosomal Database Project (RDP) release 18 (Cole et al., 2014).

For the wide-transcriptomic analysis, up to 72 RNA-seq libraries were sequenced on an Illumina NOVASEQ 6000 platform as 2 × 150 nucleotides PE read format according to the manufacturer's protocol at the NOVOGENE company (Cambridge, United Kingdom). Quality analysis was performed with FastQC, and libraries were filtered with Trimmomatic v0.40 (Bolger et al., 2014), removing reads with adaptor contamination, >10% of Ns in the sequence, and with a quality <20 in 50% of the bases. Then, libraries were mapped against the CSIC gilthead sea bream draft genome as reference library (Pérez-Sánchez et al., 2019) using HISAT2 v2.0.5 (Kim et al., 2019). Unique transcripts hit counts were calculated by FeatureCounts v1.5.0-p3 (Liao et al., 2019). Raw sequencing data files were uploaded to the Sequence Read Archive (SRA) under Bioproject accession number PRJNA901860 (BioSample DNA accession numbers: SAMN31727064–135; Biosample RNA accession numbers: SAMN31727136–207).

2.8. Statistics

Biometry and morphological analyses were performed using the SPSS Statistical Software System (SPSS, Chicago, IL, USA) and Prism (GraphPad Software Inc., CA, USA). Normality of data was verified by Shapiro-Wilk test. Differences in biometrical data and goblet cells (GCs) area among groups were analysed by two-way ANOVA, with diet and genetic background stablished as fixed factors. Within genetic groups, differences in diet were analysed by One-way ANOVA (Tukey's post-hoc test; $p < 0.05$). Differences in the level of inflammation, eosinophilic granular cells (EGCs), lymphocytes and rodlet cells infiltration were analysed by Two-way ANOVA ($p < 0.05$).

Microbial rarefaction curves, coverage ratio, species richness, and alpha diversity indexes were obtained using the R package *phyloseq* (McMurdie and Holmes, 2013). Differences in richness, alpha diversity indexes, and phylum abundance were determined by Kruskal-Wallis test (Dunn's post-test; $p < 0.05$). Differences in beta diversity across groups were tested by PERMANOVA analysis (10,000 random permutations; $p < 0.05$) (Dixon, 2003). For RNA-seq analyses, differential expressed (DE) transcripts were retrieved using DESeq2 ($p < 0.05$) (Love et al., 2014). A partial least-squares discriminant analysis (PLS-DA) using EZinfo v3.0 (Umetrics, Umeå, Sweden) and a subsequent sample clustering were used to study the separation of the microbial and transcriptomic data. Outliers were reported at a Hotelling T^2 distance >0.99. The model fitness (R2Y) and prediction (Q2) ability was assessed by a validation test (500 random permutations; pR2Y < 0.05; pQ2 < 0.05) using the *ropls* R package (Thévenot et al., 2015). The dispersion of the multidimensional PLS-DA points was measured by means of the standard distance deviation (SDD; a radius for plotting a circle around the data centre) and standard deviational ellipse (SDE; measure of the heterogeneity of the spatial distribution of data) implemented in the *aspace* R package (Buliung and Remmel, 2008; Kołacz and Grzegorzewski, 2016). Finally, we selected the minimum variable importance in the projection (VIP) value of each model achieving the complete clustering of the conditions (Wold et al., 2001; Segata et al., 2011). OTUs and genes overcoming a VIP ≥ 1 for microbiota data and a VIP ≥ 1–1.4 for RNA-seq data were determined to be mainly driving the group separation and used in subsequent analyses.

Picrust2 (Douglas et al., 2020) was used to normalize the amplicon data with 16S rRNA gene copy number and to infer metagenomic contents (FDR < 0.05). Correlation between microbiota abundances (from all of the obtained taxa) and transcript expression values (from discriminant VIP ≥ 1–1.4 transcripts) were calculated using a Spearman correlation analysis (Weiss et al., 2016) and considered as significant at a $p < 0.001$. After this procedure, two lists of genes (correlated and not correlated with microbiota) were created and two independent GO-BP over-representation (FDR < 0.05) tests were performed using the *goseq* R package (Young et al., 2010). GO-BP hierarchies and supra-categories were retrieved using GOATOOLS (Klopfenstein et al., 2018). Genes allocated to each supra-category were located in the list of VIPs (1–1.4) of the three additive-specific PLS-DA and if a minimum of 50% of genes came exclusively from one specific VIPs list, then the supra-category was considered as additive-specific. Statistically significant (FDR < 0.05, Confidence Score > 0.7) protein-protein associations were retrieved with The Search Tool for the Retrieval of Interacting Genes (STRING)

database (Szklarczyk et al., 2019). All the network visualizations were performed with Cytoscape v2.8 (Smoot et al., 2011).

3. Results

3.1. Growth performance

Data on growth performance, considering two main periods along the experiment are shown in Table 2. Within the first growth period (first 4 weeks of the trial; after 2 weeks of CTRL diet acclimation and 2 weeks of additive HD administration; 34 days in total), neither the diet or the genetic background induced significant differences (Two-way ANOVA; $p > 0.05$) between groups in any of the measured parameters. At the end of the experimental period (after 10 weeks of additive LD administration; 63 days in total), no significant differences were found in the fish survival rate, and the diet did not exert a significant effect on any of the measured parameters. However, GS fish had statistically significant (Two-way ANOVA; $p < 0.05$) higher values of body weight,

Table 2
Effects of dietary treatment on growth and feeding performance of selected for growth (GS) and reference (REF) gilthead sea bream juveniles fed to visual satiety during the experimental period (97 days) with CTRL, OA, PHY and PROB diets. The p-values of the columns Diet (D), Genotype (G) and Diet x Genotype (D x G) are the result of two-way ANOVA. Different superscript letters within a genetics group indicate significant differences between diets (One-way ANOVA, Tukey's post-test, $p < 0.05$).

	REF				GS				Two-way ANOVA		
	CTRL	PROB	OA	PHY	CTRL	PROB	OA	PHY	D	G	D x G
Period 1 (First 4 weeks of the trial; after 2 weeks of CTRL diet acclimation and 2 weeks of additive HD administration; day 0 - day 34)											
Survival (%)	94.12 ± 7.78	99.02 ± 1.70	99.02 ± 1.70	99.02 ± 1.70	95.10 ± 6.12	100.00 ± 0.00	99.02 ± 1.70	99.02 ± 1.70	0.121	0.753	0.991
Initial total biomass (g)	566.4 ± 9.0	551.4 ± 10.6	555.2 ± 6.2	564.8 ± 5.6	559.8 ± 6.9	570.7 ± 12.9	572.1 ± 17.5	571.2 ± 3.3	0.538	0.175	0.798
Final total biomass (g)	1188.4 ± 131.7	1204.7 ± 62.8	1218.7 ± 16.7	1215.3 ± 61.9	1188.0 ± 140.8	1294.8 ± 37.1	1256.1 ± 65.8	1273.1 ± 11.1	0.541	0.174	0.808
Initial Body Weight (g)	16.66 ± 1.77	16.22 ± 1.81	16.33 ± 1.82	16.61 ± 1.81	16.46 ± 2.00	16.79 ± 1.94	16.83 ± 2.05	16.80 ± 2.07	0.69	0.08	0.137
Final Body Weight (g)	37.14 ± 4.23	35.78 ± 4.39	36.20 ± 3.93	36.10 ± 4.08	36.74 ± 4.67	37.35 ± 4.41	37.31 ± 4.42	37.81 ± 4.86	0.965	0.09	0.521
Final total Length (mm)	13.24 ± 0.58	12.99 ± 0.63	13.02 ± 0.57	13.07 ± 0.56	13.13 ± 0.51	13.18 ± 0.52	13.13 ± 0.57	13.09 ± 0.64	0.527	0.189	0.53
Weight gain (g/fish)	20.43 ± 0.96	19.55 ± 1.25	19.87 ± 0.12	19.47 ± 1.35	20.19 ± 1.96	20.57 ± 1.16	20.48 ± 1.26	21.03 ± 0.85	0.986	0.156	0.637
Relative Weight gain (%)	122.56 ± 3.76	120.55 ± 7.75	121.69 ± 1.75	117.24 ± 8.97	122.62 ± 11.40	122.43 ± 4.21	121.66 ± 4.87	125.15 ± 4.56	0.985	0.376	0.701
Specific Growth Rate	2.35 ± 0.05	2.32 ± 0.10	2.34 ± 0.02	2.28 ± 0.12	2.35 ± 0.15	2.35 ± 0.06	2.34 ± 0.07	2.39 ± 0.06	0.985	0.378	0.686
Daily growth index	2.29 ± 0.07	2.24 ± 0.11	2.27 ± 0.02	2.21 ± 0.13	2.29 ± 0.17	2.30 ± 0.08	2.29 ± 0.09	2.34 ± 0.07	0.99	0.256	0.685
Feed efficiency	0.96 ± 0.05	0.92 ± 0.08	0.94 ± 0.02	0.93 ± 0.01	0.97 ± 0.12	0.97 ± 0.07	0.98 ± 0.03	1.03 ± 0.06	0.735	0.067	0.664
Feed Conversion Ratio	1.04 ± 0.05	1.09 ± 0.09	1.07 ± 0.02	1.07 ± 0.01	1.03 ± 0.13	1.04 ± 0.08	1.02 ± 0.03	0.97 ± 0.06	0.738	0.085	0.654
Thermal Growth Rate (‰)	0.96 ± 0.03	0.94 ± 0.05	0.95 ± 0.01	0.93 ± 0.05	0.96 ± 0.07	0.96 ± 0.04	0.96 ± 0.03	0.98 ± 0.03	0.416	0.313	0.411
Fulton's Condition Factor	1.60 ± 0.04	1.63 ± 0.05	1.69 ± 0.1	1.67 ± 0.1	1.62 ± 0.04	1.63 ± 0.07	1.65 ± 0.01	1.69 ± 0.02	0.293	0.716	0.142
Period 2 (After 10 weeks of additive LD administration; day 34 - day 97)											
Survival (%)	98.41 ± 2.75	97.38 ± 2.27	96.15 ± 3.85	98.72 ± 2.22	95.45 ± 7.88	100.00 ± 0.00	100.00 ± 0.00	97.43 ± 2.22	0.998	0.703	0.235
Initial total biomass (g)	893.17 ± 113.43	912.03 ± 63.60	928.23 ± 24.77	927.90 ± 48.20	900.23 ± 128.65	983.53 ± 32.20	957.60 ± 56.78	978.23 ± 11.64	0.516	0.194	0.878
Final total biomass (g)	1866.00 ± 256.59	1839.33 ± 74.38	1907.33 ± 135.03	1948.00 ± 192.50	1978.00 ± 358.42	2327.33 ± 83.34	2146.67 ± 63.63	2134.00 ± 26.23	0.489	**0.003**	0.343
Initial Body Weight (g)	37.14 ± 4.23	35.78 ± 4.39	36.20 ± 3.93	36.10 ± 4.08	36.74 ± 4.67	37.35 ± 4.41	37.31 ± 4.42	37.81 ± 4.86	0.701	0.095	0.580
Final Body Weight (g)	78.71 ± 10.58^{a}	73.13 ± 10.57^{b}	77.32 ± 11.52^{ab}	76.89 ± 10.57^{ab}	84.77 ± 12.67	87.28 ± 11.45	83.64 ± 11.17	85.36 ± 12.08	0.626	**<0.001**	**0.006**
Final total Length (mm)	17.25 ± 0.79^{a}	16.75 ± 0.78^{b}	16.91 ± 0.66^{b}	17.01 ± 0.72^{ab}	17.50 ± 0.76	17.61 ± 0.73	17.32 ± 0.73	17.46 ± 0.72	0.243	**<0.001**	**0.003**
Weight gain (g/fish)	41.67 ± 0.54	37.64 ± 1.72	41.12 ± 4.07	40.69 ± 5.46	47.71 ± 1.28	50.35 ± 2.02	46.34 ± 1.25	47.24 ± 1.97	0.938	**<0.001**	0.115
Relative Weight gain (%)	112.13 ± 3.37	106.29 ± 9.49	113.70 ± 11.38	112.48 ± 13.41	129.79 ± 9.92	136.68 ± 10.16	124.42 ± 6.55	124.04 ± 8.17	0.924	**<0.001**	0.288
Specific Growth Rate	1.20 ± 0.03	1.15 ± 0.07	1.20 ± 0.08	1.19 ± 0.10	1.32 ± 0.07	1.37 ± 0.07	1.28 ± 0.05	1.28 ± 0.06	0.944	**<0.001**	0.307
Daily growth index	1.51 ± 0.03	1.42 ± 0.08	1.51 ± 0.12	1.50 ± 0.15	1.69 ± 0.07	1.76 ± 0.08	1.64 ± 0.05	1.65 ± 0.07	0.961	**<0.001**	0.201
Feed efficiency	0.65 ± 0.04	0.61 ± 0.02	0.64 ± 0.09	0.66 ± 0.11	0.74 ± 0.03^{b}	0.84 ± 0.04^{a}	0.73 ± 0.02^{b}	0.74 ± 0.05^{b}	0.738	**<0.001**	0.104
Feed Conversion Ratio	1.53 ± 0.09	1.64 ± 0.05	1.57 ± 0.20	1.53 ± 0.26	1.35 ± 0.05^{a}	1.19 ± 0.06^{b}	1.36 ± 0.04^{a}	1.35 ± 0.08^{a}	0.939	**<0.001**	0.230
Thermal Growth Rate (‰)	0.64 ± 0.01	0.60 ± 0.03	0.64 ± 0.05	0.63 ± 0.07	0.71 ± 0.03	0.74 ± 0.03	0.69 ± 0.02	0.70 ± 0.03	0.921	**<0.001**	0.224
Fulton's Condition Factor	1.54 ± 0.01	1.56 ± 0.02	1.60 ± 0.08	1.56 ± 0.04	1.58 ± 0.01	1.60 ± 0.01	1.61 ± 0.01	1.60 ± 0.02	0.538	0.076	0.629

furcal and total length, weight gain and relative weight gain, specific growth rate, daily growth index, feed efficiency and thermal growth rate, as well as lower values of feed conversion ratio than REF fish. In REF fish, the PROB-fed group showed lower final body weight (73.13 g) and total length (16.75 cm) than CTRL fish (78.71 g; 17.25 cm). In GS fish, the PROB-fed fish showed a higher feed efficiency (0.84) and lower feed conversion ratio (1.19) than the other experimental groups (0.73–0.74; 1.35–1.36).

3.2. Gut morphology

Morphological evaluation of MGG-stained sections of gilthead sea bream AI and PI segments showed an intact intestinal epithelial barrier, a general well-organized folding pattern and a lack of cell debris in all examined fish. The display of goblet cell along the folds in AI and PI was the typical of this fish species, presenting a higher density in the upper area (mid and apical fold region) rather than in the basal area. The total area of goblet cells in the AI (109.10 ± 12.25 μm^2) was larger ($p < 0.0001$) than in the PI (87.91 ± 11.21 μm^2) (Table 3). Fish genotype did not affect the total area of AI and PI goblet cells ($p > 0.05$), either when compared by area range (30–100 μm^2; 100–200 μm^2; 200–500 μm^2) or by cell % distribution within each area range (Table 3). In contrast, the morphometric characteristics of goblet cells in the AI varied depending on the dietary treatment fed. Fish fed the PROB diet had smaller ($p < 0.05$) goblet cells than fish fed the CTRL diet (Table 3), mainly due to: (i) a reduction on the mean area of goblet cells ranging between 100 and 200 μm^2; (ii) a higher presence (%) of cells ranging from 30 to 100 μm^2 (Fig. 2a *vs* Fig. 2b), and (iii) a lower presence (%) of cells ranging from 200 to 500 μm^2 (Fig. 2c *vs* Fig. 2d). For the PI, no significant effect of the dietary treatment or fish genotype was detected, but a significant ($p < 0.05$) interaction between diet and genotype was found for 200–500 μm^2 area range (Table 3).

As with the goblet cells pattern, the level of inflammation or the density/distribution of granulocytes and lymphocytes in AI and PI (mucosa, *lamina propia* and submucosa) was not affected by the fish genotype (Table S2). In contrast, the density of AI submucosa granulocytes was higher in fish fed PHY and OA based diets than in those fed the CTRL and PROB diets (Fig. 2e-f; Table S2). Similarly, the PI of fish fed PHY and OA diets had increased *lamina propria* and submucosa general inflammation pattern as well as the density of eosinophilic granule cells than fish fed the PROB and CTRL diets (Fig. 2g-h; Table S2). Several lymphocytic foci were observed in the mucosa and *lamina propria* of both intestinal regions, however its incidence was similar in both fish genotypes and all diet groups (Fig. 2h).

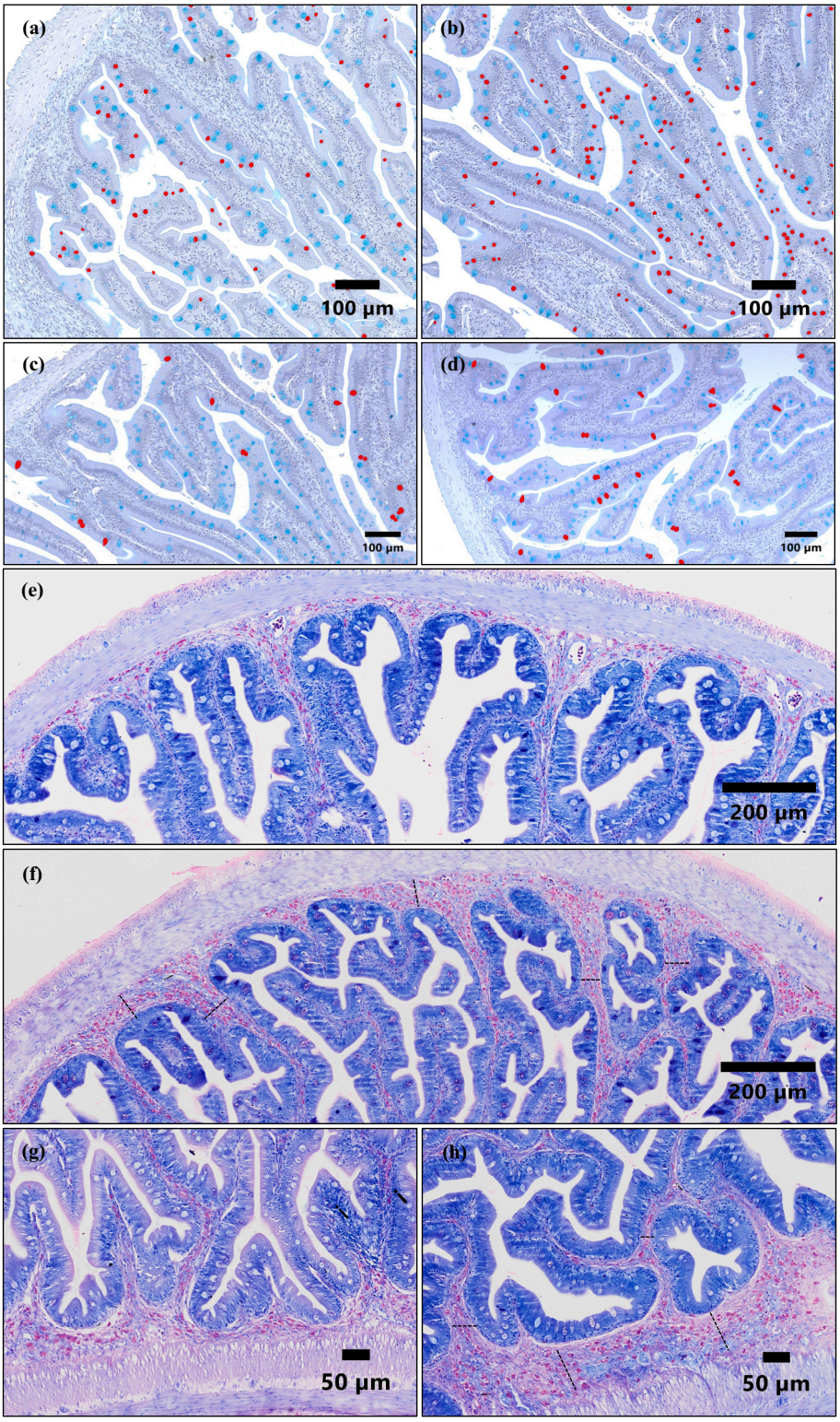
Table 3
Goblet cells area in the anterior (AI) and posterior (PI) intestine of reference (REF) and selected for growth (GS) gilthead sea bream juveniles fed the experimental diets (CTRL, PHY, OA, PROB).

	GS				REF				Two-way ANOVA (p)		
	CTRL	PHY	OA	PROB	CTRL	PHY	OA	PROB	Gen.	Diet	Diet x Gen.
AI											
Total cell area (μm^2)	117.36 ± 15.95	112.83 ± 9.43	121.59 ± 10.81	99.88 ± 9.14	127.18 ± 6.72	118.30 ± 6.45	99.30 ± 5.95	102.94 ± 3.98	n.s.	**0.01**	**0.03**
Range 30–100 μm^2											
Cell area (μm^2)	61.39 ± 1.92	61.53 ± 0.86	61.53 ± 0.81	60.10 ± 0.54	61.35 ± 0.85	61.65 ± 0.60	61.19 ± 2.00	62.91 ± 1.18	n.s.	n.s.	n.s.
Cell %	48.55 ± 8.88	51.47 ± 3.98	47.39 ± 6.59	60.15 ± 4.35	45.12 ± 3.64	48.05 ± 2.32	58.79 ± 5.07	56.16 ± 2.28	n.s.	**0.009**	**0.048**
Range 100–200 μm^2											
Cell area (μm^2)	142.45 ± 3.09	141.80 ± 1.85	143.35 ± 2.37	138.68 ± 2.88	144.34 ± 2.51	143.17 ± 1.25	136.66 ± 2.18	137.60 ± 1.45	n.s.	**0.004**	**0.02**
Cell %	39.42 ± 3.70	37.44 ± 2.91	37.41 ± 3.38	33.26 ± 1.86	38.18 ± 1.45	38.72 ± 0.76	34.45 ± 4.74	37.30 ± 2.38	n.s.	n.s.	n.s.
Range 200–500 μm^2											
Cell area (μm^2)	241.60 ± 10.77	235.37 ± 2.73	236.26 ± 3.67	235.17 ± 5.41	244.69 ± 10.35	236.49 ± 1.06	231.89 ± 0.45	236.20 ± 7.76	n.s.	n.s.	n.s.
Cell %	12.02 ± 5.51	11.08 ± 5.02	15.19 ± 6.48	6.45 ± 2.48	16.69 ± 3.09	13.21 ± 2.49	6.75 ± 0.69	6.54 ± 0.17	n.s.	**0.02**	n.s.
PI											
Total cell area (μm^2)	86.30 ± 13.00	89.09 ± 12.13	90.84 ± 15.00	85.73 ± 12.37	102.89 ± 5.99	98.34 ± 3.33	83.90 ± 7.11	78.18 ± 12.98	n.s.	n.s.	n.s.
Range 30–100 μm^2											
Cell area (μm^2)	60.21 ± 2.30	60.03 ± 2.01	60.57 ± 2.06	59.43 ± 1.86	60.78 ± 1.11	60.89 ± 0.87	59.87 ± 1.30	58.07 ± 3.69	n.s.	n.s.	n.s.
Cell %	56.30 ± 7.26	60.57 ± 2.25	70.23 ± 7.24	74.64 ± 9.84	68.84 ± 11.23	65.26 ± 5.71	64.14 ± 10.09	70.16 ± 9.19	n.s.	n.s.	n.s.
Range 100–200 μm^2											
Cell area (μm^2)	133.44 ± 3.72	134.78 ± 4.23	134.60 ± 5.84	133.68 ± 3.74	138.56 ± 0.48	137.70 ± 0.95	133.65 ± 1.41	131.37 ± 4.40	n.s.	n.s.	n.s.
Cell %	34.18 ± 2.84	32.98 ± 1.40	26.97 ± 5.72	22.79 ± 8.73	27.66 ± 8.77	29.84 ± 2.57	31.47 ± 6.42	26.10 ± 6.53	n.s.	n.s.	n.s.
Range 200–500 μm^2											
Cell area (μm^2)	228.62 ± 2.21	230.53 ± 4.69	228.92 ± 3.79	230.12 ± 2.64	235.43 ± 3.19	232.02 ± 3.51	229.03 ± 4.12	222.69 ± 6.33	n.s.	n.s.	**0.048**
Cell %	9.52 ± 4.96	6.45 ± 1.35	3.18 ± 1.60	2.57 ± 1.29	3.50 ± 2.48	4.90 ± 3.26	4.39 ± 3.68	3.74 ± 2.76	n.s.	n.s.	n.s.

Diets: CTRL (control diet), PHY (phytogenic), OA (organic acid), PROB (probiotic). Anterior intestine (AI; 1.5 cm immediately after the pyloric caeca) and posterior intestine (PI; 1 cm sections after the ileorectal valve). *Data presented as mean ± SD. Two-way ANOVA analyses($p < 0.05$). n.s. = No significant.*

3.3. Microbiota richness, alpha diversity index and population dispersion patterns

Microbiota sequencing yielded ~4.5 M high quality reads (62,594 mean reads per sample) (Table S3) that were assigned to 1157 OTUs at a 97% identity threshold. Rarefaction curves approximated saturation and showed a good coverage of the bacterial community (Fig. S1), mostly represented at the phylum level by Proteobacteria (57–68%), Firmicutes (21–26%) and Actinobacteria (7–15%) (Fig. S2). At a closer look, GS fish showed a significant decrease in richness (Chao1 and ACE values, $p < 0.05$) and alpha diversity (Shannon and Simpson values, $p < 0.05$) (Fig. 3a), which in turn resulted in a clear distance separation of GS and REF fish by discriminant analysis (R2Y = 84%; $p < 0.01$; Q2 = 20%; $p < 0.05$). In combination with this, a different population dispersion



(caption on next page)

Fig. 2. Detailed micrographs of gilthead sea bream intestine fed the different functional additives at the end of the feeding trial stained with Alcian Blue (ph = 2.5) and May-Grünwald Giemsa. For anterior intestine (AI), the lower presence of smaller goblet cells (30–100 μm^2) pattern is represented in Fig. 2a and corresponds to REF fish fed the control diet. The AI higher density of 30–100 μm^2 goblet cells pattern is represented in Fig. 2b and corresponds to REF fish fed PROB diet. For posterior intestine (PI), the larger goblet cells (200–500 μm^2) low density pattern is represented in Fig. 2c and corresponds to REF fish fed the PROB diet. The PI increased density of 200–500 μm^2 goblet cells is represented in Fig. 2d and corresponds to REF fish fed the CTRL diet. Fig. 2e and Fig. 2g represent, respectively, the AI and the PI morphological pattern of fish showing low signs of submucosa or *lamina propria* engrossment (2 level) and correspond to GS and REF fish fed the CTRL and PROB diets. Fig. 2f and Fig. 2h represent the AI and PI morphological pattern of fish submucosa and *lamina propria* with the higher inflammatory status (level 3), corresponding to GS and REF fish fed the PHY and OA diets. Note the thickening of the submucosa and *lamina propria* (———) and the higher presence of infiltrated granulocytes (➤), which was particularly evident in fish fed OA and PHYTO diets, regardless of the genotype. Fig. 2g details an infiltrated lymphocytes (*) focus in the *lamina propria* and mucosa. The scattered foci of lymphocytes were not associated to a specific genotype or diet fed. Fig (a-d), scale bar 100 μm. Fig. (e-f), scale bar = 200 μm. Fig. (g-h), scale bar = 500 μm. CTRL (control diet), PHY (phytogenic), OA (organic acid), PROB (probiotic). Anterior intestine (AI; 1.5 cm immediately after the pyloric caeca) and posterior intestine (PI; 1 cm sections after the ileorectal valve). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pattern was found for each genetic background, as evidenced the measures of standard distance deviation that were 2–3 times lower in GS fish (SDD = 28.04; SDE = 10.23) than in REF fish (SDD = 74.59; SDE = 21.93) (Fig. 3b). In the resulting PLS-DA model, three fish from the REF group were identified as outliers (Hoteling's $T^2 > 0.99$) and discarded.

3.4. Consistency of microbial differentiation patterns across specific actions of feed additives and genetics

For a given genetic background, a microbiota differentiation effect was evidenced for all feed additives by means of PLS-DA (Fig. 4), which rendered a correct classification of all individuals in each group as it was also evidenced by heatmap clustering analysis (Fig. S3a-c). However, this discriminating effect was modulated by each specific additive and genetic background as proven by the results of the PERMANOVA beta-diversity test (Table S4), reflecting the number of taxa that are unique to each of the biological systems being compared. Thus, with the addition of PHY, a high percentage of variance (R2Y = 91%) was explained by the constructed PLS-DA model, but the percentage of explained variance Q2 (0–11%) was low, regardless of the genetic background as it was mostly driven by low abundant taxa representing <3% of intestinal adherent bacteria (Fig. 4a, d). The same was found with the OA diet in GS fish, but in the case of REF fish the percentage of explained variance was high for both the observed (R2Y = 98%, $p < 0.01$) and explained variance (Q2 = 57%, $p < 0.01$) (Fig. 4b, e; Fig. S4a). In the same way, the PROB diet was able to exert a clear regulation of intestinal resident bacteria regardless of the genetic background, with always high percentage values of explained variance in both GS (R2Y = 93%, $p = 0.01$; Q2 = 43%, $p < 0.01$) and REF (R2Y = 93%, $p < 0.01$; Q2 = 35%; $p < 0.01$) fish (Fig. 4c, f; Fig. S4b, c).

Regarding the fully validated discriminant models, a VIP ≥ 1 threshold discerned up to 82 (REF-OA), 66 (REF-PROB) and 39 (GS-PROB) discriminant OTUs (Table S5). For these three groups of fish, the top-abundant (≥ 0.7% in CTRL or diet supplemented diet) discriminant OTUs are shown in Fig. 5, triggering the OA supplementation in REF fish the increase in the *Pseudomonas*, *Photobacterium*, *Micrococcus*, and *Peptoniphilus* genera, and the decrease in *Streptococcus*, *Staphylococcus*, *Rubellimicrobium* and *Propionibacterium* (Fig. 5a). In the case of the PROB diet (Fig. 5b, c), some bacteria decreased (*Acinetobacter* and *Vibrio*), while others increased (*Stenotrophomonas* and *Bacillus*), regardless of genetics. By contrast, *P. damselae*, and *Pseudomonas* genus increased in the REF fish, and decreased in the GS fish. Likewise, the genus *Micrococcus* only increased in the GS fish, whereas the genera *Ralstonia* and *Kocuria*, with >18% of abundance, vastly predominated in the adherent microbiota of the GS-PROB fish.

3.5. Bacterial inferred pathways

Aiming to assess the biological significance of the observed changes in microbiota composition, a pathway analysis was conducted with the inferred metagenomes of the OTUs that drove the separation by feed additive (Fig. S5). The results showed that 12 pathways were significantly changed when comparing REF-OA and REF-CTRL fish (Fig. S5a), being Reductive TCA cycle, Acetone/butanol/ethanol (ABE) fermentation and Menaquinol biosynthesis among the most over-represented processes, and the Catechol degradation and Mevalonate pathway among the most under-represented ones. Pathway analyses also showed a total of 19 significant differentially represented pathways when comparing GS-PROB and GS-CTRL, highlighting the over-representation of the Nitrate reduction VI pathway and the under-representation of the Peptidoglycan biosynthesis (Fig. S5b). In contrast, only 3 pathways were changed between REF-PROB and REF-CTRL fish (Fig. S5c).

3.6. Host intestinal transcriptomics

Approximately 4800 million PE reads were obtained from the RNA-seq sequenced samples, with an average of ~65 million reads per sample (Table S3). Up to 89% of these pre-processed reads were mapped against the reference genome, and unique hits counts were associated to 35,215 intestinal transcripts, corresponding to 15,684 unique descriptions (UD). Differential expression analysis discerned 8321 transcripts (6812 UD) significantly changing ($p < 0.05$) among comparisons. Within them, 3477 DE transcripts (2409 UD) marked the difference between GS-PHY and GS-CTRL fish, further decreasing to 2549 DE transcripts (2170 UD) when comparing REF-PHY and REF-CTRL fish (Fig. S6a, d). A total of 1749 (1498 UD) and 2310 (1920 UD) transcripts were differentially regulated between GS-OA and GS-CTRL fish, and between REF-OA and REF-CTRL, respectively (Fig. S6b, e). The PROB supplementation altered 2496 transcripts (2096 UD) in the GS fish and 2513 transcripts (2162 UD) in the REF fish (Fig. S6c, f).

To increase the statistical robustness of the gene expression analysis, the initial set of DE transcripts were used to fed six different PLS-DA models (Fig. 6). All the PLS-DA were statistically validated by permutation tests (pR2Y < 0.05; pQ2 < 0.05; Fig. S4d-i), with a correct classification of all individuals by heatmap clustering when a VIP ≥ 1–1.4 threshold was applied (Fig. S3d-i). Such procedure mined 460 (406 UD) and 1202 (11,056 UD) transcripts separating the GS and REF fish fed the CTRL and PHY diet, respectively (Fig. 6a, d). The GS-OA fish were split from the GS-CTRL fish by 880 transcripts (807 UD), whereas 258 transcripts (242 UD) split REF-OA and REF-CTRL fish (Fig. 6b, e). A total of 1141 discriminant transcripts (1017 UD) were found between GS-PROB and GS-CTRL, while 1205 transcripts (1084 UD) drove the discrimination between REF-PROB and REF-CTRL fish (Fig. 6c, f). Such approach retrieved as a whole 5192 DE transcripts (4507 UD) that were then used in the subsequent correlation analyses.

3.7. Host transcriptomics and microbiota correlated changes

A total of 1227 associations ($p < 0.001$) were established between OTUs (169) and DE host (939) transcripts of discriminant value (Table S6). This set of DE genes discerned 22 over-represented GO-BP terms, allocating158 DE transcripts (136 UD) that were joined in 9 supra-categories (Fig. 7a). The remaining 4253 DE host transcripts

(without any significant association with gut microbiota) discerned 90 GO-BP unique terms, allocating 865 DE transcripts (680 UD) that were identified as over-represented and clustered in 14 supra-categories (Fig. 7b).

Regarding specifically each feed additive, the host-transcriptomic effects of PHY diet were not correlated with gut microbiota changes and they become associated to 3 specific GO supra-categories, including genes related to Lipid metabolic process (54 DE transcripts; 54 UD), Cellular process (10 DE transcripts; 10 UD), and Smooth muscle contraction (4 PHY DE transcripts; 4 UD) (Fig. 7c; Fig. S7a). The transcriptionally mediated changes of the OA diet were related to the Neutrophil degranulation (19 OA-specific DE transcripts; 19 UD) GO supra-category, which only correlated with changes in bacteria taxa. In the PROB diet, up to 3 specific GO supra-categories were identified as PROB non-microbial associated processes: Response to insulin (28 DE transcripts; 28 UD), Endocytosis (27 DE transcripts; 27 UD) and Epithelial cell differentiation and proliferation (14 DE transcripts; 14 UD) (Fig. 7c, d; Fig. S7b). The PROB bacterial associated processes were related with Translation (21 DE transcripts; 21 UD) and Ribosome (13 DE transcripts; 13 UD) GO supra-categories, while the SRP protein targeting supra-category contained DE transcripts that remained either associated (13; 13 UD) or uncorrelated (13; 13 UD) with gut microbiota (Fig. S7c).

3.8. Linking host over-represented processes with transcriptomics and gut microbiota interactions

The protein-protein network analysis was used for filtering the list of genes within the above-mentioned GO supra-categories. In the case of PHY, a major network was depicted comprising 38 DE transcripts, including apolipoproteins (*apoa1*, *apoc2*) and transferases (*dgat2*, *got2*, *sptcl3*) that were downregulated by the additive, regardless of the genetic background. Both in GS and REF fish, the opposite pattern was found for phospholipases (*plb1*, *pla2g10*). However, only in GS fish, PHY diet triggered the down-regulation of *nr1d2* (lipid metabolism repressor) and β-oxidation markers (*acsl5*, *decr2*, *acadvl*, *aldh3a2*, *scp2*), together with the up-regulation of markers of sphingolipid metabolism (*kdsr*, *degs1*, *elovl1*, *sgpp1*, *plekha8*) and cholesterol and bile salts metabolism (*abcg8*, *abca1*, *nr1h3*, *nr1h4* and *cel*), as well as markers of epithelial integrity and intestinal villi arrangement (*chmp2a*, *chmp2b*, *vps4b*, *cdhr2*, *cdhr5*) (Fig. 8a).

In this analysis, the supplementation with the OA additive was represented by 5 transcripts (*atp6ap2*, *atg7*, *dync1h1*, *lamp2*, *vcp*) of the GO supra-category Neutrophil degranulation. These markers were only differentially regulated in REF fish, and four out of the total five were associated to changes with abundant gut discriminant taxa: *Staphylococcus*, *Neisseria*, *Streptococcus* and *Vibrio* (Fig. 8b).

The outcomes of the protein-protein network analysis for the PROB additive remarked the down-regulation of 32 transcripts overlapping in the Translation, Ribosome and SRP-protein targeting GO supra-categories, which were mainly involved in protein synthesis (markers of 40/60S ribosomal proteins and eukaryotic elongation factors) and proteolysis ubiquitination (*uba52*). From these 32 markers, 17 were down-regulated only in the GS fish, whereas 9 were differentially regulated only in REF fish, and 6 in fish from both genetic backgrounds. Some of these genes were correlated with four abundant discriminant taxa: *Chromohalobacter*, *Enhydrobacter*, *Vibrio* and *Acinetobacter* (Fig. 8c, d). The remaining genes in the PROB network did not show a clear expression pattern, with the exception of the up-regulated response in GS fish of markers of epithelial integrity (*ezr*, *ncstn*, *neurog3*, *plec*) and insulin signalling (*atp6ap1*, *atp6v0a1*, *atp6v0a2*, *atp6v0b*, *atp6v0c*, *atp6v1h*) (Fig. 8c).

4. Discussion

The microbiome is traditionally viewed as a non-genetic

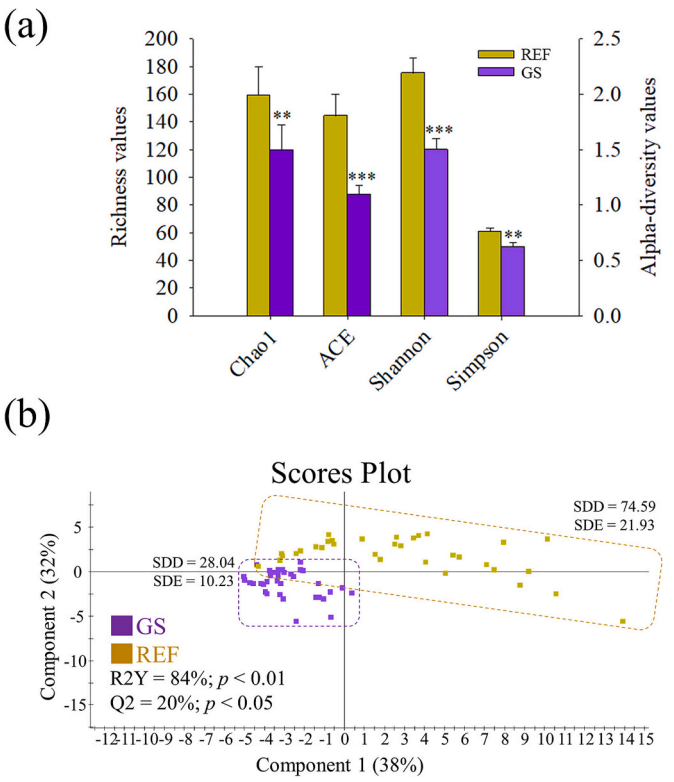


Fig. 3. Alpha-diversity, phylum composition and homogeneity of anterior intestine adherent microbiota of growth-selected (GS) and reference (REF) gilthead sea bream juveniles. (a) Species richness estimates (Chao1 and ACE, left y axis) and diversity indexes (Shannon and Simpson, right y axis) of 72 fish of GS and REF groups (36 fish per group). Asterisks indicate statistical differences between genetic groups (Kruskal-Wallis test; Dunn's post-test; **: $p < 0.01$; ***: $p < 0.001$). (b) Two-dimensional partial least-square discriminant analysis (PLS-DA) score plot representing the distribution of 69 GS and REF samples (excluding outliers; Hoteling's $T^2 > 0.99$) in the first two components of the model. Numbers next to dashed boxes represent the standard deviation distance (SDD) and the standard deviational ellipse (SDE) measures for quantifying the dispersal of the multi-dimensional PLS-DA data points.

environmental factor that shapes most host traits, though it has a genetic basis that can change host fitness (Henry et al., 2021). As part of this host-microbiota partnership, the selective breeding can lead to the establishment of a stable microbial gut community that will contribute to confer enough host fitness to cope with diet and environmental challenges (Ley et al., 2006; Goodrich et al., 2017; Gould et al., 2018; Mueller and Sachs, 2015). Likewise, GS fish in this study had a more cohesive gut microbiota, as shown by its reduced population dispersion (inter-individual variability) (Fig. 3b), which was far to be an inconvenience since these genetically improved fish shared in this (Table 2) and previous studies an overall improvement of growth performance (Montero et al., 2023; Naya-Català et al., 2022a; Perera et al., 2019) together with changes in behaviour and swimming activity (Perera et al., 2021). In any case, as pointed out in other animal models, effects of microbial inheritance and genetics on host phenotypes are highly dependent on the ecological context, and the incorporation of microbial variation into quantitative genetic models will provide fundamental novel insights into how selection operates across ecological and evolutionary scales (Henry et al., 2021), which becomes especially relevant in a context of global change. Certainly, in comparison to REF fish, the microbiome of GS fish will be prone to change its metabolism rather than composition when fish are coping with diet and ambient stressors (Piazzon et al., 2020; Naya-Català et al., 2022a, 2022b). However, the magnitude and direction of these microbiome shifts are largely dependent not only on host genetics, but also on the nature of nutrient (feed

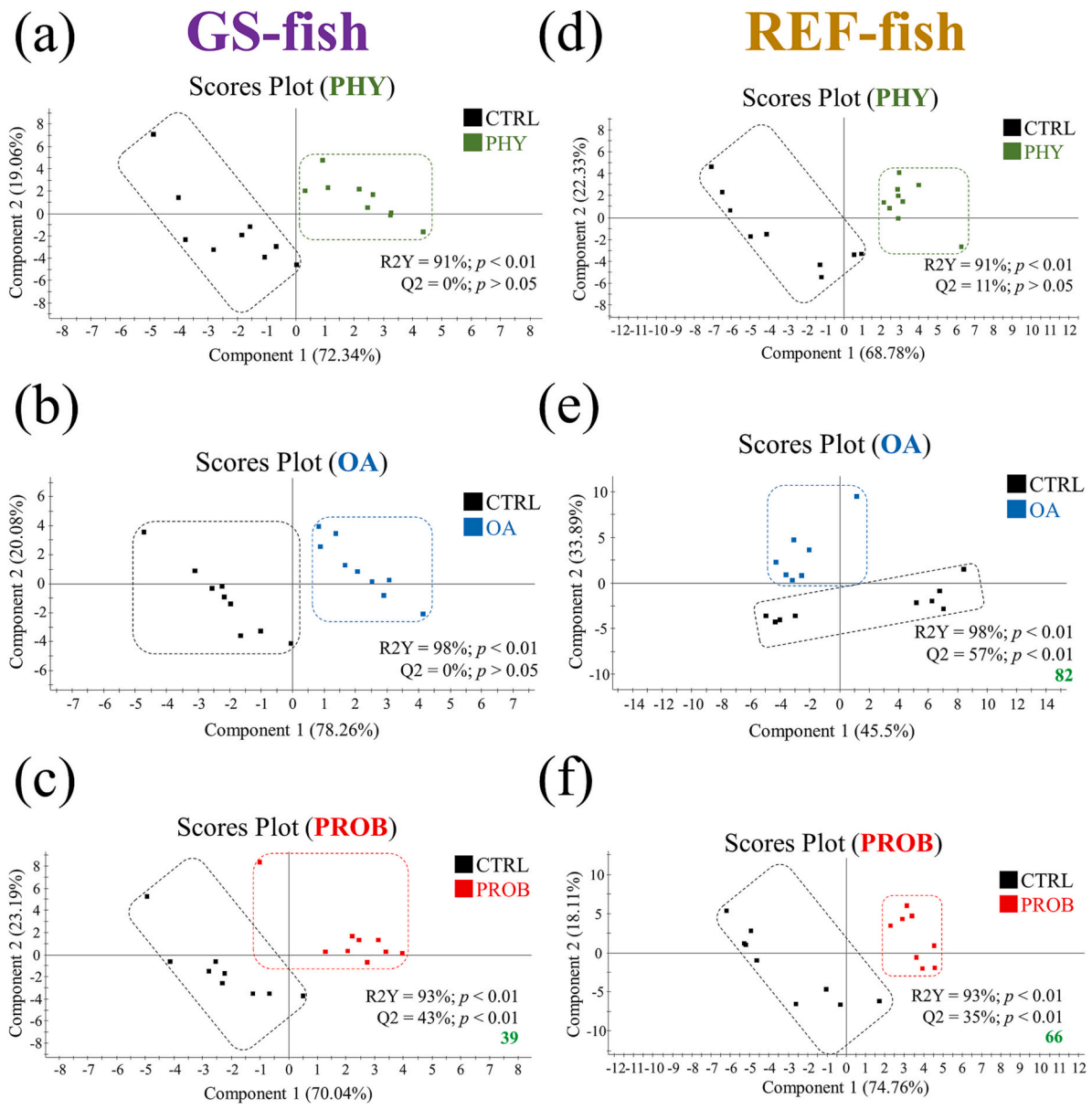


Fig. 4. PLS-DA score plots showing the effects of the additive-supplemented diets (PHY, OA, PROB) on anterior intestine adherent microbiota of growth-selected (GS) and reference (REF) gilthead sea bream juveniles. Panels represent the distribution of samples in the first two components of the model comparing (a) GS-PHY *vs.* GS-CTRL, (b) GS-OA *vs.* GS-CTRL, (c) GS-PROB *vs.* GS-CTRL, (d) REF-PHY *vs.* REF-CTRL, (e) REF-OA *vs.* REF-CTRL, and (f) REF-PROB *vs.* REF-CTRL. Green numbers in the bottom right of the plots indicate the number of OTUs identified to significantly drive the separation between the groups (VIP $\geq$ 1). Validation plots for the significant models can be found in Supplementary Fig. 5A (c), 5B (e) and 5C (f). CTRL (control diet), PHY (phytogenic), OA (organic acid), PROB (probiotic). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

additive) stimuli, in line with recent studies in European sea bass (Rimoldi et al., 2023). Thus, regardless of feed additive and genetic background, discriminant analysis was able to discern a pronounced effect on microbiome composition that explained a high percentage of the observed variance (R2Y = 91–98%) (Fig. 4). Nevertheless, a relative high percentage of the predicted variance was only explained in the case of OA-REF fish, and PROB-GS and PROB-REF fish. By contrast, the PHY diet did not induce considerable changes on gut microbiota, regardless of the genetic background, probably due to the low number and abundance of the bacteria taxa with a discriminant value at a high statistical threshold level in the PLS-DA (Fig. 4a, d). Indeed, a number of studies in gilthead sea bream and other farmed fish claimed the beneficial effects of phytobiotics on several gut health markers, including gut microbiota (Firmino et al., 2021b; Rabelo-Ruiz et al., 2022; Rimoldi et al., 2020; Salomón et al., 2022), though most of them are not validated by permutation tests in wide-metagenomic approaches. In agreement with this, we found herein that the OA diet drove changes in the gut microbiota of REF fish but not in GS fish (Fig. 4b, e), which shared discriminant bacteria taxa that remained below the 4% of abundance (Fig. 5a). According to this, the PROB diet reshaped the gut microbiota of both GS fish and REF fish, depicting changes in relatively abundant discriminant bacteria taxa that decreased from 66 in PROB-REF fish to 39 in PROB-GS fish (Fig. 4c, f). Despite this, the phenotypic effects were apparently amplified in GS fish because the inferred metagenome supported changes in almost 19 metabolic pathways, whereas only 3 metabolic pathways were predicted to be substantially altered in REF fish (Fig. S5). Summing up, genetic selection in the PROGENSA® program co-selects for a plastic gut microbiome that might confer a selective advantage with changes in main dietary ingredients (Piazzon et al., 2020; Naya-Català et al., 2022a, 2022b), but also making GS fish more responsive to

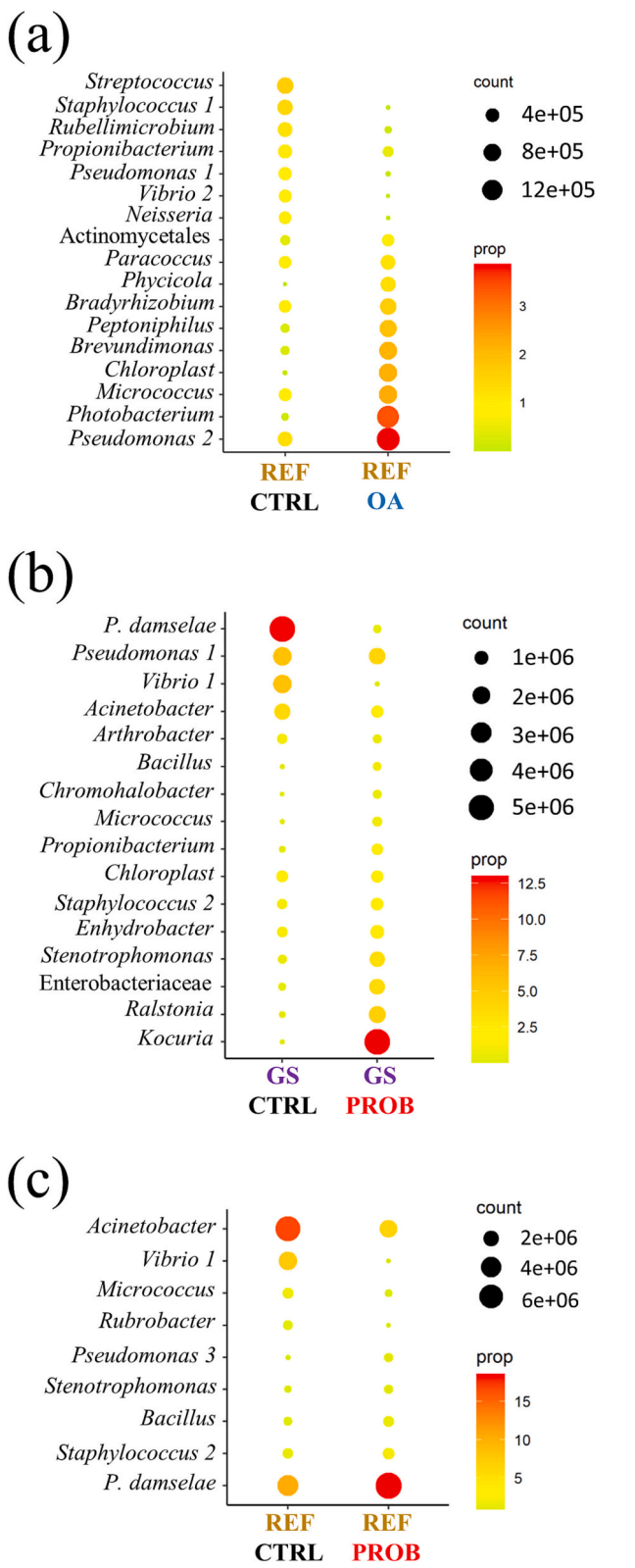


Fig. 5. Dot plots showing the main taxa (VIP ≥ 1 and > 0.7% abundance) changing in the anterior intestine adherent microbiota. Panels include the main bacteria involved in the separation of (a) REF-OA *vs.* REF-CTRL (Fig. 6E), (b) GS-PROB *vs.* GS-CTRL (Fig. 6C), and (c) REF-PROB *vs.* REF-CTRL (Fig. 6F). The size of the dots represents the mean normalized counts in each group. The colour scale represents the abundance, in percentage, of each genus within each group. CTRL (control diet), PHY (phytogenic), OA (organic acid), PROB (probiotic).

a given functional feed, which became herein especially evident in the case of the probiotic-based additive.

Phytobiotics in livestock have been often related with the transcriptional modification of several markers of lipid metabolism (Aanyu et al., 2020; Flees et al., 2021; Liu et al., 2022) and mucosal integrity (Firmino et al., 2021a; Serradell et al., 2022; Torrecillas et al., 2021; Wendner et al., 2023). In this work, PHY additive drove a considerable shift of intestinal transcriptional patterns of GS and REF fish, although as indicated before, they mostly uncorrelated with gut microbiota (Fig. 8a). Of note, the most abundant hubs in the protein-protein interaction network were represented by markers of lipid metabolism and epithelia integrity, but intriguingly 31 genes out of 38 were varying in GS-PHY fish and not in REF-PHY fish. As a result of this, GS-PHY fish specifically presented an up-regulation of the fatty acid binding protein 2 (*fabp2*) and cadherin related family members (*cdhr2* and *cdhr5*), together with a down-regulation of β-oxidation markers (*acsl5*, *decr2*, *acadvl*, *aldh3a2*, *scp2*). The same trend was previously found with fast-growing strains of gilthead sea bream (Simó-Mirabet et al., 2018), which would reflect some physiological advantage of fish fed the PHY diet, although we failed herein to relate it with a significant improvement of growth performance. In any case, the changing expression pattern of cadherin genes, also observed in phytogenic-fed sea bass without affecting the intestinal ultrastructure (Torrecillas et al., 2019), supported changes in the regulation of the villus arrangement and epithelium integrity (LeBrasseur, 2005), which would be mediated, at least in part, through villin-1 (*vil1*) (Wang et al., 2008), charged multivesicular body proteins (*chmp2a, chmp2b*), and vacuolar sorting-associated proteins (*vps4b*) (Göser et al., 2020), all of them up-regulated in GS-PHY fish. This also applies to markers of sphingolipids metabolism, including, among others, essential enzymes for the *de novo* synthesis of these essential components of cell membranes and signal transduction complexes (*kdsr*, *elovl1*, *sgpp1*, *plekha8*) (Yamaji and Hanada, 2015). The association of genetic improvement with the up-regulation of sphingolipid metabolism has already been established within the selection program PROGENSA® (Naya-Català et al., 2022b), but herein it was specifically associated to a given feed additive and genetic background, providing additional evidence for the key role of sphingolipid metabolism in our genetic selection process. Lastly, dietary supplementation of bile salts and artificial emulsifiers are able to improve the overall performance of farmed fish and gilthead sea bream in particular (Ruiz et al., 2023a, 2023b), and intriguingly we found herein that main players in the biliary cholesterol section and absorption system (*abcg8*, *abca1*, *nr1h3*) were again specifically up-regulated in GS-PHY fish, highly supporting that the transcriptome of this fish should be more suited to cope with and improved growth and feed intake.

In the present study, a main effect of dietary OA supplementation was related to a reduced abundance of *Streptococcus, Staphylococcus, Neisseria,* and *Vibrio* in REF fish (Fig. 5a). Metabolites derived from these potentially pathogenic bacteria taxa might trigger the activity of host neutrophils (Kamada et al., 2013; Zhang and Frenette, 2019), which fits well with the granulocyte infiltration observed in the AI of REF-OA fish, although this pattern was also observed in fish fed with the PHY additive (Fig. 2). Certainly, at the transcriptional level, this group of fish showed an over-representation of genes classified in the GO supra-category Neutrophil degranulation (Fig. 7d) with up to four genes (*dync1h1*, *lamp2*, *atg7, atp6ap2*) related with the neutrophil granule trading (Paludan et al., 2021) that were correlated with changes in gut microbiota (Fig. 8b). Although the exact mechanism cannot be assured, it might be hypothesized that these transcriptional markers would depict the fine-tuning of the granulocyte-mediated inflammatory response. At the same time, the inferred bacterial metagenome highlights the potential for an increased ABE fermentation (Fig. S5), mainly made by solventogenic Clostridial bacteria in anaerobic culture conditions (Turton et al., 1983), which would be supported herein by the raise of the Clostridiales genus *Peptoniphillus* (2% increase in REF-OA fish). Butyrate in particular has been linked to gut health and homeostasis in a number of animal

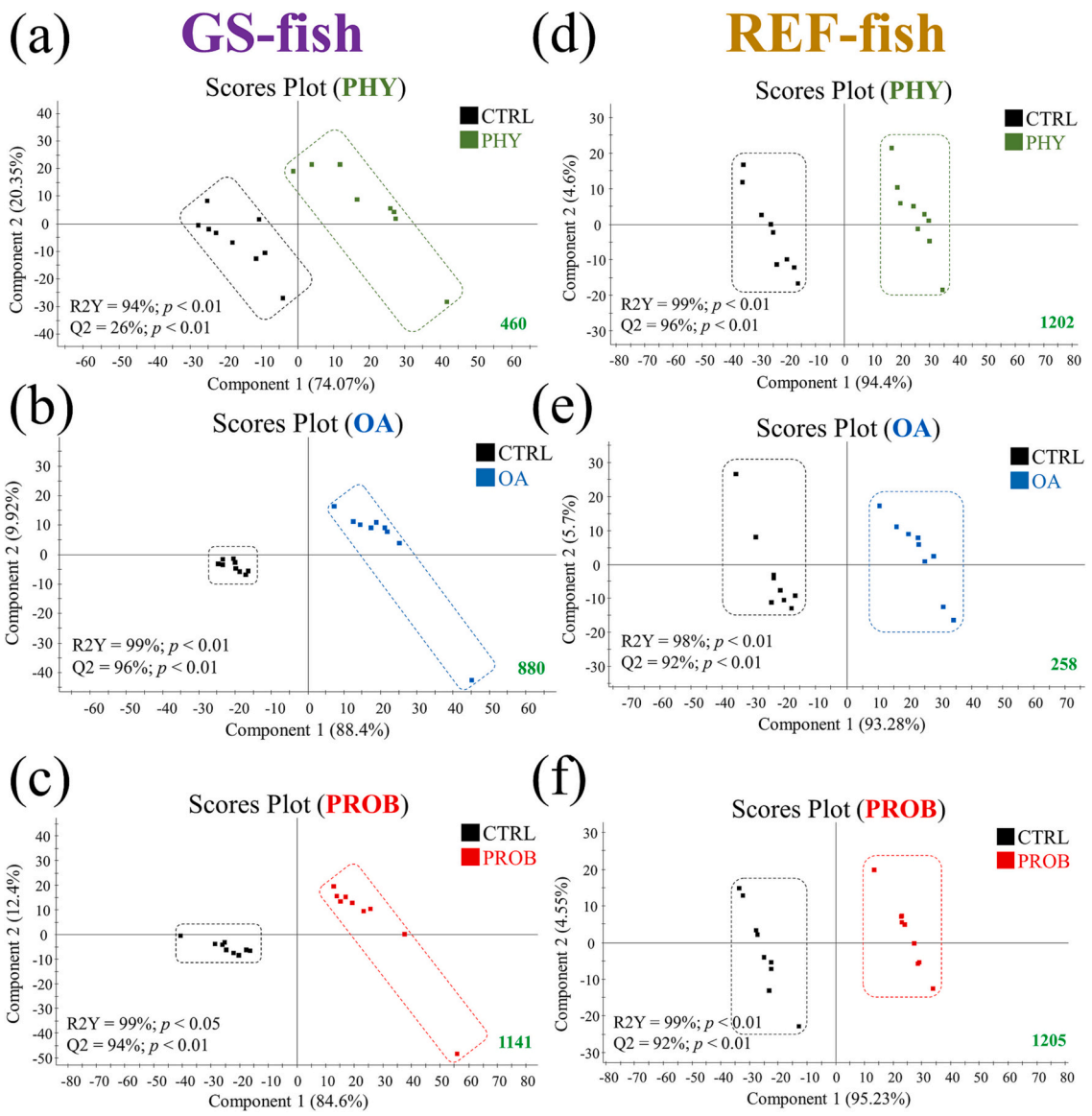


Fig. 6. PLS-DA score plots showing the effects of the additive-supplemented diets (PHY, OA, PROB) on anterior intestine transcriptional patterns of growth-selected (GS) and reference (REF) gilthead sea bream juveniles. Panels represent the distribution of samples in the first two components of the model comparing (a) GS-PHY *vs.* GS-CTRL, (b) GS-OA *vs.* GS-CTRL, (c) GS-PROB *vs.* GS-CTRL, (d) REF-PHY *vs.* REF-CTRL, (e) REF-OA *vs.* REF-CTRL, and (f) REF-PROB *vs.* REF-CTRL. Numbers in the bottom right of the plots indicate the number of transcripts identified to significantly drive the separation between the groups (VIP ≥ 1–1.4). Validation plots for the shown models can be found in Supplementary Fig. 5D (a), 5E (b), 5F (c), 5G (d), 5H (e), 5I (f). CTRL (control diet), PHY (phytogenic), OA (organic acid), PROB (probiotic).

models, including humans (Lin et al., 2021) and gilthead sea bream (Estensoro et al., 2016; Piazzon et al., 2017). However, more short fatty acids are not necessarily better, and the OA diet rendered an impaired performance in REF, which did not occur in genetically improved fish (Table S2). Vitamin surplus is perhaps another example of the importance of appropriate levels of dietary nutrients, and both deficiency and excess in dietary vitamin K3 negatively affects larvae performance and bone health in gilthead sea bream (Sivagurunathan et al., 2023). How this type of metabolic dysfunction can be extensive to our experimental model remains elusive, but it is noteworthy that the inferred metagenome of REF-OA fish also shares an over-representation of microbiota processes related with the biosynthesis of menaquinol (a precursor of the vitamin K) (Fig. S5). In other words, when the organism is not ready to achieve a high rate of reaction, the provision of powerful fuels can trigger the collapse rather than the improvement of the biological system.

Bacillus-based probiotics represent promising ingredients to endorse the intestinal health and growth of aquaculture species (Gopi et al., 2022; Tran et al., 2023), including gilthead sea bream (Simó-Mirabet et al., 2017; Zaineldin et al., 2018). In agreement with this, we found that signs of inflammation and granulocyte infiltration in the submucosa and *lamina propria* of the AI and PI of fish fed PHY and OA additives were not found in PROB fish (Fig. 2). This occurred in coincidence with the establishment of *Bacillus* genus (>2.5%) in the adherent mucosa of PROB fish (Fig. 5b, c), though a significant growth improvement was limited to GS fish (Table 2), reducing significantly fish growth performance in PROB-REF compared to fish fed CTRL diet. Certainly, the microbiome of GS-PROB shared a higher abundance of *Kocuria* and *Micrococcus* genera, which promote an improved digestion and a proper reduction of nitrate to nitrite (Ferysiuk and Wójciak, 2020; Rocha and Laranjinha, 2020; Tamme et al., 2006). In line with these results, the inferred bacterial metagenome of GS-PROB fish supported a higher activity of the Nitrate reduction pathway (Fig. S5b), which has been associated to a correct endothelial function, vascular relaxation, and/or

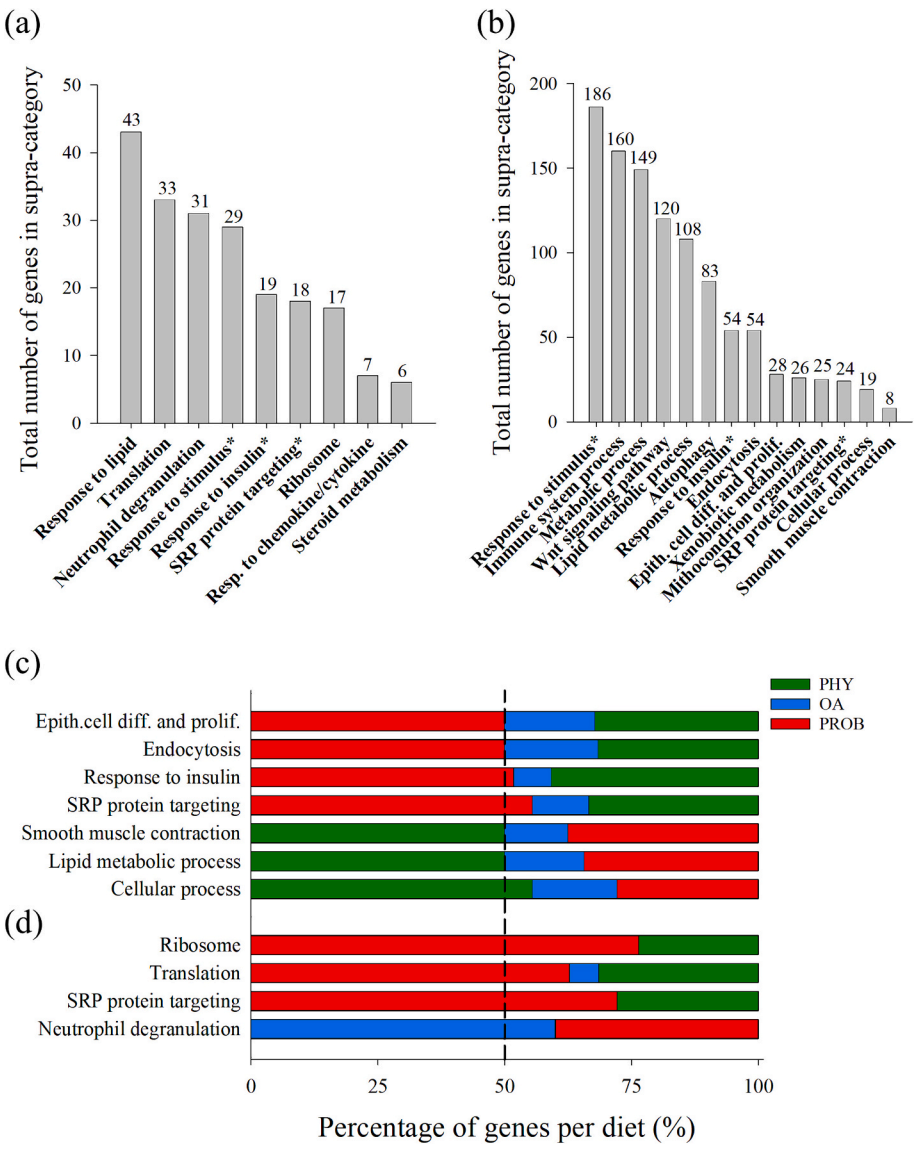


Fig. 7. Intestinal over-represented processes in gilthead sea bream anterior intestine with the additive-supplement inclusion. Bar plots depicting the results of the over-representation analyses and supra-category retrieval, together with the corresponding number of genes in each supra-category, over the fraction of genes correlated (a) and not correlated (b) with microbiota. Asterisks highlight the supra-categories present in both bar plots. Horizontal bar chart showing the additive-specific supra-categories not correlated (c) and correlated (d) with microbiota and the corresponding percentage of genes that each additive supply.

inhibition of the platelet aggregation (Machha and Schechter, 2011). The transcriptional signatures also appeared improved by the PROB diet in GS fish (Fig. 8c), with the protein interaction plot showing a concordant up-regulation of V-ATPases (*atp6ap1, atp6v-0a1, −0a2, −0b, −0c, −1 h*) that act translocating protons from cytosol to intracellular compartments or extracellular space, maintaining pH homeostasis at the cellular and the whole organism level (Collaco et al., 2013). This also applies to key components of Wnt (*ezr, neurog3*) and Notch (*ncstn, plec*) intracellular signalling pathways, and their up regulation in GS fish would constitute primary driving activities assisting intestinal stem cells in maintenance and proliferation (Cotter et al., 2015; Kaemmerer et al., 2019). The association between Notch and the goblet cell differentiation pathway (Zecchini et al., 2005) could also partly explain the differences in the intestinal pattern of GS-PROB fish, which showed a higher percentage of smaller goblet cells than fish fed the CTRL diet in comparison to other experimental additives (Table 3). The interaction plot also disclosed a consistent down-regulation of a number of markers of protein synthesis and ubiquitination in GS-PROB fish, which might, as a whole, support a reduced epithelial turnover and improved intestinal barrier function, also highlighted for the GS and REF comparison in a recently published study (Naya-Català et al., 2022b). By last, *Chromohalobacter*, *Enhydrobacter*, *Vibrio*, and *Acinetobacter* genera were correlated with protein metabolism markers, and inferred pathways rendered the down-regulation of microbiota-derived glycine degradation and the up-regulation of valine and isoleucine biosynthesis. (Fig. 8d; Fig. S5). The role of these specific bacteria taxa in fish protein metabolism remains elusive, but new recent evidence shows a strong link between gut microbiota and amino acid metabolism, which is translated in two different ways: i) influencing the entry of amino acids into the portal circulation for whole-body use, and ii) by the capacity of some colonizing bacteria to facilitate the *de novo* synthesis of branched amino acids (Beaumont et al., 2022; Hayashi et al., 2021; Lin et al., 2017).

5. Conclusions

In summary, genetic selection for growth induced a drastic reduction of the gut microbiota variability among individuals, coupled to an improved growth and histological responses with the use of feed

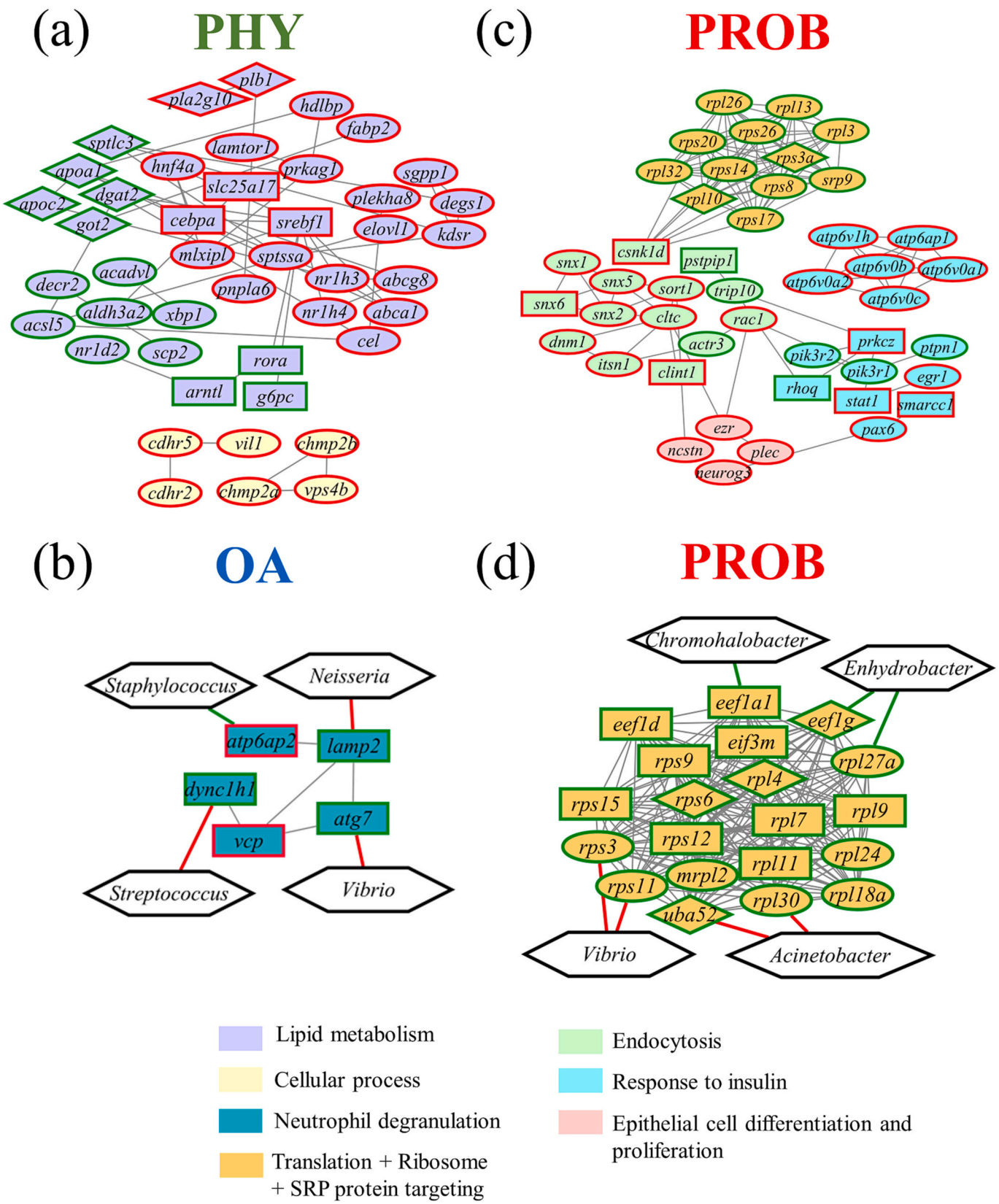


Fig. 8. Protein–protein interaction plots and expression patterns of (a) PHY-, (b) OA-, and PROB-specific supra-categories not correlated (c) and correlated (d) with microbiota. Grey edges between nodes show significant protein-protein relations (FDR < 0.05; STRING confidence score > 0.7). Geometrical forms indicate if a gene is differentially expressed in GS (ellipses), REF (squares) or both (diamonds) fish genetic backgrounds. Border colors indicate up-regulation (red) or down-regulation (green) of the gene in the Additive *vs.* CTRL comparison. White hexagons represent abundant (>0.7%) microbiota. Colored edges show significant positive (red edges) and negative (green edges) correlations (Spearman, $p < 0.001$). CTRL (control diet), PHY (phytogenic), OA (organic acid), PROB (probiotic). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

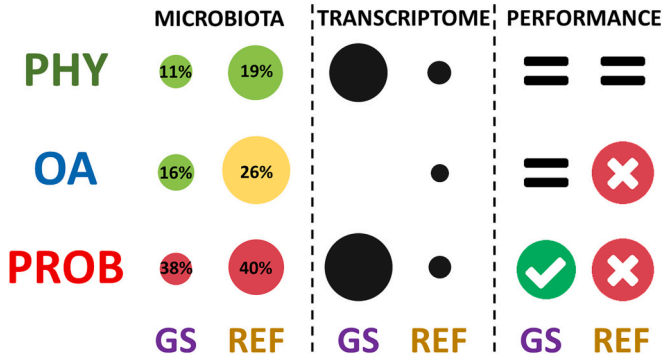


Fig. 9. Schematic representation of the overall effects of the three functional additives on the microbiome, transcriptome, and performance of genetically selected and reference gilthead sea bream. Circle sizes represent the amount of discriminant (VIP $\geq$ 1) taxa and protein-protein interacting DE transcripts obtained along the study. GS (growth-selected fish), REF (reference fish) (REF) CTRL (control diet), PHY (phytogenic), OA (organic acid), PROB (probiotic).

additives in comparison to the reference fish. The specific mode of action of the additives upon host transcriptomics and gut microbiota was also highly dependent on the genetic background (Fig. 9). Thus, PHY primarily shaped the transcriptome of GS fish, whereas OA changed the gut microbiota of REF fish. PROB caused an opposite pattern in GS and REF in terms of weight gain and triggered changes in both host transcriptome and gut microbiota of GS and REF fish. Altogether, selective breeding shapes a more responsive gut transcriptome in combination with a more stable and plastic gut microbiota in fish fed supplement diets. Metagenomics and transcriptomics integration makes sense towards the success of innovative breeding and nutritional programs, as the generated knowledge herein can be of practical use to know which processes are mostly susceptible to be targeted in a given genome-environment interaction to improve the resilience of farmed fish.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.740770.

CRediT authorship contribution statement

Fernando Naya-Català: Data curation, Formal analysis, Investigation, Software, Visualization, Writing – original draft, Writing – review & editing. **Silvia Torrecillas:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Resources, Supervision, Writing – original draft, Writing – review & editing. **M. Carla Piazzon:** Data curation, Formal analysis, Investigation, Software, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Samira Sarih:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Josep Calduch-Giner:** Investigation, Writing – review & editing. **Ramón Fontanillas:** Resources, Writing – review & editing. **Barbara Hostins:** Resources, Writing – review & editing. **Ariadna Sitjà-Bobadilla:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing. **Félix Acosta:** Investigation, Writing – review & editing. **Jaume Pérez-Sánchez:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Daniel Montero:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

Barbara Hostins reports a relationship with INVE Aquaculture that includes: employment. Ramon Fontanillas reports a relationship with Skretting Aquaculture Research Centre that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Raw sequencing data are available at NCBI's Sequence Read Archive under accession PRJNA901860 (BioSample DNA accession numbers: SAMN31727064-135; Biosample RNA accession numbers: SAMN31727136-207).

Acknowledgements

This work was supported by the European Union's Horizon 2020 research and innovation programme (AquaIMPACT- Genomic and nutritional innovations for genetically superior farmed fish to improve efficiency in European aquaculture) [grant agreement number 818367]; the MCIN with funding from European Union NextGenerationEU [PRTR-C17·I1] and by Generalitat Valenciana [THINKINAZUL/2021/024]; the European Social Fund (ESF) & ACOND/2022 Generalitat Valenciana [RYC2018-024049-I]. Authors want to thank Dr. Afonso, from Grupo de Investigación en Acuicultura (GIA), IU-ECOAQUA, Universidad de Las Palmas de Gran Canaria, for the PROGENSA® breeding program. We acknowledge the support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

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