



# Genetically superior European sea bass (*Dicentrarchus labrax*) and nutritional innovations: Effects of functional feeds on fish immune response, disease resistance, and gut microbiota

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## ABSTRACT

The objective of this study was to determine if selected fish genotypes could benefit from the use of functional additives in novel aqua feed formulations to improve growth performance, gut microbiota, immune response, and disease resistance in fish. Two batches of juvenile European sea bass selected for high growth (HG; selected sires x selected dams), and wild types (WT; wild sires x selected females) were fed a “future diet” coated with three different functional additives for 12 weeks as follows: (i) 2 weeks with a high dose, followed by (ii) 10 weeks with a low dose. The functional additives tested were a mixture of probiotics (PROB), organic acids (ORG), and phytogens (PHYTO). A pathogen challenge test (*Vibrio anguillarum*) and a stress condition (overcrowding) were performed after each dose. At the end of the feeding experiment, fish from the HG group performed better than fish from the WT group in terms of body weight, relative growth, SGR, and DGI. The results of the two challenge tests performed after two weeks of high dose and ten weeks of low dose showed a significant effect of diet on fish survival. GALT-associated gene expression analysis revealed an interaction between the genotype and diet for *il-1β* in the distal gut. Finally, regarding the gut microbiota, discriminant analysis showed no clear separation between fish fed the future diet and those fed the same diet with experimental additives. Nevertheless, the relative abundance of certain taxa varied between experimental groups. For example, fish fed the ORG diet had higher relative abundance of *Streptococcus* in both genotypes, whereas fish fed the PHYTO diet had higher abundance of Lactobacillales. In contrast, fish fed PROB had lower abundance of *Pseudomonas* and *Acinetobacter*.

## 1. Introduction

The development of the aquafeed industry is the first step in expanding aquaculture production. New strategies are needed to deal with the limited resources of wild fish traditionally used to produce raw materials such as fish meal (FM) and fish oil (FO). One of the biggest challenges in aquaculture is to find alternative and more sustainable feed ingredients that can replace FM and FO without compromising fish

health and growth performance. To date, a wide range of raw materials have been successfully used in fish feeds without demonstrated negative effects on growth performance. These include plants (Hardy, 2010; Huyben et al., 2020; Torrecillas et al., 2017), animal by-products (Fontinha et al., 2021; Rimoldi et al., 2018b), insects (Rimoldi et al., 2021; Terova et al., 2021), unicellular microbes (Rimoldi et al., 2020a), and microalgae (Sarker et al., 2020). However, it is also true that optimal feeding strategies in intensive aquaculture systems should be

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evaluated in conjunction with fish gut health. In particular, replacing FM and FO in aqua feeds with plant products may affect the health of carnivorous farmed fish by altering gut morphology and microbiota and modifying local gut and systemic fish immunity (Huyben et al., 2020; Martin and Król, 2017; Simó-Mirabet et al., 2018; Torrecillas et al., 2017). Indeed, insufficient nutritional value of the diet is the main factor affecting the gut microbiota, which provides essential health benefits to the host, especially by regulating immune homeostasis (Wu and Wu, 2012). Although gut immunity of fish is less developed than that of higher vertebrates, the gut immune components of teleosts contain both effector and inducer sites (Bruce et al., 2017). Unlike mammals, the gut immune system of fish lacks lymphoid tissue aggregates such as Peyer's patches, but instead consists of diffuse gut-associated lymphoid tissue (GALT) in which the induction and effector sites of the lamina propria are indistinguishable (Rombout Jan et al., 2011; Salinas et al., 2011). Similar to higher vertebrates, GALT contains mucosal immune cells such as lymphocytes, plasma cells, granulocytes, and macrophages that make this mucosal tissue an active immune organ. Intestinal microbiota plays a central role in the development of GALT (Dawood, 2021; Wu and Wu, 2012). Experiments in germ-free animal models have demonstrated the critical role of the commensal gut microbiota in priming neutrophils, enhancing disease resistance to pathogens, and in the initiation and progression of intestinal inflammation (Galindo-Villegas et al., 2012; Montalban-Arques et al., 2015; Tlaskalova-Hogenova et al., 2014). In fact, germ-free animals show a deficient development of the immune system and have an immature GALT (Ha et al., 2014).

In addition, beneficial bacteria break down and ferment indigestible dietary fiber, resulting in the production of large amounts of short-chain fatty acids (SCFAs). Among SCFAs, butyrate has received particular attention due to its multiple beneficial effects on the health of the intestinal tract and peripheral tissues of vertebrates, including fish. Butyrate has anti-inflammatory effects on the colon and stimulates the immune system of fish (Piazzon et al., 2017; Rimoldi et al., 2016; Terova et al., 2016). For instance, in grass carp (*Ctenopharyngodon idella*), administration of sodium butyrate leads to upregulation immune-related gene expression and downregulation of inflammatory and proinflammatory genes at the intestinal level (Tian et al., 2017). It is therefore undeniable that the content and diversity of the intestinal microbiota are strictly related to the general health of the host and its ability to resist infections and environmental stressors.

One strategy for improving intestinal immunity and ameliorating the composition of the commensal microbiota is the use of functional additives. The most important of these are probiotics, prebiotics, phytonutrients, and organic acids. Several studies have shown that functional diets containing different bioactive compounds can improve growth (Rimoldi et al., 2018a), gut health (Estensoro et al., 2016; Nimalan et al., 2022; Rimoldi et al., 2020a, 2020b; Torrecillas et al., 2018, 2019), and immunity of fish (Fernández-Montero et al., 2021; Moroni et al., 2021; Piazzon et al., 2017; Serradell et al., 2020; Terova et al., 2016) and shrimp (Kesselring et al., 2021); thereby mitigating the negative side effects caused by the replacement of marine ingredients in the diet.

Most probiotics used in aquaculture belong to the Firmicutes phylum, particularly lactic acid-producing bacteria (LAB) and *Bacillus* spp. The modes of action of probiotics include exclusion of pathogens, improvement of feed digestion, absorption of macro- and micro-nutrients, improvement of immune response, and production of antimicrobial and functional compounds (El-Saadony et al., 2021; Simón et al., 2021). Recently, we showed that dietary administration of the probiotic strain *Lactococcus lactis* subsp. *lactis* SL242 stimulates the expression of interleukins *il-10* and *il-12*, and modulates the sea bream (*Sparus aurata*) gut microbiota even without the colonization of the probiotic in the host's intestinal mucosa (Moroni et al., 2021).

In recent years, our research has contributed significantly to the knowledge of the efficacy of additives in aquafeeds for the two most important species for aquaculture in the Mediterranean Sea: European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*). In

particular, we found a positive effect of prebiotic and phyto-genic additives on both immune response and gut microbiota in sea bass fed low FM and FO diets (Rimoldi et al., 2020b; Torrecillas et al., 2021, 2019). Our findings suggest that dietary intake of galactomannan oligosaccharides and phytonutrients induces changes in the gut microbiota, modulates the expression of oxidative enzyme-related genes, and attenuates the stress response of fish. However, proper development of the aquaculture sector involves not only effective replacement of marine raw materials with novel substitutes, but also a successful breeding program to improve growth and feed utilization. Several strains of fish that can tolerate novel feeds with low (Belghit et al., 2019; Boudry et al., 2021; Gjedrem et al., 2012) or even zero amounts (Abernathy et al., 2017; Brezas and Hardy, 2020; Callet et al., 2017) of marine raw materials have already been developed.

Accordingly, the present study aimed to verify whether selected fish genotypes can benefit from the use of functional additives in aquafeeds by addressing the limitations of FM/FO availability in terms of improving fish growth performance, gut health, and disease resistance. To this end, three different types of additives (probiotics, organic acids, and phytonutrients) were separately added to the feed and tested on two batches of juvenile European sea bass: selected high growth fish (HG) and wild types (WT). Fish growth performance, immune-related gene expression, gut microbiota composition, and disease resistance were evaluated for these two fish strains.

## 2. Materials and methods

### 2.1. Ethical statement

The animal experiments complied with the European Union Council Directives (2010/63/EU) for the use of experimental animals. All protocols used in the present study were approved by the Bioethics Committee of the University of Las Palmas de Gran Canaria (OEBA ULPGC 11/2020).

### 2.2. Fish and diets

Two batches of juvenile European sea bass, selected high growth population (HG; 32 selected sires x 7 selected dams), and "wildtype" population (WT; 33 wild sires x 7 selected females) produced at MARBEC-IFREMER, were reared in the facilities of the Parque Científico-Tecnológico Marino (PCTM) at the University of Las Palmas de Gran Canaria (Telde, Canary Islands, Spain). The mating scheme of the genotypes HG and WT was described in detail by Montero et al. (2023). Briefly, seven dams were derived from experimental broodstocks selected for growth for three generations. Eggs were collected by stripping and pooled equally between dams. The batch HG was obtained by *in vitro* fertilization with thawed semen from 33 selected sires (breeding company EMG Ecloserie Marine de Gravelines) for multi-trait selection (growth, morphology, and low muscle fat content) over seven generations (>35 years). The genotype WT was bred using sperm from 32 wild European sea bass from the western Mediterranean caught in the Gulf of Lion. One-day-old hatched larvae from each batch of fish (HG or WT) were pooled in equal numbers from each dam and sent to the University of Las Palmas de Gran Canaria (ULPGC; Las Palmas de Gran Canaria, Spain). Selected (HG) or reference fish (WT) were maintained under similar conditions during the pre-weaning, weaning, and early juvenile phases. At 294 days post-hatching (dph), after the juveniles had reached a mean body mass of 10 g, they were nutritionally challenged. The fish were fed a "future diet" that represented our control diet, in which the total amount of FO was replaced by a combination of poultry oil (PO) and DHA-algae oil and 50% of FM by poultry meal (PM) (Skretting ARC, Norway), up to an initial experimental size of 16 g. The formulation and proximate composition of the "future" or control diet are shown in Table 1. Proprietary functional additives were produced by INVE Aquaculture (Belgium) and consisted of a probiotic mixture (PROB), a

**Table 1**

Ingredients and proximal composition of the reference “Future” diet (CTRL).

| Ingredients (%)                            |       |
|--|-------|
| Corn gluten                                | 5.0   |
| Hi Pro Soybean meal <sup>a</sup>           | 6.0   |
| Wheat gluten                               | 10.2  |
| Faba bean dehulled <sup>b</sup>            | 8.0   |
| Wheat                                      | 19.95 |
| Soy protein concentrate <sup>c</sup>       | 15.0  |
| Fish oil <sup>d</sup>                      | 0.0   |
| Fish meal <sup>e</sup>                     | 10.0  |
| Rapeseed oil                               | 8.98  |
| Phosphate                                  | 0.35  |
| Vitamin & mineral mix <sup>f</sup>         | 0.3   |
| Poultry meal <sup>g</sup>                  | 10.0  |
| Poultry oil <sup>h</sup>                   | 1.37  |
| DHA oil <sup>i</sup>                       | 2.75  |
| Lecithin                                   | 2.0   |
| <b>Proximal composition (% dry matter)</b> |       |
| Moisture                                   | 6.25  |
| Crude protein                              | 51.18 |
| Crude fat                                  | 17.67 |
| Ash  | 4.57  |

Yttrium premix: 0.1%

<sup>a</sup> Soya bean meal: CJ Selecta S.A (Brasil)<sup>b</sup> Faba beans: Cefetra BV (The Netherlands)<sup>c</sup> Soya protein concentrate: CJ Selecta S.A (Brasil)<sup>d</sup> Fish oil: Copeinca, S. A. (Perú)<sup>e</sup> Fish meal: Norsildmel AS (Norway)<sup>f</sup> Mineral and Vitamin premix: Trouw Nutrition (The Netherlands)<sup>g</sup> Poultry meal: Sonac (Belgium)<sup>h</sup> Poultry oil: Sonac (Belgium)<sup>i</sup> DHA: Veramaris (Evonik)

mixture of organic acids (ORG), and natural plant extracts (PHYTO). Specifically, the probiotic mixture (INVE, Belgium) contained three *Bacillus* species: *B. subtilis*, *B. licheniformis*, and *B. pumilus* at a total bacterial concentration of  $2 \times 10^{10}$  CFU (colony forming units)/g of product and with each of the bacterial species in an equal ratio. The organic acid booster was formulated based on the knowledge and known benefits of butyric acid and consisted of 70% butyric acid sodium salt of (GBM CMR, Sanluc, Belgium), while the phytogetic booster consisted of a robustness-enhancing additive based on 16% natural plant extracts of garlic combined with medium-chain fatty acid sources (Aquagarlic P Protec, Domca, Spain). After the acclimation period, fish were randomly distributed to 24 tanks of 500 l (34 fish/tank; 12 tanks per genotype;  $19.0 \pm 0.4$  g) and fed until visual satiation with the future diet (CTRL) or with the future diet containing three different functional additives (INVE Aquaculture, Belgium): (i) 2 weeks with high dose, followed by (ii) 10 weeks with low dose (Fig. 1). The experimental feed additives

were hand-oil-top-coated at two doses: low and high. For the PROB feed, the high and low doses were set at 10 g/kg and 2 g/kg, respectively. For the ORG feed, 7.5 g/kg corresponded to the high dose and 3 g/kg to the low dose, while natural plant extracts (PHYTO) were coated at 7.5 g/kg and 5 g/kg.

Growth performance parameters were calculated for both feeding periods (high and low doses). Before each sampling, fish were fasted for 24 h, anaesthetized with clove oil (4 ml clove oil / 100 l water) and measured individually. Growth parameters were calculated as follows:

Daily growth index (DGI) = [(final weight<sup>1/3</sup> - initial weight<sup>1/3</sup>)/number of days x 100];

Specific growth rate (SGR) = [Ln (final weight) - Ln (initial weight)] / number of days x 100].

At each sampling point, 3 fish per tank (three tanks/feeding treatment) were euthanized with an overdose of anaesthetic (clove oil) and samples were collected at the end of the feeding experiment (12 weeks) for gut microbiota characterization (autochthonous) and gene expression analysis. After each dose feeding, a pathogen (*Vibrio anguillarum*) challenge test was performed in conjunction with stress conditions (overcrowding), as previously described (Serradell et al., 2020), to investigate the potential of additives to improve the resistance of fish exposed to both infection and stress conditions. Briefly, this stress test consisted of keeping fish submerged in 25.6-litre cages (40 cm x 20 cm) for one week (three tanks/feeding treatment) for one week and exposing them to *V. anguillarum* (CI) ( $10^5$  CFU per fish) by anal inoculation. For each feeding treatment, the cages were distributed to 6 cylindrical conical 500-liter tanks in a recirculating aquaculture system (RAS) supplied with filtered water at a temperature of 22 °C. The water renewal rate of RAS was one per hour (500 l/hour), while aeration consisted of pumping air and, if necessary, automatically adding oxygen. Fish mortality was monitored during the experiment and relative survival (RPS) was calculated at the end of the experiment using the following equation: RPS = [1 - mortality of fish fed the diet with additives (%) / mortality of fish fed the control diet (%)] x 100. Fish were fed the respective diets during the challenge test and fish survival was recorded daily.

### 2.3. Metabarcoding analysis of gut microbial communities

#### 2.3.1. Sampling and bacterial DNA extraction

At the end of the feeding experiment (12 weeks), intestinal samples were collected from six fish in each feed/batch group were collected (48 samples in total). Feces were removed from each intestine by careful squeezing, and mucosa-associated microbiota (autochthonous microbiota) was obtained by scraping the intestinal mucosa (without pyloric ceca) with a sterile cotton swab. The tip of the swab was immediately

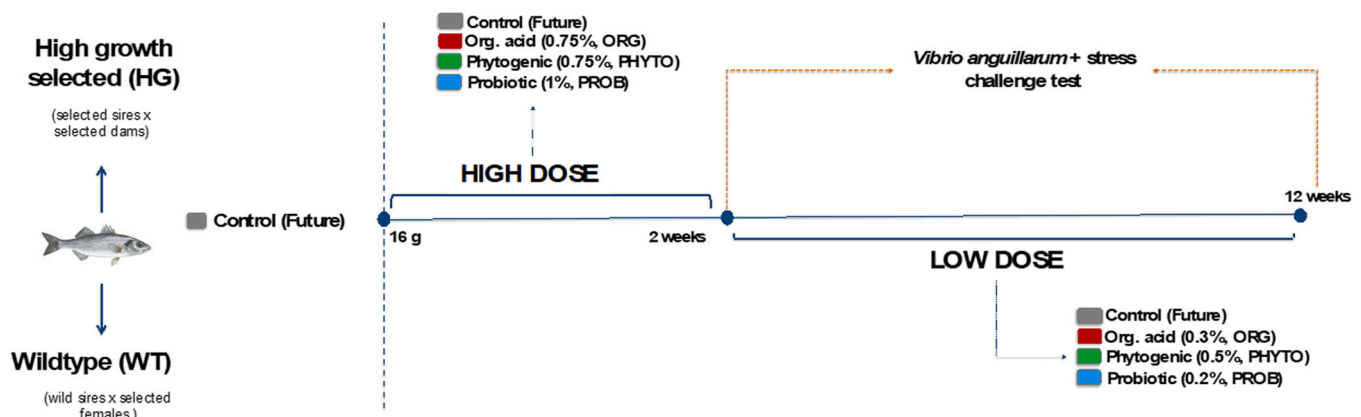


Fig. 1. Graphical schematic of the experimental design.

immersed in 300  $\mu$ l Xpedition Lysis/Stabilization Solution (Zymo Research, Italy) and vortexed to facilitate bacterial release (Rimoldi et al., 2019). The tips of the swab and the solution were stored at 4 °C until DNA extraction.

DNA was extracted from 250  $\mu$ l of gut bacterial suspension and from 200 mg of each feed (three aliquots/feed) using a DNeasy PowerSoil® Pro kit (Qiagen, Milan, Italy), according to the manufacturer's instructions. The concentration and purity of DNA were measured using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Milan, Italy). Bacterial DNA was stored at – 20 °C until amplicon library preparation.

### 2.3.2. 16S library preparation and MiSeq amplicon sequencing

Library preparation and sequencing on the Illumina MiSeq platform (Illumina, Italy) were performed by GalSeq SRL (Milan, Italy). The details of the methodology used for 16 S rRNA gene library preparation and sequencing have been reported previously (Terova et al., 2021). To identify the bacterial taxa in the gut, the hypervariable region V4 of the bacterial 16 S rRNA gene was amplified using aliquots of the isolated DNA from each sample and the oligonucleotides 515 F:5'-GTGY CAGCMGCCGCGGTAA-3' and 806 R:5'-GGACTACNVGGTWTCTAAT-3'. The expected size of the PCR products on the Agilent 2100 Bioanalyzer lane was ~ 400 bp. Amplicon libraries were quantified by qPCR, pooled at equimolar concentrations, diluted at 6 pM, multiplexed, and sequenced on an Illumina MiSeq instrument using a pair-ended sequencing strategy (2 × 250). All sequences were submitted to the European Nucleotide Archive (EBI ENA).

### 2.3.3. Bioinformatic analysis of raw sequencing data

The obtained reads were quality filtered (Q > 30), merged, and processed with the QIIME 2™ (v. 2018.4) pipeline using default settings (Bolyen et al., 2019). The remaining high-quality reads were dereplicated, and singletons and chimeric sequences were removed using the QIIME DADA2 denoise-paired command. The output of the DADA2 pipeline was a feature table of amplicon sequence variants (ASV table) in which the number of times each ASV occurred in each sample was recorded.

Taxonomic assignment was based on the Silva database (<http://www.arb-silva.de>) at the genus level. All ASVs assigned to chloroplasts and mitochondria were removed from the analysis because they were of eukaryotic origin. The alpha (within a single sample) and beta (between samples) diversity of bacterial communities were calculated. In particular, alpha diversity indices (Chao 1, Faith PD, observed ASVs, Shannon, and Simpson) were evaluated at the same level of rarefaction. Beta diversity was calculated using the weighted (presence/absence/abundance) and unweighted (presence/absence) UniFrac distance matrices (Lozupone and Knight, 2005; Lozupone et al., 2007).

To visualize the core microbiota (ASVs present in at least four of the six samples per diet/batch group), a Venn diagram was created using the Venny 2.1 tool (<https://bioinfo.cnb.csic.es/tools/venny/index.html>).

### 2.3.4. Predictive functional analysis of gut bacterial communities

The functional profile of the gut microbiome was predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt1) (Langille et al., 2013). The 16 S rRNA gene data were referenced according to the Greengenes 13.8 database, and the resulting data were used for prediction analysis with PICRUSt via the Kyoto Encyclopedia of Genes and Genomes (KEGG). The identified KEGG orthology pathways were sorted into functional categories based on the KEGG pathway subsystem at hierarchical level 3. The PICRUSt1 output files (profile and metadata) were uploaded to the Statistical Analysis of Metagenomic Profiles software package (STAMP) (Parks et al., 2014) to generate extended error plots for each pairwise comparison. Welch's two-tailed t-test was used to determine differences between the two groups with 95% confidence.

## 2.4. Gene expression analysis

For quantitative gene expression analysis, 0.5 cm samples of the proximal and distal intestinal regions (n = 6 fish /diet) were collected at the end of the feeding experiment and stored in RNAlater™ stabilization solution (ThermoFisher, Milan Italy) until they were delivered to the laboratory of the Department of Biotechnology and Life Sciences (Varese, Italy), where they were stored at – 80 °C until molecular analysis.

Total RNA was automatically extracted from intestinal samples using the Maxwell® 16 LEV simplyRNA kit (Promega, Milan, Italy) in combination with the Maxwell® 16 instrument (Promega, Milan, Italy). The amount and purity of extracted RNA were determined spectrophotometrically using a NanoDrop™ 2000c spectrophotometer (Thermo Scientific, Milan, Italy). After extraction, 100 ng of RNA was subjected to qPCR using an iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad, Milan, Italy). This kit uses a combination of iScript™ RNase H + reverse transcriptase (to generate complementary DNA) and antibody hot-start iTAQ DNA polymerase to perform SYBR® Green real-time reactions in one step. PCR efficiency is improved over a wide dynamic range and under different conditions. All primer sequences used for quantification of target genes are listed in Supplementary Table 1. The qPCR reactions were run in triplicate on a Bio-Rad® CFX96™ system under the following conditions: 10 min at 50 °C, 1 min at 95 °C, followed by 40 cycles consisting of 10 s at 95 °C and 30 s at 60 °C, followed by a melting curve (65–95 °C). Ct values were analyzed with Bio-Rad CFX Maestro software (Bio-Rad, Milan, Italy) using the 2<sup>- $\Delta\Delta$ CT</sup> method (Livak and Schmittgen, 2001).  $\beta$ -actin was chosen as the housekeeping gene, and Ct values were transformed to a relative amount, using the lowest Ct value as the calibrator.

## 2.5. Statistics

All data were tested for normality and homogeneity of variance. Differences were considered statistically significant at  $p \leq 0.05$ . For quantitative data, individual effects of diet and genomic batch were analysed using two-way ANOVA. To ensure the assumptions required for the parametric tests, the relative abundance values (%) of the bacterial taxa were angle transformed. Analysis of the microbial dataset was performed only for those taxa whose overall abundance exceeded 1% up to the order level and 0.5% at the family and genus levels. The PERMANOVA test for beta diversity (999 permutations) was applied to compare the dissimilarity of microbial communities between groups using UniFrac distance matrices. The dissimilarities between experimental groups and the corresponding VIP values were calculated with Partial Least Squares Discriminant Analysis (PLS-DA) using R software. The remaining analyses were performed with Statistical Software System v21.0 (SPSS, Chicago, IL, USA) and PAST3 software (Hammer et al., 2001).

## 3. Results

### 3.1. Fish growth performance

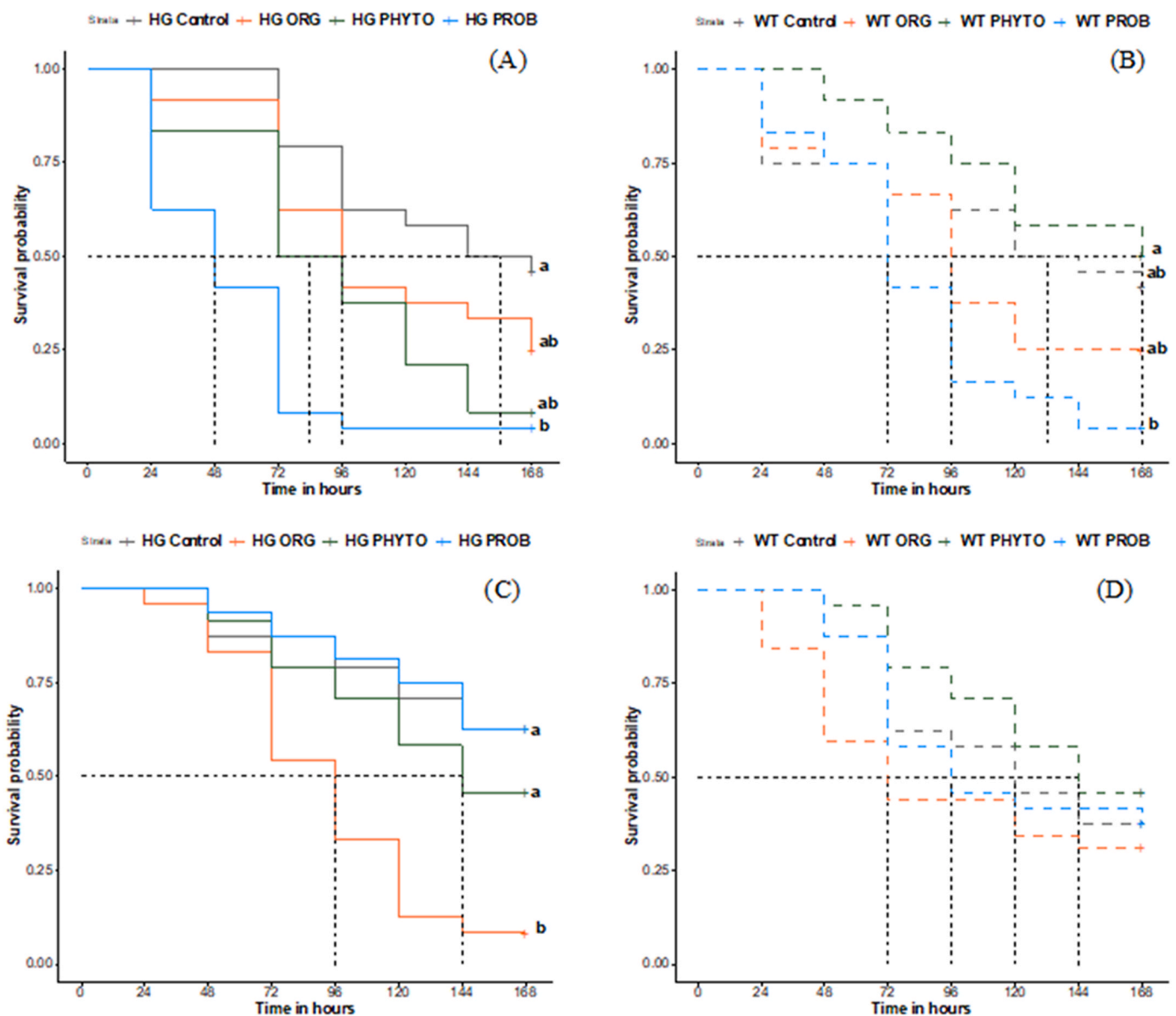
From the two-week feeding period (period of high-dose supplementation) to the end of the feeding experiment (period of high-dose + low-dose supplementation), fish from HG performed better ( $p < 0.05$ ) than fish from WT in terms of body weight, relative growth, SGR, and DGI. Relative weight gain and SGR ranged from 365% to 400% and 1.3–1.4% in the HG groups to 290–310% and 1–1.1% in the WT fish group, respectively (Table 2). Functional feeding treatments only affected the performance of the HG fish at the end of the feeding trial, with fish fed the ORG diet having lower ( $p < 0.05$ ) final body weight than fish fed the control diet, but similar to fish fed the PROB and PHYTO diets (Table 2).

The resistance of fish to *V. anguillarum* after feeding the experimental functional additives during the feeding experiment is shown in Fig. 2. At

**Table 2**

Growth performance and feed utilization of wild (WT) and selected for growth (HG) European sea bass fed the experimental diets. W: mean body weight, WG: relative weight gain; SGR: specific growth rate; DGI: daily growth index.

|        | weeks | HG                          |                             |                            |                             | WT              |               |               |                 | Significance |          |          |
|--------|-------|-----------------------------|-----------------------------|----------------------------|-----------------------------|-----------------|---------------|---------------|-----------------|--------------|----------|----------|
|        |       | CTRL                        | PROB                        | ORG                        | PHYTO                       | CTRL            | PROB          | ORG           | PHYTO           | Diet         | Genotype | D*G      |
| W (g)  | 0     | 19.2 ± 0.3                  | 19 ± 0.2                    | 19.5 ± 0.1                 | 18.5 ± 0.7                  | 18.8 ± 0.3      | 18.9 ± 0.2    | 19.1 ± 0.4    | 19 ± 0.5        | ns           | ns       | ns       |
|        | 2     | 34.2 ± 3.1                  | 37.8 ± 4.4                  | 36.7 ± 0.5                 | 36.1 ± 2.1                  | 32 ± 3.5        | 32.7 ± 3.3    | 29.7 ± 1.7    | 30.8 ± 3        | ns           | p < 0.05 | ns       |
|        | 12    | 79.7 <sup>a</sup><br>± 11.3 | 74.2 <sup>ab</sup><br>± 4.8 | 71.2 <sup>b</sup><br>± 4.4 | 74.7 <sup>ab</sup><br>± 2.6 | 55.9 ± 6.4      | 59.2 ± 2.3    | 57.2 ± 3.6    | 59.4 ± 2        | ns           | p < 0.05 | p < 0.05 |
| WG (%) | 12    | 400.4<br>± 53.7             | 388.1<br>± 24.9             | 364.3<br>± 2.8             | 401.6<br>± 27.8             | 289.8<br>± 37.1 | 307.9<br>± 10 | 302.1<br>± 16 | 311.6<br>± 15.9 | ns           | p < 0.05 | ns       |
| SGR    | 12    | 1.4 ± 0.1                   | 1.3 ± 0.1                   | 1.3 ± 0.01                 | 1.4 ± 0.1                   | 1 ± 0.1         | 1.1 ± 0.01    | 1.1 ± 0.1     | 1.1 ± 0.01      | ns           | p < 0.05 | ns       |
| DGI    | 12    | 18.8 ± 3.6                  | 17.9 ± 1.6                  | 16.9 ± 0.1                 | 18.2 ± 1.1                  | 11.6 ± 2.1      | 13 ± 0.7      | 12.5 ± 1.1    | 13.1 ± 0.7      | ns           | p < 0.05 | ns       |



**Fig. 2.** Cumulative survival (%) of European sea bass (*Dicentrarchus labrax*) in the challenge test against *V. anguillarum* combined with confinement stress after 2 weeks of high dose for genotypes HG (A) and WT (B) and 12 weeks of feeding (2 weeks of high dose + 10 weeks of low dose) for genotypes HG (C) and WT (D). Different letters indicate statistical differences ( $p < 0.05$ ; Kaplan-Meier survival). HG, genetically selected genotype; WT, wild-type genotype of European sea bass. Diets: probiotic mixture (PROB), organic acid mixture (ORG), phytochemical (PHYTO).

the end of the high-dose supplementation period (two weeks; Figs. 2A, 2B), the Kaplan-Meier curve showed a significant diet effect ( $F=3.469$ ,  $p = 0.0411$ ), indicating that fish fed a high dose PROB had a lower ( $p < 0.05$ ) probability of survival than fish fed the CTRL diet, regardless of the batch of fish. After long-term supplementation (2 weeks high dose +10 weeks low dose), the results differed between fish families (Figs. 2C, 2D). HG fish fed the ORG diet showed the lowest survival rate ( $p < 0.05$ ) compared to the other treatments (Fig. 2C), while no significant differences were found between the experimental groups in WT fish, indicating an effect of genotype on the use of the tested additives (Fig. 2D).

### 3.2. Gut expression level of inflammation- and immune- related genes

In the proximal intestine, administration of any of the tested additives (PROB, PHYTO, and ORG) did not affect the expression of target genes compared to control fish. However, there was a significant effect of genotype batch ( $p < 0.05$ ); expression of *cd4*, *cox-2*, and *il-10* was downregulated in HG compared to WT fish, regardless of diet (Fig. 3). In contrast, neither diet nor genotype affected transcript levels of the *mhc-II*, *il-1 $\beta$* , and *tnf- $\alpha$*  genes in the proximal intestinal tract (data not shown). In contrast, there was an interaction effect between genotype and diet on *il-1 $\beta$*  gene expression in the distal intestine (Fig. 4). Selected fish fed a probiotic-supplemented diet showed upregulation of *il-1 $\beta$*  compared with the same batch fed CTRL or OA. No differences were found between experimental feeding groups in the expression levels of *cd4*, *tnf- $\alpha$* , and *il-10* (data not shown), while the genes *mhc-II* and *cox-2* showed a batch effect and were significantly ( $p < 0.05$ ) higher expressed in HG and WT fish, respectively (Fig. 4). However, there is no evidence of a correlation between gut microbiome profiles and host GALT gene expression.

### 3.3. Metabarcoding analysis outputs

All Illumina sequence files (FASTQ format) were deposited in the public database of the European Nucleotide Archive (EBI ENA) under

accession code **PRJEB61519**.

A total of 840,441 reads were correctly classified using the database SILVA database. The Good's coverage value for all samples was  $\geq 0.99$ , indicating that sequencing coverage was achieved and the ASVs found were representative of the microbial communities in feed and gut. To calculate the alpha diversity metrics, the feed and gut mucosal samples were normalized at a sequencing depth of 10,000 reads. The alpha diversity metrics are shown in Table 3. Statistical analysis using a two-way ANOVA showed no significant differences in species richness and diversity between the groups.

Beta diversity analysis revealed no overall effect of genetic batch and/or diet on microbial community profiles (Fig. 5A, B). Permutational multivariate analysis using Permanova's test with 999 permutations on weighted ( $F = 0.02$ ;  $p = 0.926$ ) and unweighted ( $F = 0.284$ ;  $p = 0.639$ ) UniFrac distance data fully confirmed the PCoA results.

Also, PLS-DA showed no clear separation between CTRL and fish fed additives, regardless of their genetic background (Fig. 6A, B). However, when we considered only the effect of genetic background, less inter-individual variability was observed at HG (Fig. 6A).

### 3.4. The feed-associate microbiota

The microbiota profile of the feed is outlined at the phylum, class, order, family, and genus levels. All samples included six different phyla, nine classes, 22 orders, 32 families, and 38 genera. However, when considering only the most abundant bacterial taxa, there were two phyla, three classes, 10 orders, 18 families, and 19 genera. The composition of the feed-associated microbiota was reported at the phylum and genus levels (Fig. 7). The relative abundances (%) of the most abundant taxa found in the feed samples and their statistical significance are shown in Supplementary Table 2. At the phylum level, the feeds showed a similar profile, with a higher proportion of Proteobacteria (63–74%) followed by Firmicutes (24–35%) (Fig. 7A). As expected, the highest abundance of the genus *Bacillus* was found in the probiotic-

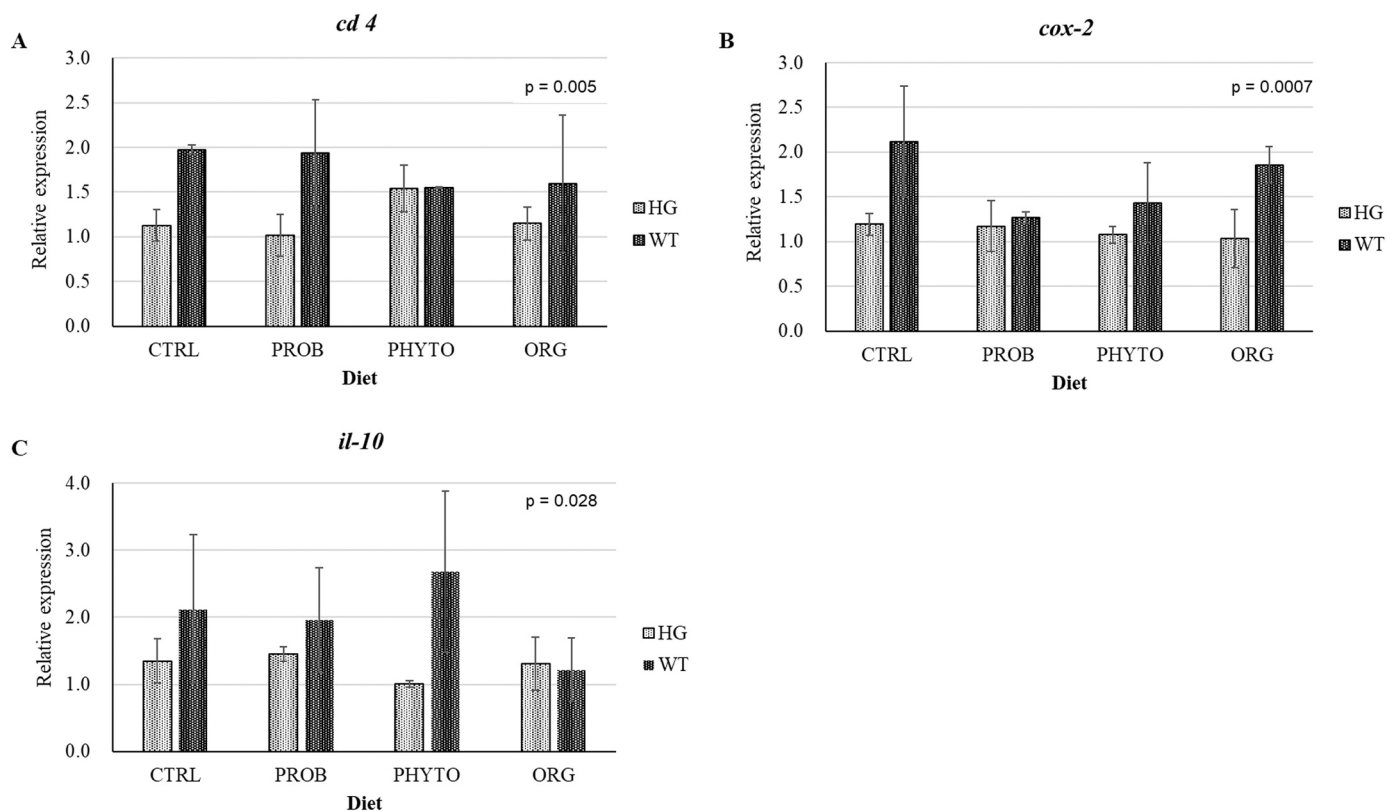


Fig. 3. Gene expression data in proximal gut. Data are mean  $\pm$  SD.

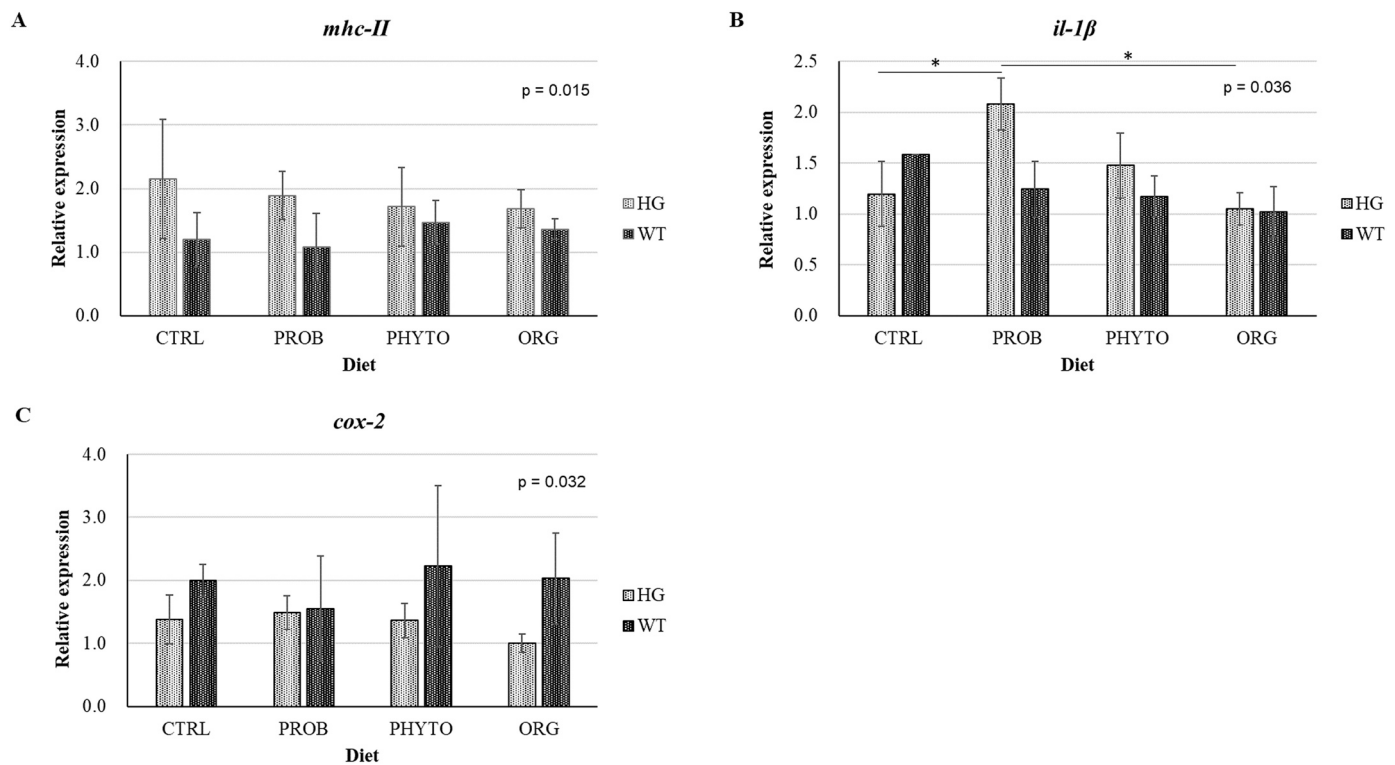


Fig. 4. Gene expression data in the distal intestine. (\*) indicates significant difference between means ( $p < 0.05$ ). Data are mean  $\pm$  SD.

Table 3

Alpha diversity metrics of mucosa-associated microbial communities. The values are reported as mean values ( $n = 6$ )  $\pm$  SD. The means were compared by two-way ANOVA test ( $p < 0.05$ ).

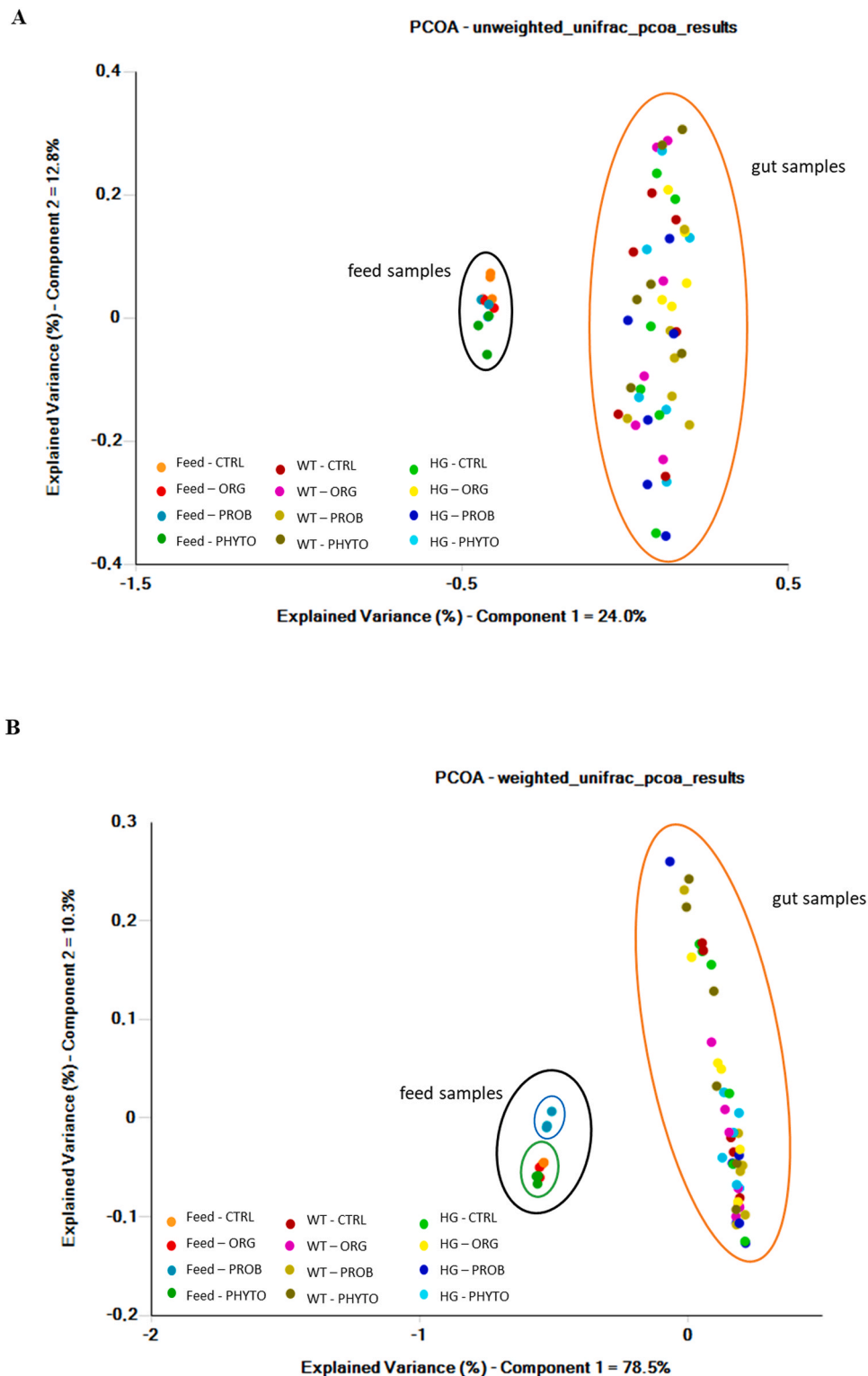
|                     |       | Chao 1          | Faith PD        | Observed OTUs   | Shannon         | Simpson         |
|---------------------|-------|-----------------|-----------------|-----------------|-----------------|-----------------|
| WT                  | CTRL  | 658 $\pm$ 281   | 9.1 $\pm$ 2.7   | 560 $\pm$ 238   | 6.2 $\pm$ 1.4   | 0.93 $\pm$ 0.04 |
|                     | ORG   | 602 $\pm$ 249   | 10.0 $\pm$ 4.2  | 500 $\pm$ 215   | 5.8 $\pm$ 1.2   | 0.92 $\pm$ 0.04 |
|                     | PROB  | 429 $\pm$ 178   | 7.3 $\pm$ 1.8   | 372 $\pm$ 169   | 5.3 $\pm$ 1.5   | 0.90 $\pm$ 0.05 |
|                     | PHYTO | 730 $\pm$ 219   | 11.0 $\pm$ 3.7  | 631 $\pm$ 209   | 6.7 $\pm$ 1.4   | 0.95 $\pm$ 0.04 |
| HG                  | CTRL  | 537 $\pm$ 293   | 8.3 $\pm$ 4.1   | 469 $\pm$ 253   | 6.0 $\pm$ 1.4   | 0.93 $\pm$ 0.05 |
|                     | ORG   | 665 $\pm$ 190   | 9.6 $\pm$ 0.9   | 558 $\pm$ 160   | 6.2 $\pm$ 1.4   | 0.93 $\pm$ 0.05 |
|                     | PROB  | 465 $\pm$ 272   | 7.4 $\pm$ 2.5   | 384 $\pm$ 238   | 5.1 $\pm$ 1.2   | 0.89 $\pm$ 0.04 |
|                     | PHYTO | 463 $\pm$ 85    | 7.9 $\pm$ 2.1   | 406 $\pm$ 82    | 5.6 $\pm$ 0.7   | 0.92 $\pm$ 0.03 |
| <b>Significance</b> |       | Diet: 0.231     | Diet: 0.211     | Diet: 0.241     | Diet: 0.231     | Diet: 0.156     |
|                     |       | Genotype: 0.254 | Genotype: 0.219 | Genotype: 0.270 | Genotype: 0.412 | Genotype: 0.617 |
|                     |       | D*G: 0.293      | D*G: 0.610      | D*G: 0.355      | D*G: 0.540      | D*G: 0.896      |

containing feed. Overall, the microbial community profile of the probiotic-containing feed differed most from that of the control.

### 3.5. Dietary gut microbiota modulation

The microbiota of the 48 intestinal mucosal samples was mainly composed of six phyla, 11 classes, 34 orders, 53 families, and 101 genera. Considering only the most representative taxa, it consisted of four phyla, six classes, 15 orders, 28 families, and 25 genera. The gut microbial community profiles for each feeding group are shown in Fig. 8 at the phylum, family, and genus levels. As expected, Firmicutes and Proteobacteria were the most abundant phyla in the sea bass gut (Fig. 8A) and accounted for more than 90% of the sequencing reads in all experimental groups (Supplementary Table 3). Regardless of the diet administered, the core microbiota was identified in both HG and WT samples. In both batches, the majority of genera were represented in all dietary groups (Fig. 9A). Even when only genotype was considered, the core microbiota was similar between the two fish strains, which shared 19 genera (Fig. 9B). Only six and four genera were exclusive to WT and HG, respectively.

The results of the two-way ANOVA showed an influence of diet and genotype on the relative abundance of certain taxa. However, the interaction effect between the two main factors (diet and genotype) was significant only in rare cases (Supplementary Table 3). When the main effects of diet and genotype were considered, the former was clearly more significant. At the phylum and class levels, statistical analysis by two-way ANOVA revealed no significant differences in relative taxa abundance. Dietary additive intake affected gut microbiota profiles at the family (Sphingomonadaceae, Moraxellaceae, Pseudomonadaceae) and genus (*Streptococcus*, *Pseudomonas*, *Sphingobium*, and *Novosphingobium*) levels. A significant interaction between diet and genotype was found at the order level for Lactobacillales, resulting in higher abundance in fish fed the PHYTO diet (Supplementary Table 3). Significant genotype-diet interactions were also observed for the family Weeksellaceae (enriched in WT-PHYTO) and for the genera *Enterovibrio* (enriched in HG-PHYTO) and *Acinetobacter* (lower in HG-PROB and WT-PROB). In general, the relative abundance of *Acinetobacter* and *Pseudomonas* was reduced in the guts of fish fed probiotics compared to controls for both strains (Supplementary Table 3, Figs. 8B, 8C). Probiotics and phytochemicals negatively affected the abundance of *Novosphingobium* and



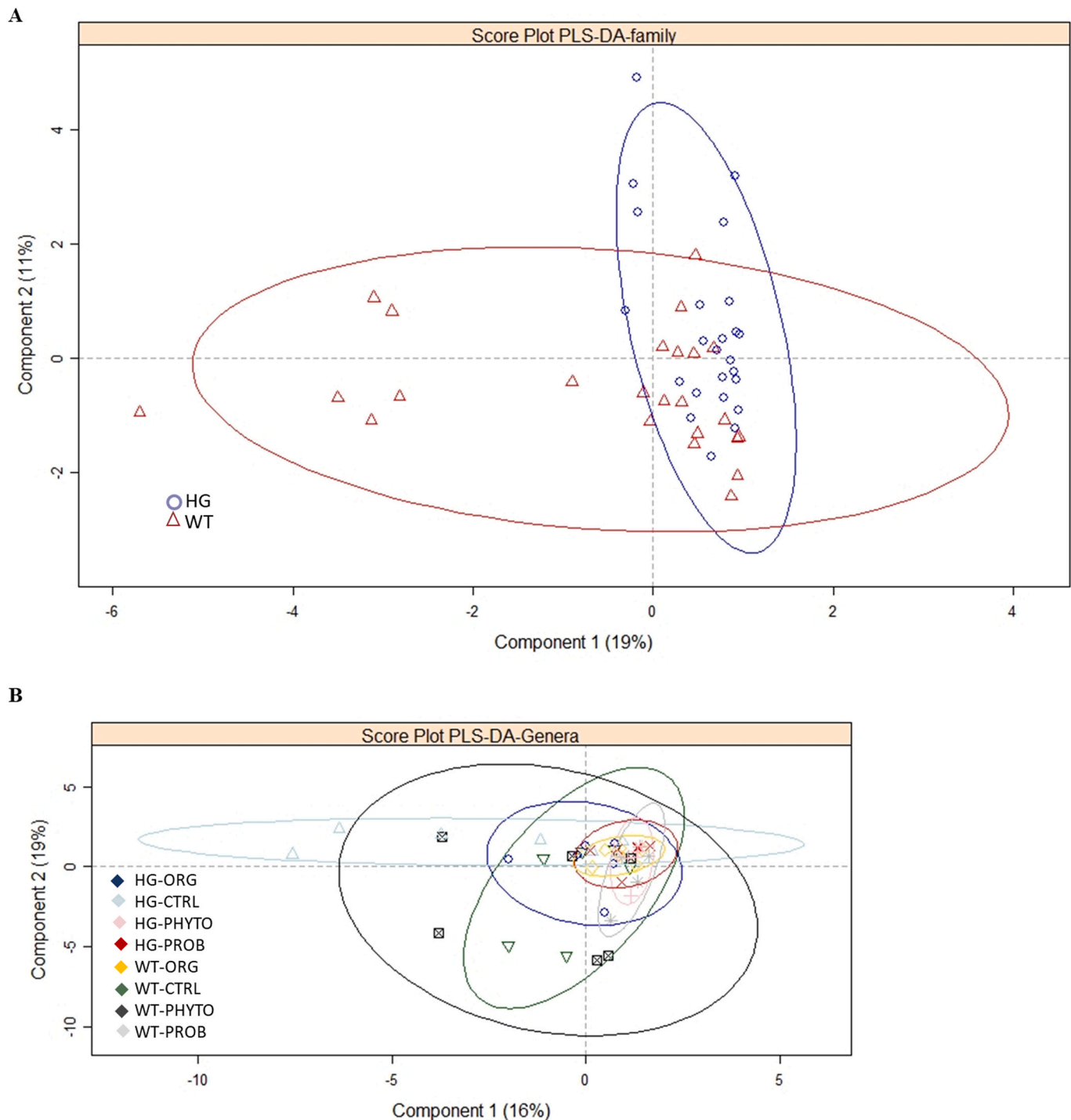
**Fig. 5.** Analysis of beta diversity between groups. (A) Unweighted and (B) weighted UniFrac PCoA plots of individual feed and gut samples from each group. Individual sample was represented as spot.

*Sphingobium* (Supplementary Table 3, Fig. 8C). Dietary supplementation with organic acids increased the relative abundance of *Streptococcus*, regardless of genotype, while bacteria of the genus *Photobacterium* were preferentially associated with WT sea bass (Supplementary Table 3, Fig. 8C).

### 3.6. Predictive functional profile of gut microbial communities

A functional profile of the gut microbiota was predicted using the PICRUSt tool. Functional analysis of the samples from WT samples showed higher abundance of carbohydrate metabolism when supplemented with organic acids compared to controls (Fig. 10A). In response to the PHYTO diet, nucleotide metabolism improved (Fig. 10B), whereas





**Fig. 6.** Partial least square-discriminant analysis (PLS-DA) based on relative abundances of bacterial genera in the gut microbiota of the final samples. GS, genetically selected genotype; WT, wild-type genotype of European sea bass.

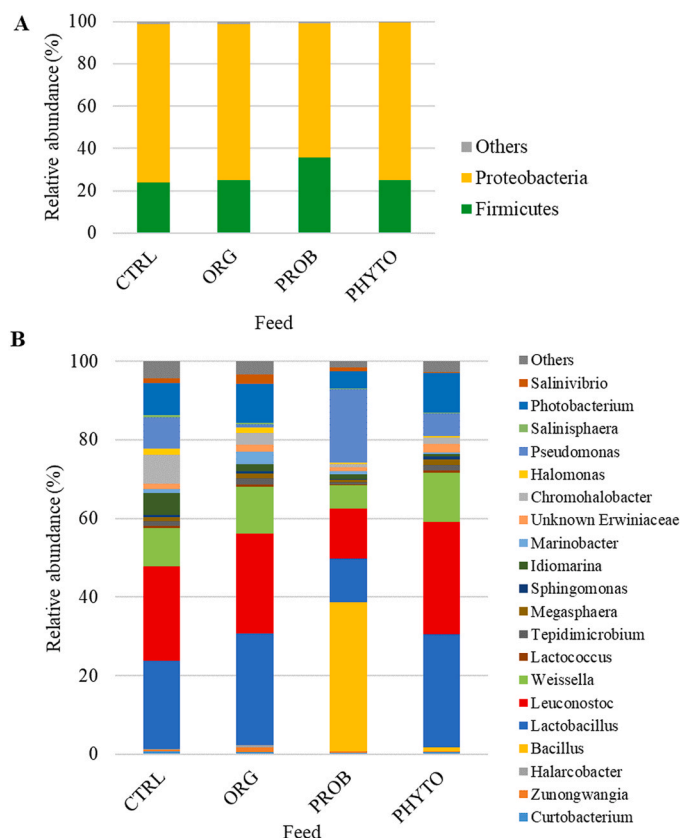
probiotic supplementation resulted in significant enrichment of DNA replication, ribosome biogenesis, and translation factor pathways (Fig. 10C).

In HG fish, metabolic pathways related to carbohydrate metabolism were more prominent in fish fed additives than in control fish. In particular, galactose metabolism and transport pathways were enriched in the gut microbiota of the HG-PHYTO (Fig. 11A) and HG-PRO (Fig. 11B) groups. In addition, the HG-PHYTO group had a higher abundance of metabolic pathways related to energy metabolism. Finally, a diet supplemented with organic acids resulted in enrichment

of bacterial carbohydrate metabolism in genetically selected sea bass (Fig. 11C).

#### 4. Discussion

The challenge of FM/FO replacement should be addressed by the aquatic feed industry with a strategy that includes a combination of technology, “complementary” raw materials, and innovation in selective breeding, rather than identifying individual substitutes (Turchini et al., 2019). In this context, the AquaIMPACT project (Horizon 2020), which



**Fig. 7.** Mean relative abundance (%) of bacteria most abundant in feed at phylum (A) and genus (B) taxonomic levels (N = 3). Only bacteria with a total abundance of 0.5% were reported. Bacteria with lower abundance were grouped together and reported as “other”.

funded the present study, aims to integrate fish breeding and nutrition strategies to improve the competitiveness of European aquaculture and ensure a high-quality end product with limited environmental impact.

The aim of this study was to investigate the effects of a future diet supplemented with various functional additives on two different genotypes of sea bass. Effects on growth, host transcriptome, and resistance to vibriosis, and gut microbiota composition, were evaluated. The composition of the gut microbiota is strictly diet-dependent and has a major impact on host health. Therefore, evaluating the effects of novel formulations and functional additives on gut microbial communities of fish is critical for validating current feeding strategies in aquaculture. To date, high-throughput sequencing is the best strategy to characterize the profile of the gut microbiota of European sea bass in response to novel feeds (Busti et al., 2020; Pérez-Pascual et al., 2020; Rimoldi et al., 2020b; Serra et al., 2021).

In agreement with our previous study conducted with the same batches of fish, the genotype HG gave the best results in terms of growth and feed intake after 10 weeks at a low dose of feed additives (Montero et al., 2023). The present experiment confirmed that selected sea bass had a higher utilization capacity for a future diet poor in marine components than WT fish. Interestingly, in the HG group, only the fish fed PROB had a lower final weight than the fish in the control group. This is contrary to the widely held notion that the use of probiotics in aquaculture can improve fish survival, growth and health. In sea bream, administration of *Lactococcus lactis* did not improve feed conversion or specific growth rates, but had a positive effect on the final body weight of fish fed higher doses of probiotics compared to the control group (Moroni et al., 2021). Similarly, growth performance of turbot (*Psetta maxima*) was significantly improved when fish were fed different *Bacillus* species (*B. subtilis*, *B. licheniformis*, and *B. siamensis*), and the best

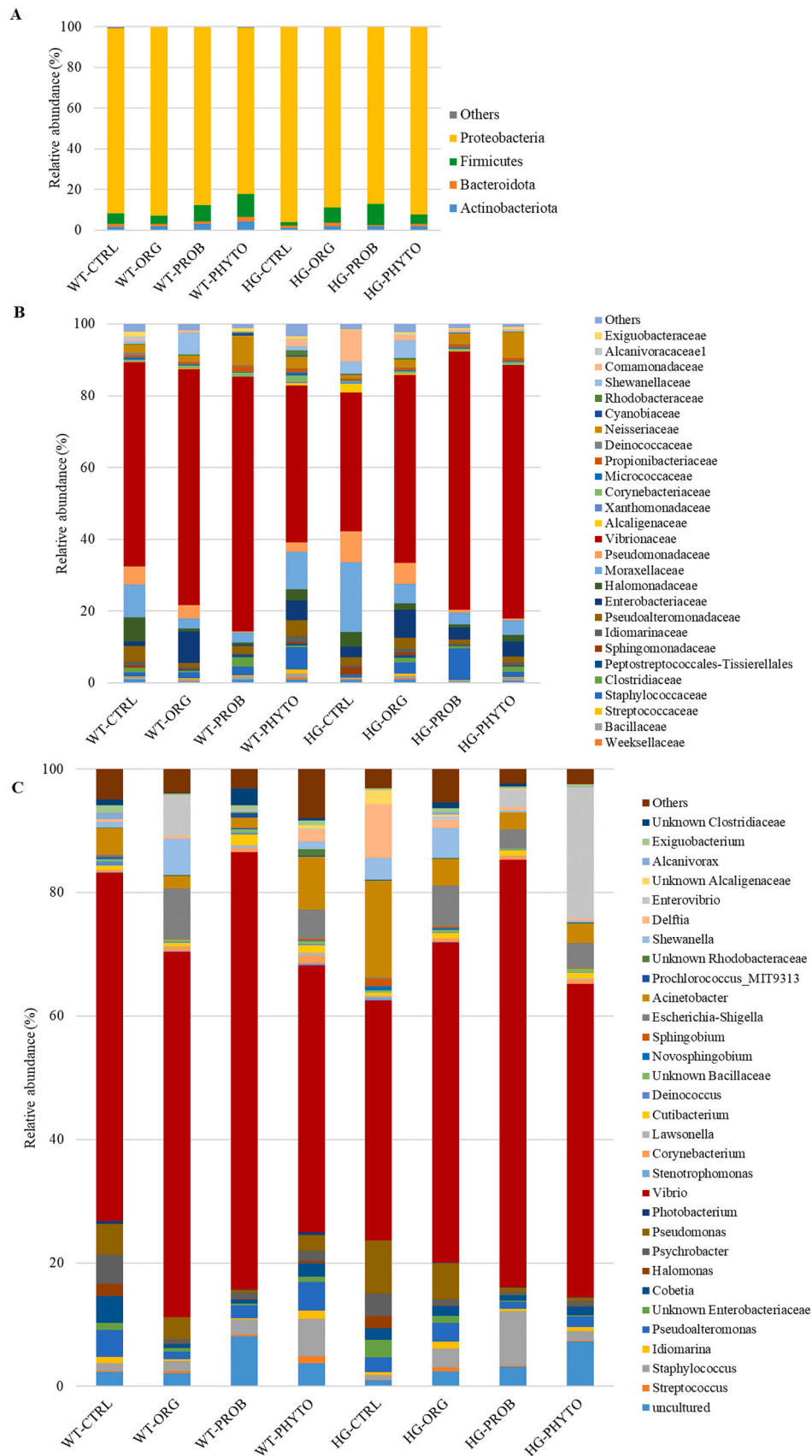
results were obtained with *B. licheniformis* supplementation (Ma et al., 2022). However, contrary to all expectations, survival of fish exposed to pathogenic *V. anguillarum* in combination with overcrowding stress was lower in fish fed high-dose probiotic supplementation than in controls, regardless of genotype. In contrast to our results, a recent study on sea bass reported that *Bacillus velezensis* in the diet improved fish survival to *V. anguillarum* infection (Monzón-Atienza et al., 2022). The negative effect of the PROB diet on fish survival was reversed in the final stress test conducted at the end of 12 weeks of feeding after the phase of low-dose administration (2 weeks high-dose + 10 weeks low-dose). It is difficult to explain this unexpected reaction. Indeed, most probiotic modes of action have been observed in *in vitro* experiments, and the efficiency of a probiotic selected *in vitro* may change significantly when administered *in vivo*. In the host, probiotic organisms are influenced by complex factors, such as gut microbial interactions and/or the nutritional environment. Furthermore, probiotics may be more or less effective depending on the dose used (Moroni et al., 2021); however, the use of probiotics with multiple strains, as in the present study, should provide more benefit to the host than probiotics with only one strain (Puvanasundram et al., 2022; Shadrack et al., 2021).

After 12 weeks of feeding, fish fed the diet ORG had the lowest survival rates regardless of genotype, while survival rates were better for selected fish than for WT with all other additives.

Organic acids have long been used as feed additives in terrestrial livestock production, especially in swine and poultry nutrition (Khan et al., 2022; Nguyen et al., 2020). Their application in aquafeed, on the other hand, is new and little researched. Organic acids have been used as feed preservatives for centuries due to their antimicrobial activities (Ng and Koh, 2017). Organic acids are widely known as antibacterial, immune enhancing, and growth promoting agents in terrestrial animals. Fabay et al. (2022) recently studied the effects and importance of dietary supplementation with organic acids in farmed fish and concluded that their beneficial effects depend on the type and dose of organic acids as well as the species of fish used. Similar to the present study, sea bream fed a specific combination of short and medium fatty acids monoglycerides showed no effect on growth performance, but economic FCR improved compared to controls (Rimoldi et al., 2018a). Similarly, organic acids combined with natural identical compounds (thymol and vanillin) at the inclusion dose tested did not promote growth, feed conversion, or feed intake in juvenile sea bass reared under normal and suboptimal environmental conditions (Busti et al., 2020). In contrast, coconut oil, which is particularly rich in lauric acid (C12), improved growth and feed intake of sea bream (Simó-Mirabet et al., 2017). In addition to the growth performance of the fish, no antibacterial effects were observed in fish fed the diet ORG, and they showed lower survival to *V. anguillarum* infection at the end of the feeding trial. Also, in a parallel experiment with sea bream, testing the same diet and functional additives, no significant effect of the diet on the growth and survival rate of the fish was observed. Even in this case, only an effect of genotype was observed; in particular, the selected fish performed better than the reference group (data not published).

Regarding the expression of immune-related genes, we found upregulation of *cd4*, *cox-2*, and *il-10* genes in the proximal intestine in a genotype-dependent manner, resulting in higher expression in HG fish, regardless of diet type. In contrast, an interaction effect between diet and genotype was observed in the distal part of the intestine, leading to an increase in *il-1 $\beta$*  expression in selected fish fed a probiotic diet.

Accordingly, significant intestinal upregulation of the pro-inflammatory cytokine *il-8* and anti-inflammatory cytokines *il-10* and *tgf- $\beta$*  was observed in sea bass at increasing levels of dietary additives, particularly citric and sorbic acids and nature-identical compounds (Busti et al., 2020). The highest expression levels of *il-10* and *il-12* were observed in sea bream fed a high-dose probiotic diet (Moroni et al., 2021). Cytokines play an important role in the innate immune response of fish and are considered the best reference genes for studying their immune response (Sakai et al., 2021). *Il-1 $\beta$*  is an important cytokine



**Fig. 8.** Mean relative abundance (%) of the most abundant bacteria in the intestinal mucosa of sea bass at the end of feeding experiment at the phylum (A), family (B), and genus (C) taxonomic levels (N = 6). Only bacteria with a total abundance of 0.5% were reported. Bacteria with lower abundance were grouped together and reported as “other”. GS, genetically selected genotype; WT, wild-type genotype of European sea bass.

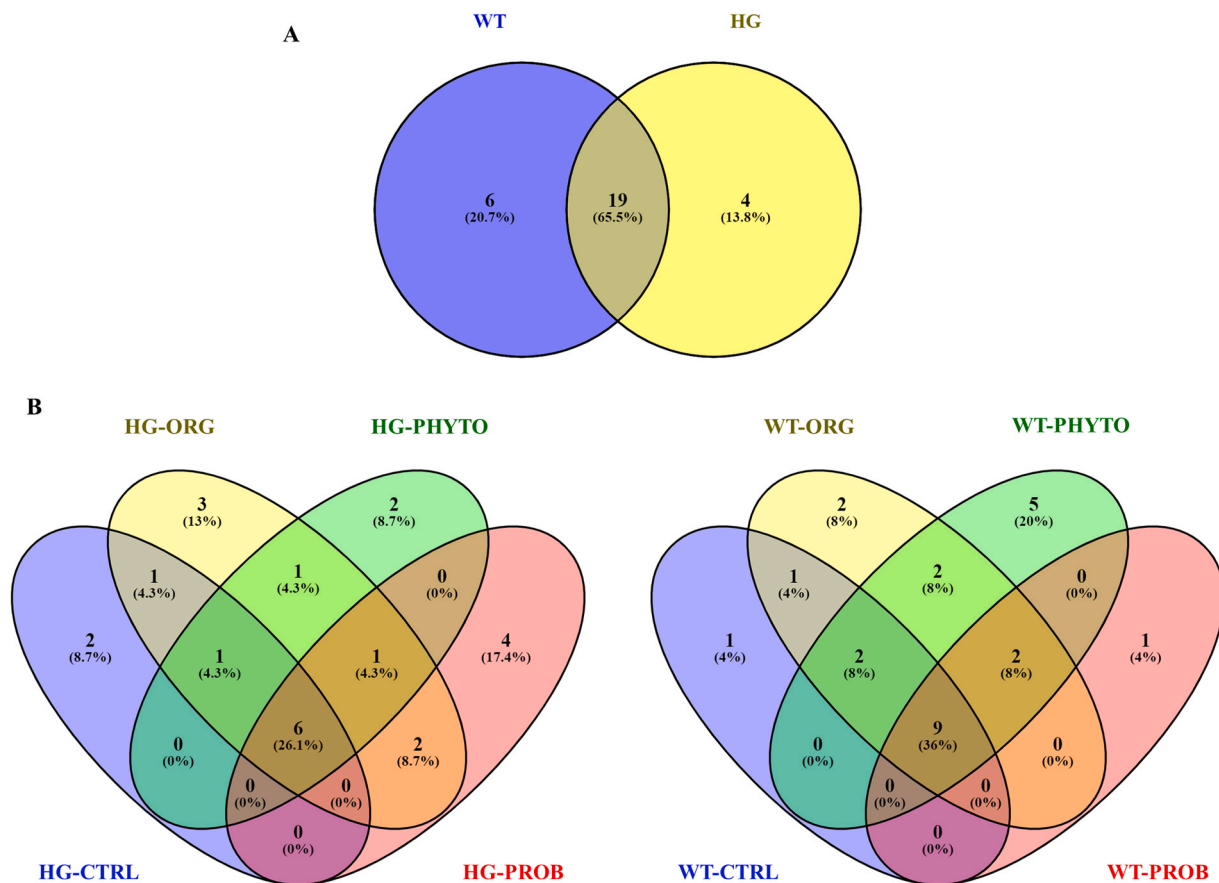


Fig. 9. Venn diagrams showing OTUs common between the two genotypes (A) or between feeding groups (B).

involved in the activation of lymphocytes, phagocytic cells, and proinflammatory signaling pathways in peripheral tissues and brain. Interestingly, in the rainbow trout intestinal epithelial cell line RTgutGC and in intestinal explants, transcript levels of *il-1 $\beta$*  and *tnf- $\alpha$*  genes were significantly increased by exposure to the two *Bacillus* strains, whereas intraperitoneal injection of *B. subtilis* in trout upregulated the anti-inflammatory interleukins *il-4* and *il-13* (Docando et al., 2022). However, *in vivo* evaluation of probiotics is usually performed by feeding fish for an extended period of time. Administration of *Bacillus* probiotics for prolonged periods has been shown to upregulate transcription of cytokines in several fish species (Abarike et al., 2018; Cerezuela et al., 2013; Monzón-Atienza et al., 2022; Telli et al., 2014).

The greater influence of genotype on immune-related gene expression is easier to explain. This could be a consequence of multi-trait selection to improve fish growth parameters. As previously reported by Montero and colleagues (Montero et al., 2023), the GS fish used in this study were also indirectly co-selected for other traits, such as better adaptation to the rearing process and breeding manipulations, but also resistance to disease.

Despite the lack of significant growth enhancement in fish fed the tested additives, a modulatory effect of both diet and genotype on the resident gut microbial communities was observed. The overall composition of the gut bacterial communities did not differ between diets or genetic backgrounds, as confirmed by the high proportion of taxa forming the core microbiota. Also, no differences were observed in species richness, biodiversity, or beta diversity between experimental groups. However, when partial least-squares analysis was performed, the HG group showed lower individual variability in terms of gut microbiota composition than the WT group. This result confirms the improved ability of the selected fish to cope with changes in diet composition previously observed in the same sea bass batches

(Torrecillas et al., 2023) and in sea bream (Naya-Català et al., 2022; Piazzon et al., 2020).

Piazzon et al. (Piazzon et al., 2020) suggested that genetic selection for growth may indirectly lead to higher plasticity of the microbiota in response to changes in diet. In turn, the responses mediated by the microbiota could similarly influence host evolution. Unfortunately, the influence of the microbiota on host evolution remains poorly understood. What is certain is that the dynamics involved are complex and include feedback loops as well as non-genomic factors. In this context, Kolodny and Schulenburg (2020) have recently described and proposed several microbiome-mediated responses and/or the evolutionary consequences of plasticity. Among them, the microbiome-mediated Baldwin effect has piqued our interest, which refers to evolution via heritable traits in the host that provide for an enriched presence of beneficial microbes, leading to improved fitness.

In agreement with previous studies, 90% of the total gut resident bacteria were represented by the phyla *Firmicutes* and *Proteobacteria* (Montero et al., 2022; Rimoldi et al., 2020b). Although discriminant analysis did not clearly separate groups, two-way ANOVA revealed some effects of diet and/or genotype on the relative abundance of certain bacterial taxa. Specifically, ORG diet increased the relative abundance of *Streptococcus* regardless of genetic background. This genus belongs to the lactic acid bacteria family (LAB), which are considered the most promising probiotics in aquaculture. Similarly, dietary organic acids in combination with nature-identical compounds appear to exert prebiotic properties, leading to moderate increases in the genera *Lactobacillus* and *Leuconostoc* in sea bass (Busti et al., 2020). In agreement with these results, we previously found a positive effect of an organic acid mixture on *Leuconostocaceae* and *Lactobacillaceae* in the gut of sea bream (Rimoldi et al., 2018a). The genus *Photobacterium* appeared to be preferentially associated with the WT background. Some species of this

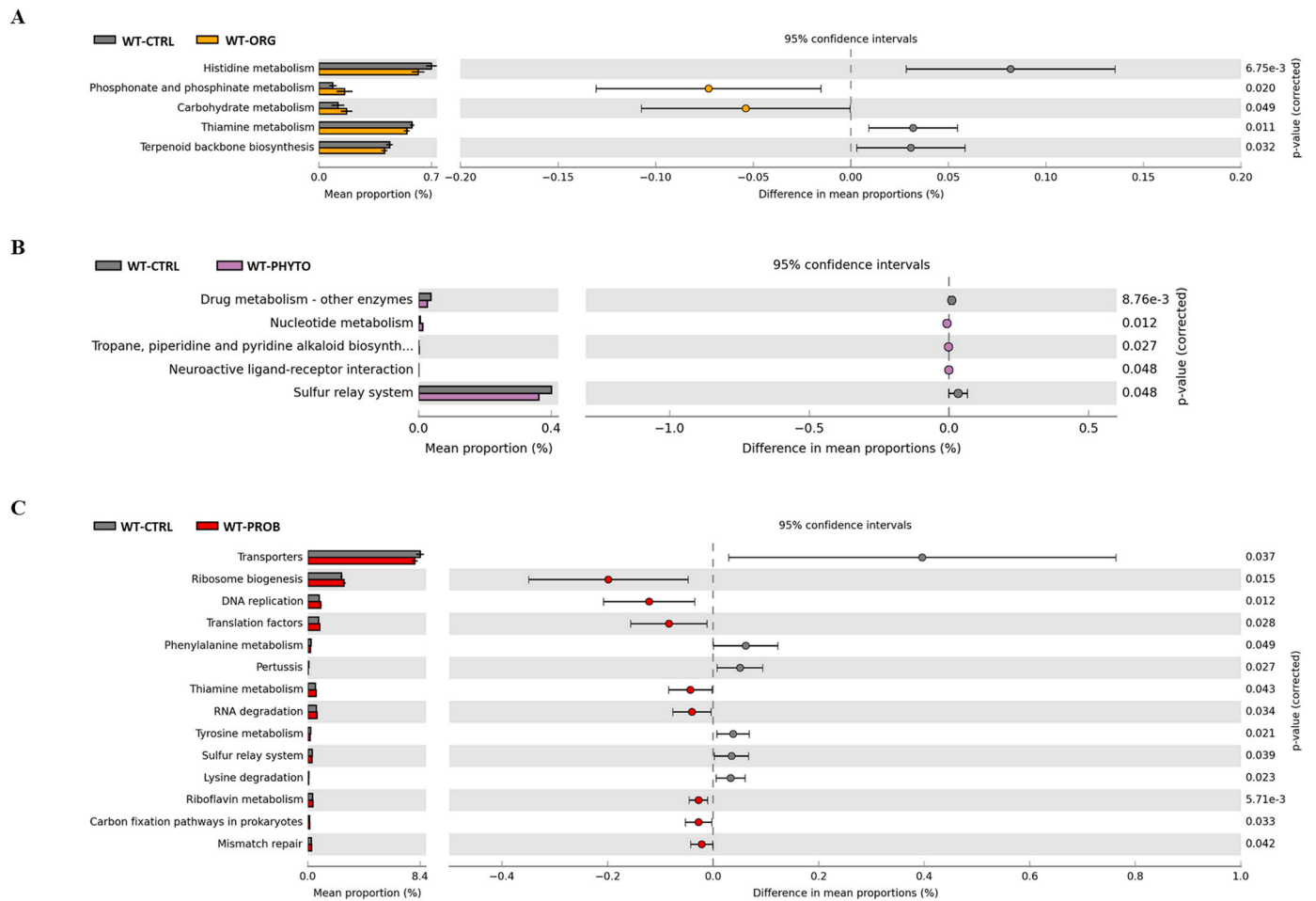


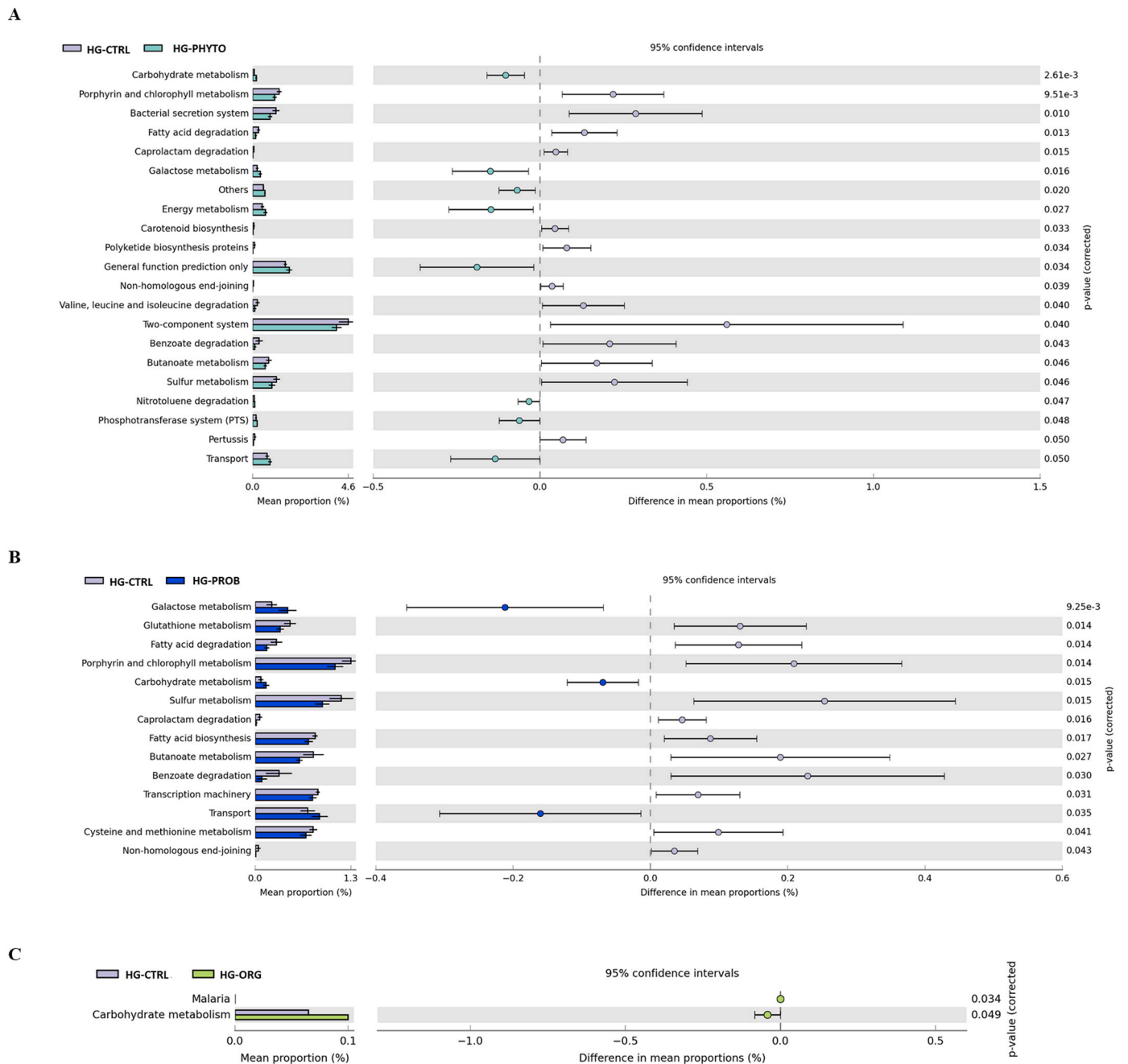
Fig. 10. PICRUST analysis results of predicted functional pathways of gut mucosal microbiota of wild-type European sea bass (WT).

genus function as mutualistic bacteria in the gut of marine fish and support chitin digestion by secreting chitinase, while others, such as *P. damselae*, are common pathogens in aquatic animals (Huang et al., 2020). In our previous experiment using the same fish genotypes but fed a control diet or a non-supplemented “future” diet, the influence of genetic background on gut microbiota composition was more evident. Several bacterial genera, such as *Psychrobacter*, *Micrococcus*, *Enhydrobacter*, *Corynebacterium*, *Cutibacterium*, *Paracoccus*, and *Stenotrophomonas*, were associated with WT fish, regardless of diet (Torrecillas et al., 2023). Similarly, the strong influence of host genetics on gut microbiota composition was highlighted in sea bream. This influence was particularly pronounced in fish fed “future” diets with some genera, such as *Rubellimicrobium*, *Reyranella*, *Lactobacillus* and *Bifidobacterium*, emerging as HG-related taxa (Naya-Català et al., 2022).

The effects of genetic and dietary interactions on gut microbiota composition were specific for each additive. Supplementation with phytonics increased the number of members of Lactobacillales and Weeksellaceae in the gut of fish from WT. This is consistent with previous studies examining the effects of dietary supplementation with galactomannan oligosaccharides and phytonics on the gut microbiota of European sea bass fed a FM/FO deficient diet, which found a reduction in potentially pathogenic taxa with an increase in Lactobacillales (Rimoldi et al., 2020b). In selected sea bass, both PHYTO and PROB diets reduced the abundance of opportunistic pathogenic genera *Novosphingobium* and *Sphingobium*. In addition, dietary supplementation with PROB showed bactericidal activity against the genera *Pseudomonas* and *Acinetobacter*, regardless of genotype. Our tested probiotic mixture contained three *Bacillus* species: *B. subtilis*, *B. licheniformis* and

*B. pumilus*. The antimicrobial properties of several *Bacillus* species have been described for various aquatic pathogenic bacteria (Olmos et al., 2020; Soltani et al., 2019; Van Doan et al., 2019). For example, *B. amyloliquefaciens* shows good antagonistic activity *in vitro* against *Aeromonas hydrophila*, *Acinetobacter sp.* and *Acinetobacter tandoii* (Kavitha et al., 2018). However, although the highest abundance of *Bacillus* was found in the PROB diet, the results did not show significant change in the relative abundance of this genus at the gut level. The finding that *Bacillus*-based probiotics are able to regulate the microbiota of European sea bass, despite not colonizing of the host intestinal mucosa is not new. This result is further evidence that probiotics regulate the gut microbiota of European sea bass and thus provide health benefits even though they do not colonize the host intestinal mucosa (Moroni et al., 2021).

An increase in *Streptococcus* was observed in fish fed organic acids, regardless of genotype. Accordingly, increased levels of Lactobacillales, mainly represented by the genera *Lactobacillus* and *Leuconostoc*, were found in sea bream fed a specific combination of short- and medium-chain monoglycerides and in sea bass fed a combination of organic acids and natural compounds in their diet (Busti et al., 2020; Rimoldi et al., 2018a). LAB includes many bacterial genera, including *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Leuconostoc*, and *Pediococcus*. These genera differ in their pathogenic potential, and it is difficult to draw a clear dividing line between beneficial and virulent species. In general, *Lactobacilli* and *Lactococci* are considered harmless, while streptococcal infections are a major cause of disease in fish. However, all LABs have developed high acid tolerance and are stable during their fermentation processes, which produce organic acids as end



**Fig. 11.** PICRUSt analysis results of predicted functional pathways of gut mucosal microbiota of genetically selected European sea bass (HG).

products. Therefore, it can be assumed that acidifiers in the diet can improve their numbers.

Finally, PICRUSt analysis showed that fish from HG had a significant increase in carbohydrate and energy metabolism when fed with PROB, ORG, and PHYTO supplemented diets, while the WT fish had an improvement in carbohydrate metabolism only when fed with ORG supplemented diet. An increase in these metabolic pathways indicates a better ability of the selected fish to utilize carbohydrates. Upregulation of bacterial metabolic pathways involved in energy supply has been previously found in other fish species, such as rainbow trout selected for growth (Biasato et al., 2022). In both cases, the selected fish responded better to dietary changes than WT fish by modulating their gut microbiota activity in response to changes in the diet.

### 5. Conclusions

In conclusion, the composition of the gut microbiota was strongly influenced by the genetic background. Genetically selected fish shared low individual variability but coped better with diet composition than WT. However, the effect of functional additives on the gut microbiota profile was both additive- and genotype-dependent. Low-dose *Bacillus*-based probiotics are most effective in modulating the resident gut microbiota and activating the gut immune system of European sea bass, even in the absence of gut mucosal colonization. In contrast, no direct beneficial effects on survival following bacterial challenge can be associated with dietary additives.

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## CRediT authorship contribution statement

**S. Rimoldi:** Methodology, Data collection, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **D. Montero:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **S. Torrecillas:** Conceptualization, Methodology, Data collection, curation, Formal analysis, Writing – original draft, Writing – review & editing. **A. Serradell:** Methodology, Data collection, Data curation, Formal analysis. **F. Acosta:** Methodology, Data collection, curation, Formal analysis. **P. Haffray:** Writing – review & editing. **B. Hostins:** Additive producer. **R. Fontanillas:** Feed producer. **F. Allal:** Methodology, Data curation. **A. Bajek:** Animal production. **G. Terova:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing.

## Declaration of Competing Interest

The authors declare no competing interests.

## Data Availability

All the sequences were submitted to the public European Nucleotide Archive (EBI ENA).

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.aqrep.2023.101747](https://doi.org/10.1016/j.aqrep.2023.101747).

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