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Genotype x nutrition interactions in European sea bass (*Dicentrarchus labrax*): Effects on gut health and intestinal microbiota

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ABSTRACT

One of the main objectives of the present study was an effective replacement of dietary fishmeal/fish oil (FM/FO) by new raw materials without negatively affecting European sea bass (*Dicentrarchus labrax*) performance and health status within a selection breeding context. The genomic selection of this species is still in its infancy if compared to salmonids and in particular, the role of *D.labrax* genome in shaping the gut microbiome, has been scarcely investigated.

Wildtype (WT) and selected (GS) sea bass were fed with two diets: a control (20% FM/ 5–9% FO), and a "future" (F) diet, in which FM was decreased to 10% being replaced by poultry meal, whereas FO was completely replaced by a blend of rapeseed, poultry, and algae oils.

The morphological evaluation of the intestine revealed a well-organized folding pattern and a conserved gut epithelial barrier for all fish groups. Despite a basal level of inflammation in the proximal intestine of WT fish, no differences were observed neither in the morphometric characteristics of goblet cells nor in the expression of GALT-related genes in response to fish genotype or diet. At distal intestine, WT fish showed a higher inflammatory status and larger goblet cells than GS fish and within the same genotype, fish fed the F diet had in general larger goblet cells. In distal intestine, a significant effect was found on the expression of 3 out of 7 target GALT genes. In particular, the expression of cytokines il-1β, tnf-α, and il-10 was different, showing an interaction effect diet x genotype.

Diet had a lower influence upon gut bacterial composition than genotype. Indeed, regardless of the diet, WT fish showed higher species richness than GS genotype and this could be a direct consequence of selective breeding for multiple traits selection including growth, external morphology for lower abdominal fat deposition and adaptation to multiple and successive feed sources and composition across generations of selection. Furthermore, the gut microbiota of GS fish shared a reduced individual variability, indicating an enhanced capacity to cope with changes in diet composition. The less changes of GS sea bass at the level of gut bacterial composition in cumulating data collected with the feeds, demonstrate a capacity to reshape their microbiota thus better adapting to the diet, but with no negative impact on their growth performances, and even a better growth. A significant genotype effect was found for specific bacterial taxa, such as *Paracoccus* genus and other genera belonging to Moraxellaceae family, which were enriched in WT fish, regardless of the diet. Interestingly, the relative abundance of *Paracoccus* genus was positively correlated with higher proinflammatory cytokine il-1β expression found in distal intestine of wildtype sea bass.

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1. Introduction

The environmental and economic sustainability of the aquafeed sector depends on an adequate and efficient response to the increasing demand of traditional fish derived raw materials as aquaculture production increases. During the last decades, the evolution of aquafeed formulations has been notable in terms of complexity and sustainability, from a use focused mainly on meals and oils from vegetal origin (Turchini et al., 2009; Montero and Izquierdo, 2010) to the current combination of them with emergent raw materials characterized by a relative low carbon footprint and reduced resource dependence. Those new raw materials include meals from terrestrial animal by- products, insect and single cells or rich long chain polyunsaturated fatty acid (LC-PUFA) algae oils, among others. Recent studies have demonstrated the suitability of those emergent raw materials as candidates to support a fish meal (FM) and fish oil (FO) reduction in current fish fed formulations (Carvalho et al., 2020; Sarker et al., 2020; Shaikhiev et al., 2020; Fontinha et al., 2021; Torrecillas et al., 2021; Estévez et al., 2022). Among them, poultry oil (PO) and poultry meal (PM) are sustainable, highly available, competitive in terms of price, and relatively resistant to auto-oxidation raw materials to use in fish feed production. PO as partial replacer of FO, despite its lack of n-3 LC-PUFA, represents an adequate source of saturated and monosaturated fatty acids for fish (Turchini et al., 2013; Hatlen et al., 2015; Campos et al., 2019a; Carvalho et al., 2020). Thus, the successful level of FO replacement by PO will be mainly conditioned by the target species essential fatty acids (EFA) requirements and developmental stage together with the level of FM or other external sources of dietary n-3 LC-PUFA. For example, its suitability as FO replacer has been demonstrated when combined with algae derived n-3 LC-PUFA rich oils, enhancing DHA deposition in gilthead sea bream compared to fish fed FO based diets (Carvalho et al., 2020) and with no detrimental effect on marine fish growth performance (Galkanda-Arachchige et al., 2020; Carvalho et al., 2020; Carvalho et al., 2021). PM from by-product origin has a relatively high protein but can present deficiencies or imbalances of certain essential amino acids (Tacon et al., 2006). PM suitability as FM replacer in marine fish aquafeeds formulations has been evaluated in a recent meta-analysis indicating the fish species and level of FM replacement as the main conditioning factors in terms of maintaining fish growth performance (Galkanda-Arachchige et al., 2020). For example, FM can be successfully replaced by PM up to 90% in Juvenile Black Sea Bass (*Centropristis striata*) (Dawson et al., 2018), up to 80% in marine Japanese Sea Bass (*Lateolabrax japonicus*) (Wang et al., 2015), up to 59% in red sea bream (*Pagrus major*) (Takagi et al., 2000) or up to 50% gilthead sea bream (*Sparus aurata*) (Nengas et al., 1999). Accordingly, an effective replacement of FM/FO by new formulas addresed to guarantee a FUTURE scenario, covering the limitations on the availability of fish-derived raw materials, without compromising fish performance and health status while maintaining feed quality and costs within a selection breeding context, is one of the main objectives of the present study. In these sense, there is still some knowledge gap on the potential side effects of novel ingredients on critical aspects, such as fish mucosal health or alterations of the fish gut microbiota homeostasis (Estruch et al., 2015; Ringø et al., 2016; Torrecillas et al., 2017; Rimoldi et al., 2018a, 2021; Huyben et al., 2020; Fontinha et al., 2021; Terova et al., 2021) and very few studies are available addressing specifically this issue. In one hand, the available studies in salmonids somehow are highly dependent on the target species, the nature of the microbiota (allochthonous vs autochthonous) and intestinal region considered. Atlantic salmon (*Salmo salar*) fed a diet in which 200 g/kg of FM was replaced by poultry by-products meal, presented a modulation of allochthonous microbiota in the distal intestine as compared to fish fed the control diet (FM: 450 g/kg), but no effect was seen on autochthonous microbial communities (Hartviksen et al., 2014). However, when 58% of FM (580 g/kg) was replaced, fish presented higher microbial richness in digesta and gut mucosa, with a more relevant effect on digesta-associated microbiota, and without effects on PCNA (proliferating cell nuclear antigen) immunoreactive cells density (Gajardo et al., 2017). In gilthead sea bream, instead, a diet with 22% of FM replacement by PM produced an increase of species richness and diversity only on allochtonous intestinal microbiota (Fontinha et al., 2021). In contrast, rainbow trout (*Oncorhynchus mykiss*) fed PM at 120–150 g/kg did not show any increase of gut microbial richness (Rimoldi et al., 2018b).

Table 1
Ingredients and proximate composition of the experimental diets with different pellet size. CTRL (control diet); F (Future diet).

Ingredients (%):	CTRL 1.8	F 1.8	CTRL 4	F 4	CTRL 6	F 6
Corn gluten	2.94	4	5	5	5	5
Hi Pro Soy bean meal [1]	7	5.82	6	6	6	6
Wheat gluten	17.2	17.47	9.04	10.2	7	9.26
Faba bean dehulled [2]	8	8	8	8	8	8
Wheat	15.54	15.23	20.25	19.95	20.82	20.65
Soy protein concentrate[3]	17	18	15	15	12.43	11
Fish oil [4]	5.11		7.03		8.95	
Fish meal [5]	20	10	20	10	20	10
Rapeseed oil	4.29	4.51	6.68	8.98	8.88	10.92
Phosphate	0.52	0.27	0.6	0.35	0.52	0.48
Vitamin & mineral mix [6]	0.3	0.3	0.3	0.3	0.3	0.3
Poultry meal [7]		10		10		10
Poultry oil [8]		2.22		1.37		2.91
DHA oil [9]		2.11		2.75		3.38
Lecithin	2	2	2	2	2	2
Proximal composition (% dry matter)						
Dry matter	93.0	93.0	93.0	93.0	93.0	93.0
Moisture	7.0	7.0	7.0	7.0	7.0	7.0
Crude protein	46.9	47.4	40.8	41.3	37.8	38.3
Crude fat	16.0	16.0	20.0	20.0	24.0	24.0
Ash	5.7	5.5	5.6	5.4	5.4	5.2

[1] Soya bean meal: CJ Selecta S.A (Brasil).
[2] Faba beans: Cefetra BV (The Netherlands).
[3] Soya protein concentrate: CJ Selecta S.A (Brasil).
[4] Fish oil: Copeinca, S. A. (Perú).
[5] Fish meal: Norsildmel AS (Norway).
[6] Mineral and Vitamin premix: Trouw Nutrition (The Netherlands).
[7] Poultry meal: Sonac (Belgium).
[8] Poultry oil: Sonac (Belgium).
[9] DHA: Veramaris (Evonik).

In parallel to studies addressed to find new dietary formulations, great research efforts have been spent for improving profitability and sustainability of cultured fish species through genetic selection to better matched to novel diets poor in marine raw materials and rich in alternative more sustainable feedstuff (Abernathy et al., 2017; Belghit et al., 2019; Boudry et al., 2021; Brezas and Hardy, 2020; Callet et al., 2017; Gjedrem et al., 2012; Overturf et al., 2013). Genetic ang genomic selection of the European sea bass (*Dicentrarchus labrax*) are still in its initial stages (Boudry et al., 2021; Vandeputte et al., 2019) when compared to salmonids (Al-Tobasei et al., 2021; Tsai et al., 2015; Vallejo et al., 2017) even if first positive application are reported for genomic selection to improve resistance to several diseases (Griot et al., 2021) In particular, the role of host genome of cultured fish species in shaping the gut microbiome, has been scarcely investigated (Blaufuss et al., 2020; Chapagain et al., 2020; Piazzon et al., 2020).

The present study aimed to investigate the potential of a multiple traits selected genotype of European sea bass versus a wildtype genotype when fed a "future diet" with low content of fish-derived raw materials (10% FM/0% FO + DHA oil) along the entire production cycle in terms of gut health status, and microbiota composition. The response to diet by genetic selection interaction on fish growth, feed efficiency, carcass traits, and fillet proximate and faty acid composition on the same

European sea bass individuals, have been just published by our group (Montero et al., 2023).

2. Materials and methods

2.1. Ethics statement

Animal manipulation procedures used through the present experiment accomplished with the guidelines for animal researcg of the European Union Directive (2010/63/EU) and Spanish legislation (RD 53/2013). The Bioethical Committee of the University of Las Palmas de Gran Canaria (Rec. code: OEBA_ULPGC_27/2019 ULPGC), avoinding any discomfort, stress and pain to the experimental fish. Handling was performed under natural clove oil anesthesia (0.2 ml/l; Guinama S.L; Spain, Ref. Mg83168), and fish were euthanized prior sampling with an overdose of natural clove oil (5 ml/l).

2.2. Experimental diets

Two isolipidic, isonitrogenous, and balanced in micro and macronutrients diets were formulated to meet the nutritional requirements of European sea bass. The control diet (CTRL) formula was based in a practical diet used for European sea bass culture, containing: 20% of FM, 5–9% of FO (depending on the fish age of development), a blend of vegetable origin meals (60–67%), and 4–8% of rapeseed oil (Table 1). The future diet (F) formula was based on the CTRL diet but 50% of FM content was replaced by PM and total FO was replaced by a blend of poultry oil and microalgae derived oil rich in docohexanoic acid (DHA) and docosapentaenoic acid (DPA) (DHA Natur Oil, Archer Daniels Midland, USA). Control and F diets were formulated for three different growing periods of fish. Feeds were manufactured by Skretting ARC Feed Technology Plant (Stavanger, Norway) at 3 pellet sizes of 1.8, 4 and 6 mm. Diets were shipped and analyzed for proximate composition at ULPGC facilities (for more details, please see Montero et al., 2023). Diets formula is shown in Table 1.

Yttrium premix: 0.1%.

2.3. Experimental conditions

The study was carried out at the Parque Científíco-Tecnológico Marino (PCTM) of the University of Las Palmas de Gran Canaria (Telde, Canary Island, Spain) under the conditions described in Montero et al. (2023).

On March 2019, two different genotypes (GS or WT) were produced in MARBEC-IFREMER facility (Palavas-les-flot, France). Spawns of 7 dams were fertilized with thawed sperm from 33 or 32 selected sires (GS) or wild sires (WT). The dams were derived from the IFREMER experimental broodstocks selected for growth for 3 generations. The 33 GS sires were derived from the commercial breeding program operated by EMG Ecloserie Marine de Gravelines breeding company since 6–7 generations (>35 years) on growth, external morphology on a wild type shape and a limitation in muscular lipid content. The 32 WT sires were originated from the Gulf of Lion (Westren Mediterranea). Eggs were equi-represented between dams, pooled and transferred into 65 Petri dishes (one per sire) to produce 231 (GS) and 224 (WT) families by in vitro fertilization. The reproductive and fertilization process is detailed in Fauvel et al. (2012) for the use of sperm crypreservation and Griot et al., 2021 for the whole fertilization process. After incubation at 14 °C, one-day-old hatched larvae were equirepresented between dams, pooled by genotype and shipped to the University of Las Palmas de Gran Canaria (ULPGC; Las Palmas de Gran Canaria, Spain) by airplane into oxygen-saturated water within transport bags that were kept in insulated boxes.

The larval production was grown following the standardized methodology of the Research group in Aquaculture at the ULPGC facilities (Betancor et al., 2011; Atalah et al., 2011) until arriving to the initial experimental age (294 dph, $t = 0$). Then, fish were randomly assigned different experimental tanks (10 tanks for each genotype and dietary group, 500 l or 1000 l cylinder-conical when fish grew) at an initial density of 50 fish per tank. The tanks were equipped with an open flow-through water system and the fish were exposed to natural photoperiod (12 L:12D). The fish were fed three times a day to apparent satiation six days a week to the end of the feeding trial (609 dph, final sampling). Those procedures were done during all the experimental period except those days affected by the general lockdown imposed by COVID19 pandemic in Spain (from March to May 2020). For sampling, fish were caught and euthanized by inmersion in an overdose of natural clove oil (Guinama S.L; Spain, Ref. Mg83168). Three to six transverse sections of proximal gut, posterior gut (pre-ileorectal valve) and rectum (post-ileorectal valve) region ($n = 10$ fish/genotype at t = 0; $n = 5$ fish genotype/diet at final sampling) were taken and fixed at 4 °C in 4% paraformaldehyde for optical examination studies. Portions of the same intestinal sections were fixed at 4 °C in 2.5% glutaraldehyde and 0.15 M HEPES buffer (pH = 7.4) for structural studies ($n = 3$ fish/tank). For molecular analysis, 0.5 cm samples of proximal and distal intestine regions (post-ileoreactal valve) ($n = 6$ fish genotype/diet at both samplings) were collected and conserved in RNA*later*™ Stabilization Solution (ThermoFisher) until delivered to the laboratory of Department of Biotechnology and Life Science (Varese, Italy) and then, were stored at −80 °C until molecular analysis. The mucosa associated microbiota (resident microbiota) was obtained by scraping the entire intestinal mucosa (n = 6 fish genotype/diet at both samplings) with a sterile cotton swab, material transferred into XpeditionTM Lysis/Stabilization Solution (Zymo Research, Irvine, CA, USA) until processing.

2.4. Morphological and mucus production studies

After 48 h in 4% paraformaldehyde at 4 °C, gut samples were dehydrated and embedded in paraffin. Sections of 4 μm were stained with hematoxylin and eosin (H&E) for optical examination, with Alcian Blue (pH = 2.5) in order to differentiate goblet cell secreting acid mucins, and with May-Grünwald/Giemsa (MGG) for studying leukocyte populations distribution (Martoja and Martoja-Pierson, 1970). Afterwards, slides were digitally scanned in a digital scanner Olympus VS120 (Optic system BX61VS, Tokyo, Japan) equipped with VC50 and VS-XM10 cameras and acquired with Olympus VS software (VS-NIS-SQL-V2.6, Tokyo, Japan). Digitalized images of gut transverse sections (Alcian Blue pH = 2.5) were used to determine gut goblet cells area (μm^2) for each intestinal region evaluated using CellSens Dimension Desktop 1.16 (Olympus Iberia, Spain) as previously described for this fish species (Torrecillas et al., 2019).

2.5. Ultrastructure study

Samples for ultrastructural studies were processed following the standard procedures of the Research Group in Aquaculture of the ULPGC for this tissue (Torrecillas et al., 2019). Samples were post-fixed at 4 °C in 2% osmium tetroxide and 2% uranyl acetate, dehydrated, and embedded individually in a resin block. Ultrathin (50 nm) sections were qualitatively evaluated with a Field Emission Scanning Electron Microscope (FESEM; Carl Zeiss, Sigma 300 VP), using the transmission mode with STEM in BF detector at the Advanced Confocal and Electron Microscopy and Electron Microscopy Research Service (SIMACE) of the ULPGC. Samples were qualitatively evaluated for intestinal membrane lining appearance, microvilli structure, cytoplasmatic electrondensity, enterocytes packaging, and infiltrated leukocytes populations.

2.6. Quantitative PCR analysis

The Maxwell® 16 LEV simplyRNA kit (Promega, Italy) was used in combination with Maxwell® 16 Instrument (Promega, Italy) for automated RNA purification from the intestinal samples. The quantity and

Table 2
Nucleotide sequences of primers used for gene expression analysis by qPCR.

Gene	Acc. N.	Primer	Nucleotide sequence (5′- 3′)	Efficiency (%)
il-1β	AJ269472	Forward	TTGTGTTTGAGCGCGGAACA	99.0
		Reverse	TGTCGGTCACGCTGCATTG	
tnf-α	DQ070246	Forward	AAACCGGCCTCTACTTCGTCTA	92.1
		Reverse	TCCCGCACTTTCCTCTTCAC	
il-10	AM268529	Forward	AGCGCTGCTAGACCAGACTGT	99.6
		Reverse	CGGCAGAACCGTGCTTAGAT	
mhc-I	AM943118	Forward	TACCTCACCCAGGAGTGCAT	107.6
		Reverse	GGAAGGTCTGTCCTCAGCAG	
mhc-II	AM113466	Forward	GCTCAGAGACGGACAGGAAG	94.4
		Reverse	CCAGGTGAGAGTGGATCTGG	
cox-2	AJ630649	Forward	AGCACTTCACCCACCAGTTC	103.2
		Reverse	AAGCTTGCCATCCTTGAAGA	
cd-4	AM849811	Forward	TCACCCCACTCATCTCATCA	102.8
		Reverse	TGATGAGATGAGTGGGGTGA	
β-actin	AY148350	Forward	TCTTCCAGCCTTCCTTCCT	92.1
		Reverse	GATGTCAACGTCGCACTTCA	

purity of extracted RNA were evaluated spectrophotometrically using a NanoDrop™ 2000c spectrophotometer (Thermo Scientific, Italy). After extraction, one hundred nanograms of total RNA was subjected to qPCR using the iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad). The primers used for the amplification of target genes have been designed for this study on the basis of the coding sequences deposited in NCBI database. The oligonucleotide sequences and their efficiency are shown in Table 2. The reaction mix contained 10 μl of iTaq™ Universal SYBR® Green reaction mix (2×), 0.25 μl iScript reverse transcriptase, 500 nM of each reverse and forward primer, and nuclease free water to a final volume of 20 μl. The qPCR reactions were run 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, then melt-curve (65–95 °C). The raw data from qPCR runs was analyzed by Bio-Rad CFX Maestro software (Bio-Rad, Italy). Genes relative expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). β-actin was chosen as housekeeping gene and the experimental group with the lowest expression value was selected as calibrator. However, the stability of three different housekeeping genes was evaluated by means of Bio-Rad CFX Maestro Software: translation elongation factor 1α (EF1α), β-actin, and α-tubulin. Of these, β-actin and eEF1α genes resulted the best in terms of stability value and we arbitrary chose the first one as housekeeping.

2.7. Microbiota analysis

2.7.1. Microbial DNA extraction

The DNeasy PowerSoil Kit (Qiagen, Italy) was used for nucleic acid extraction, following the manufacturer's instructions with few modifications at the lysis step, as previously described by Rimoldi et al. (Rimoldi et al., 2020). The starting material was represented by 200 mg of feed (3 aliquots/feed) or 250 μL of gut bacteria suspension from each sampled fish. DNA concentration was measured by NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Italy) and stored at −20 °C until amplicon library preparation. As the negative control of the extraction procedure, a sample with only lysis buffer was processed in parallel with samples. The absence of bacterial DNA contamination in negative control was verified both by spectrophotometer absorbance and PCR using the oligonucleotides 515F: 5′-GTGYCAGCMGCCGCGGTAA-3′ and 806R: 5′-GGACTACNVGGTWTCTAAT-3′ specific for V4 hypervariable region.

2.7.2. Illumina 16S gene library preparation and Miseq sequencing

Library preparation and sequencing were performed by the GalSeq srl company (Italy), applying the Illumina protocol "16S Metagenomic Sequencing Library Preparation for Illumina MiSeq System" (#15044223 rev. B) and using an Illumina MiSeq platform. Details of methodology applied for generation of 16S rRNA gene libraries and their sequencing were previously reported by Rimoldi et al. (2018b). For the characterization of microbial communities, the hypervariable region V4 of 16S rRNA gene was amplified, using the oligonucleotides 515F: 5′-GTGYCAGCMGCCGCGGTAA-3′ and 806R: 5′-GGACTACNVGGTWTCTAAT-3′. The expected size of PCR products on Agilent 2100 Bioanalyzer trace was ~400 bp. Amplicon libraries were quantified by qPCR, pooled at equimolar concentrations, diluted at 6 pM, multiplexed and sequenced on an Illumina MiSeq device with pair-ended sequencing (2 × 250) strategy. All sequences were submitted to public European Nucleotide Archive (EBI ENA).

2.7.3. Sequencing data analysis

The raw sequences were analyzed with QIIME 2™ (v. 2018.4) pipeline at the default setting (Bolyen et al., 2019). During the preprocessing step, paired-end sequencing reads were adapter trimmed, quality filtered (Q > 30), and merged. Remaining reads were dereplicated; singletons and chimeric sequences were removed using QIIME DADA2 denoise-paired command. The end product was an amplicon sequence variant (ASV) table, which recorded the number of times each ASV was observed in each sample. The Silva database (http://www.arb-silva.de) was used for taxonomic assignment of the ASV. The taxonomical classification was performed down to genus level. ASV assigned to chloroplasts and mitochondria were removed from the analysis as of eukaryotic origin. Alpha (within a single sample) and beta diversity (between samples) of bacterial communities were performed using QIIME alpha-phylogenetic and beta-phylogenetic commands.

The alpha diversity indices (Chao 1, Faith PD, Observed ASVs, Shannon, and Simpson) were calculated at the same level of rarefaction, i.e. considering the sample size with lower number of sequences. Beta diversity was calculated using both the weighted (presence / absence / abundance) and unweighted (presence / absence) UniFrac distance matrix. The UniFrac distances between the microbial communities of the individual samples were visualized by means of two-dimensional scatter plots from Principal Coordinate Analysis (PCoA) (Lozupone and Knight, 2005; Lozupone et al., 2007).

The common core microbiome (OTUs shared and found in at least four out of the six samples per dietary group) was identified and visualized by a Venn diagram drawn using the web tool Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/index.html).

2.7.4. Functional analysis of bacterial communities

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al., 2013) was used to perform predictive pathway analysis. For taxonomic classification QIIME2 feature-classifier classify-sklearn function based on Greengenes (v.13.8) reference database was applied. A corresponding biom table was generated and used as input for PICRUSt as described in details by Rimoldi et al. (Rimoldi et al., 2021). The PICRUSt output files (profile and metadata) were uploaded to the Statistical Analysis of Metagenomic Profiles (STAMP) software package (Parks et al., 2014) and extended error plots were created. A Welch's two-side *t*-test with 95% confidence was applied to identify differences in microbial metabolic pathways between two groups.

2.8. Statistics

All data were tested for normality and homogeneity of variance. Differences were considered significant at $p < 0.05$. Morphological findings based on a range scale evaluation were analyzed by Kruskal-Wallis and Mann–Whitney *U* test. For quantitative data, the individual effects of diet and genotype factors were analyzed by two-way ANOVA analyses. At $t = 0$, a student's t-test was applied for testing differences between families. In order to meet the assumptions required by parametric tests, the relative abundance data (%) of bacterial taxa were angular transformed. Only taxa with an overall abundance higher than

Table 3
Scoring of mucosa, *lamina propria* and submucosa inflammatory status as well as infiltrated leukocytes subpopulations in proximal gut, posterior gut (pre-ileorectal valve) and rectum (post-ileorectal valve) for the two European sea bass (*D. labrax*) genotypes (GS and WT) at the end of the long-term feeding trial.

			GS		WT	
			Control	Future	Control	Future
Proximal gut	Mucosa	Inflammation	1–2	1–2	2	1–2
		Granulocytes	1–2	1	1	1
		Lymphocytes	2	2	2–3	2
		Foamy cells	2[a]	2[a]	2-3[b]	2-3[b]
		Rodlet cells	2–3	2–3	2–3	2–3
	Lamina propria	Inflammation	1–2 [a]	2 [a]	2 [b]	2 [b]
		Granulocytes	1–2 [a]	1–2 [a]	2 [b]	2 [b]
		Lymphocytes	2 [a]	2 [a]	2–3 [b]	2–3 [b]
		Foamy cells	2	2	1–2	2
	Submucosa	Inflammation	1	1	1	1
		Granulocytes	1	1	1	1
		Lymphocytes	1	1	1	1
Posterior gut (pre-ileo valve segement)	Mucosa	Inflammation	2	1–2	2	1–2
		Granulocytes	1	1–2	1	1
		Lymphocytes	2	2	2	1–2
		Foamy cells	1–2	1–2	1–2	1–2
		Rodlet cells	1–2	1–2	1–2	1–2
	Lamina propria	Inflammation	1	1	1–2	1
		Granulocytes	1	1	1	1
		Lymphocytes	2–3 [a]	2 [a]	2 [b]	2 [b]
		Foamy cells	1	1	1	1
	Submucosa	Inflammation	1	1	1	1
		Granulocytes	1	1	1	1
		Lymphocytes	2	2	2	2
Rectum (post-ileo valve segment)	Mucosa	Inflammation	1–2	2	1–2	1–2
		Granulocytes	1	1	1	1
		Lymphocytes	2	2	2	1–2
		Vacuolization	1–2 [a]	2 [b]	1–2 [a]	2–3 [b]
		Hypertrophic vacuoles	1 [a]	2 [b]	1 [a]	2 [b]
	Lamina propria	Inflammation	2	2	1	1–2
		Granulocytes	1	1	1	1
		Lymphocytes	2	2	2	2
	Submucosa	Inflammation	1–2	1–2	1–2	2
		Granulocytes	2	1–2	1	1
		Lymphocytes	1–2	2	2	2

1 (low incidence/presence); 2 (medium incidence/presence); 3 (high incidence/presence). GS = selected genotype of European sea bass for high growth; WT = wildtype European sea bass genotype. Control = control diet based in a 20% Fish meal/5% Fish oil; Future = alternative diet based in a 10% Fish meal/ 0% Fish oil. Different different letters indicate differences ($p < 0.05$) associated to genotypes/dietary groups. Kruskal-Wallis, Mann–Whitney *U* tests.

1% at order level and 0.5% at family and genus level analyzed. Non-parametric two-way PERMANOVA test with 999 permutations was applied to assess beta-diversity dissimilarities. The dissimilarities between experimental groups were also evaluated by applying partial least squares discriminant analysis (PLS-DA) using R Project for Statistical Computing software. The contribution of the different taxa to the group separation was determined by the minimum variable importance in the projection (VIP) values.

Statistical analyses of principal components (PCA) were carried out with the R Project for Statistical Computing software, using the statistical packages "FactoMiner 2.4" for data analysis and "Factoextra 1.0.7" for graphical representation. As a prerequisite for the analysis, data normality was verified through multivariate skewness and Kurtois analysis. The rest of analyses were performed using the SPSS Statistical Software System v21.0 (SPSS, Chicago, IL, USA), GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA, USA) and PAST3 software (Hammer et al., 2001).

3. Results

3.1. Morphological studies

Morphological evaluation of MGG-stained sections revealed a well-organized folding pattern and a conserved intestinal epithelial barrier for all fish experimental groups.

After the feeding trial, for the proximal gut region, a trend to higher level of general inflammatory status on the WT fish *lamina propria* compared to GS fish group was observed, regardless of the diet (Table 3). Despite both, wildtype and selected fish, presented a similar distribution pattern of EGCs within the *lamina propria* and mucosa regions, WT fish were characterized by higher incidence of lymphocytic focuses (Table 3; Fig. 1A-B), as well as higher presence of foamy-like phagocytic cells infiltrated in the mucosa (Table 3; Fig. 1C-D). Foamy cells presented PAS+ material in the cytoplasmatic vacuoles, probably in relation with the removal of apoptotic enterocytes, but also lipids which gave them a foamy appearance (Fig. 1E). Proximal gut submucosa inflammatory pattern was not affected by genotype or diet supplementation (Table 3). A high presence of rodlet cells was detected in all proximal gut regions studied, regardless of the genotype or dietary group studied. Rodlet cells presence was not limited to the basal region (Fig. 1F), but they were also detected in a relative mid-high density along the mid and upper fold regions (Fig. 1G-H).

In general terms, the posterior gut (pre-ileorectal valve region) was not affected by genotypes or diets (Table 3; Fig. 2A), and likewise proximal gut, no evident differences were found regarding EGCs distribution pattern. However, an increase in the presence of *lamina propria* lymphocytes was associated to GS genotype fish (Table 3; Fig. 2B) compared to the WT genotype group (Table 3; Fig. 2C). A high variation on the posterior gut inflammatory status was observed between different fish and different fold regions, founding inflammatory areas in the mucosa or/and in the submucosa of some fish (Fig. 2B). No differences between the dietary groups were observed on the distribution pattern or number of rodlet cells and phagocytes in this intestinal region (Table 3).

For the post-ileorectal valve segment, regarding the general

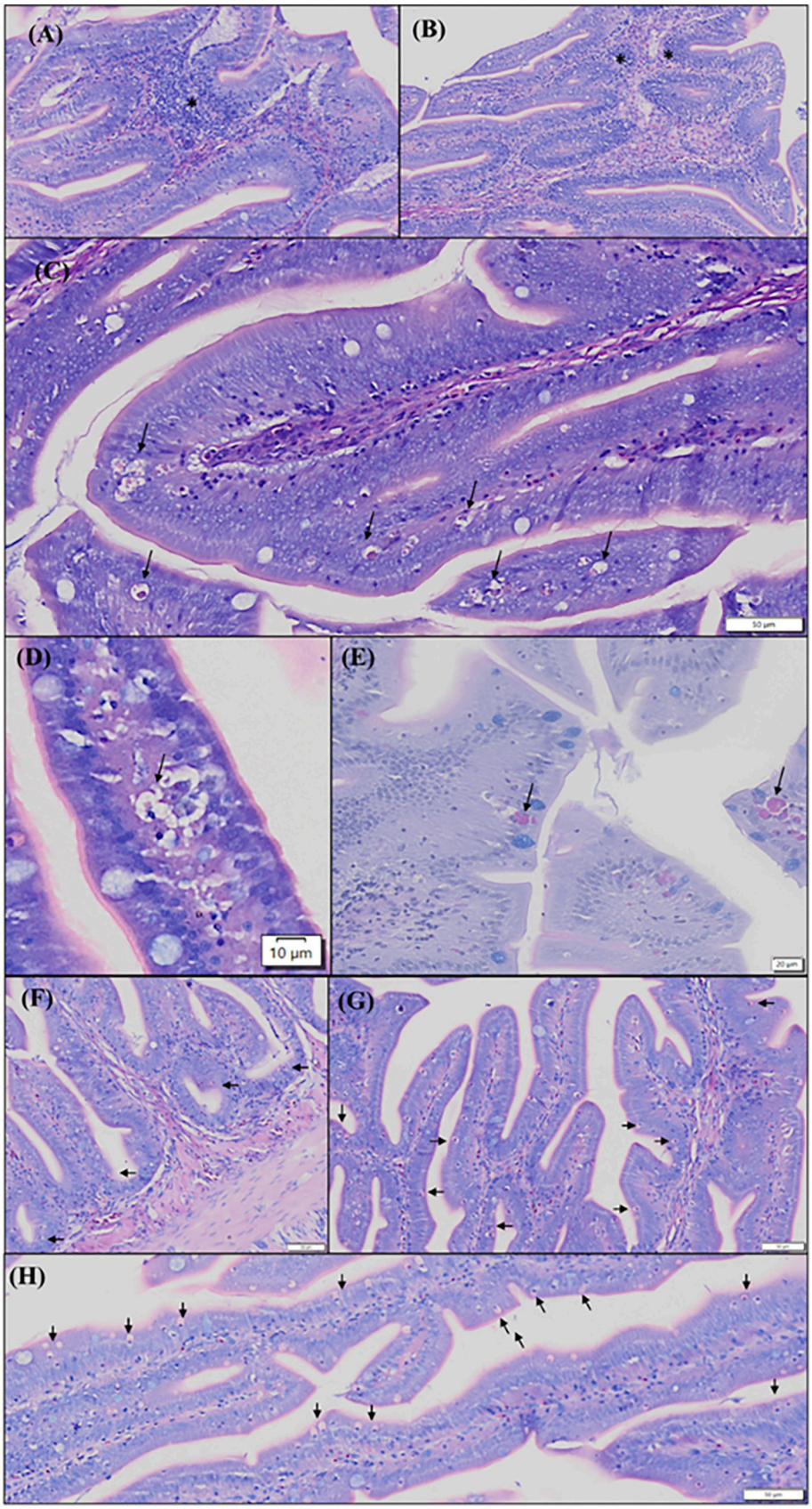

Fig. 1. Detailed micrographs of infiltrated granulocytes, lymphocytes, foamy fagocitic-like cells and rodlet cells present in European sea bass (*D. labrax*) proximal gut intestinal mucosa stained with May-Grünwald Giemsa and Alcian Blue-PAS. (1A-B) Detail of lymphocytic focuses detected in the mucosa and lamina propria (*). Scale bar 100 µm. (1C—D) Detail of foamy fagocitic-like cells present in the mucosa (→), which presented PAS+ material inside the cytoplasmatic vacuoles (1E). Scale bar 50, 10 and 20 µm. (1F—H) Detail of rodlet cells presence pattern in the fold basal region (1F), and along the fold (1G-1H). Observe the high density of rodlet cells present not only in the basal region of the proximal gut fold but also in the mid and apical fold region (1G-1H). Scale bar 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

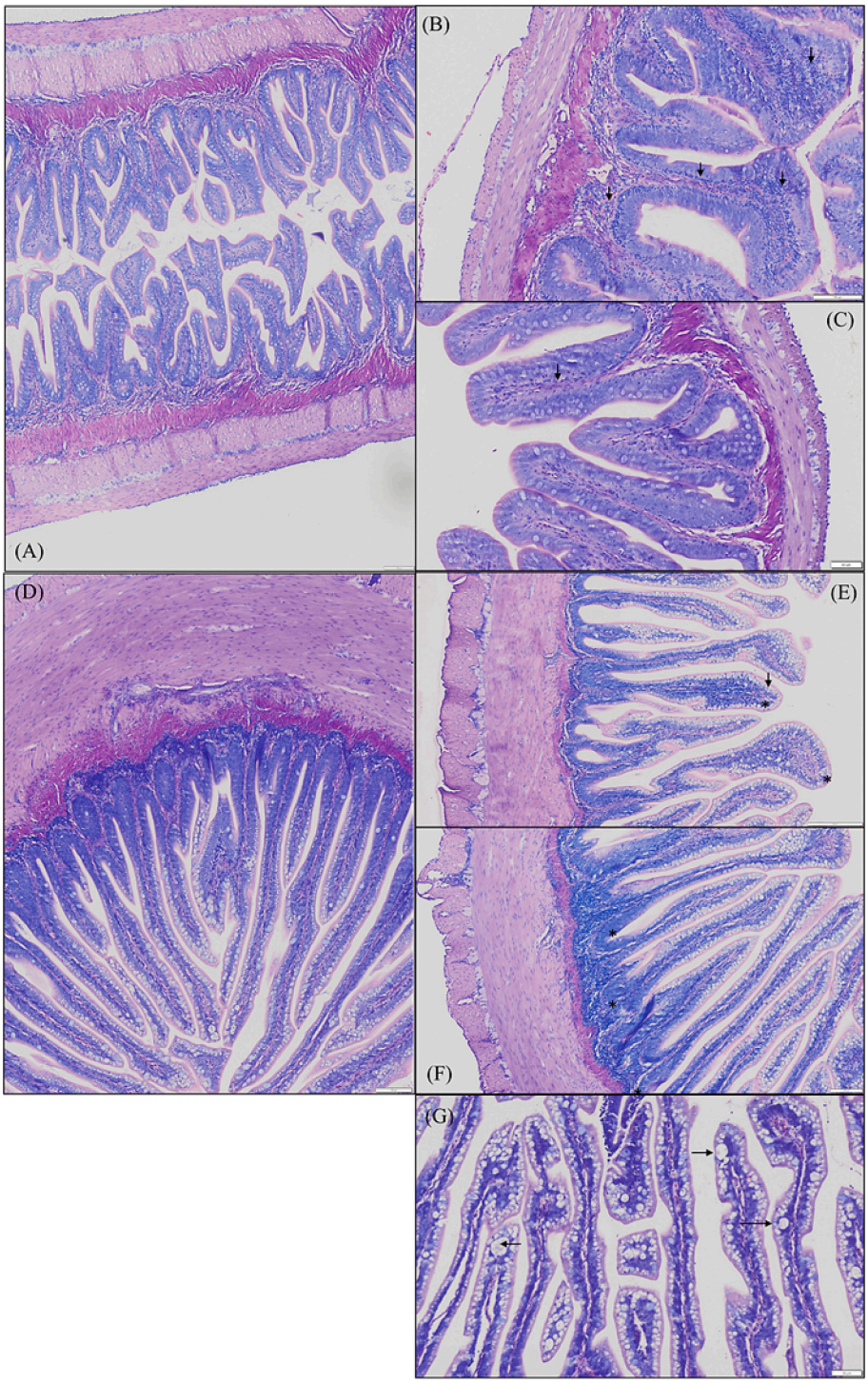

Fig. 2. Detailed micrographs of European sea bass (*D. labrax*) pre-ileorectal and post-ileovalve gut region stained with May-Grünwald Giems. (2A) Detail of the general morphological pattern observed for pre-ileorectal intestinal region. (2B) Detail of lymphocytic focuses detected in the pre-ileorectal intestinal region mucosa, *lamina propria* and submucosa (→), but not associated to a fish genotype or dietary treatment fed in relation to a non-inflammatory porterior gut pattern (2C)·(2D) Detail of the general morphological pattern observed for post-ileorectal valve region area. Detail of lymphocytic focuses detected in the *lamina propria* and infiltrated intraepithelial lymphocytes (IELs) in the mucosa (→)(2E), but not associated to any specific genotype or diet. (2F) Detail of a higher inflamation status of the submucosa in WT fish (*). (2G) Detail of hypertrophic vacuoles present in fish fed Future diet (→), regardeless of the genotype origin. Scales bar 100 μm and 50 μm.

inflammatory status in the mucosa and *lamina propria,* no differences associated to genotype or diet were detected (Table3; Fig. 2D). As observed for the pre-ileorectal valve segment, a high variability between individuals as well as the presence of inflammatory focuses in the *lamina propria*, mucosa (Fig. 2E) and submucosa (Fig. 2F) were identified. Certainly, the submucosa region of WT fish presented a trend to a higher inflammatory status, particularly a in terms of density of lymphocytes (Table 3; Fig. 2E), compared to GS fish (Fig. 2D vs 2E). In addition, fish fed future diet presented an increased presence of vacuoles, some of them with signs of hypertrophy, which were not observed in fish fed the control diet (Table 3; Fig. 3G).

3.2. Mucus production studies

In the proximal gut, no differences were detected on goblet cells area for neither genotype nor diet ($n = 7000$ cells /fish, 5 fish/ diet/genotype, respectively) (Fig. 3A), ranging approximately from 50 to 75 μm^2. On the contrary, the distal region goblet cells area was affected by both variables depending on the region evaluated. In the pre-ileorectal valve segment (posterior gut) fish belonging to the WT group presented larger ($p < 0.05$) goblet cells than selected fish, regardless of the diet fed (Fig. 3B, C), whereas for the post-ileorectal valve region (rectum) fish fed the future diet presented larger goblet cells (Fig. 3C; $P < 0.1$) than

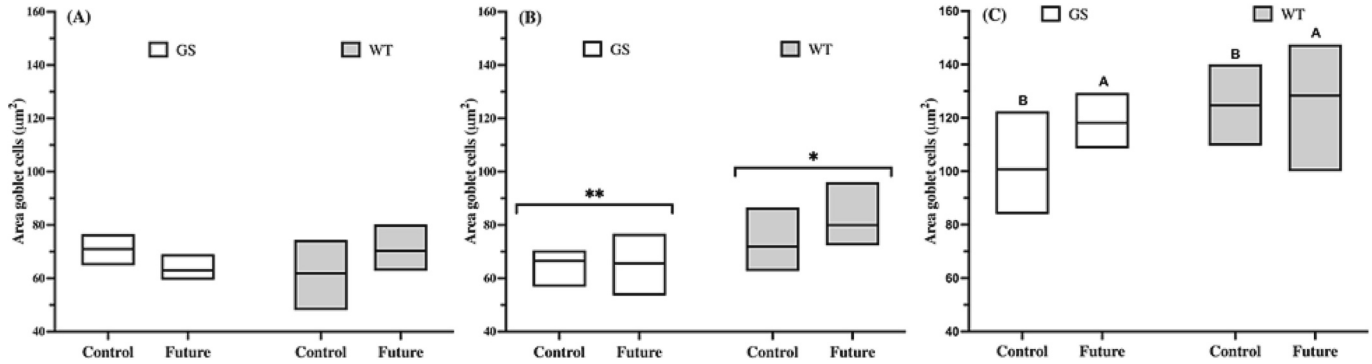

Fig. 3. Area (µm^2) of (A) proximal gut, (B) posterior gut (pre-ileorectal valve segment) and (C) rectum (post-ileorectal valve segment) goblet cells of European sea bass (*D. labrax*) at the end of the long-term feeding trial. GS, genetically selected genotype; WT = wildtype European sea bass genotype. Control = control diet based in a 20% Fish meal/5% Fish oil; Future diet based in a 10% Fish meal/ 0% Fish oil. Data represent the mean ± SD. Two-way ANOVA, different symbols indicate significant differences at $p \leq 0.05$, different capital letters indicate significant differences at $p \leq 0.1$.

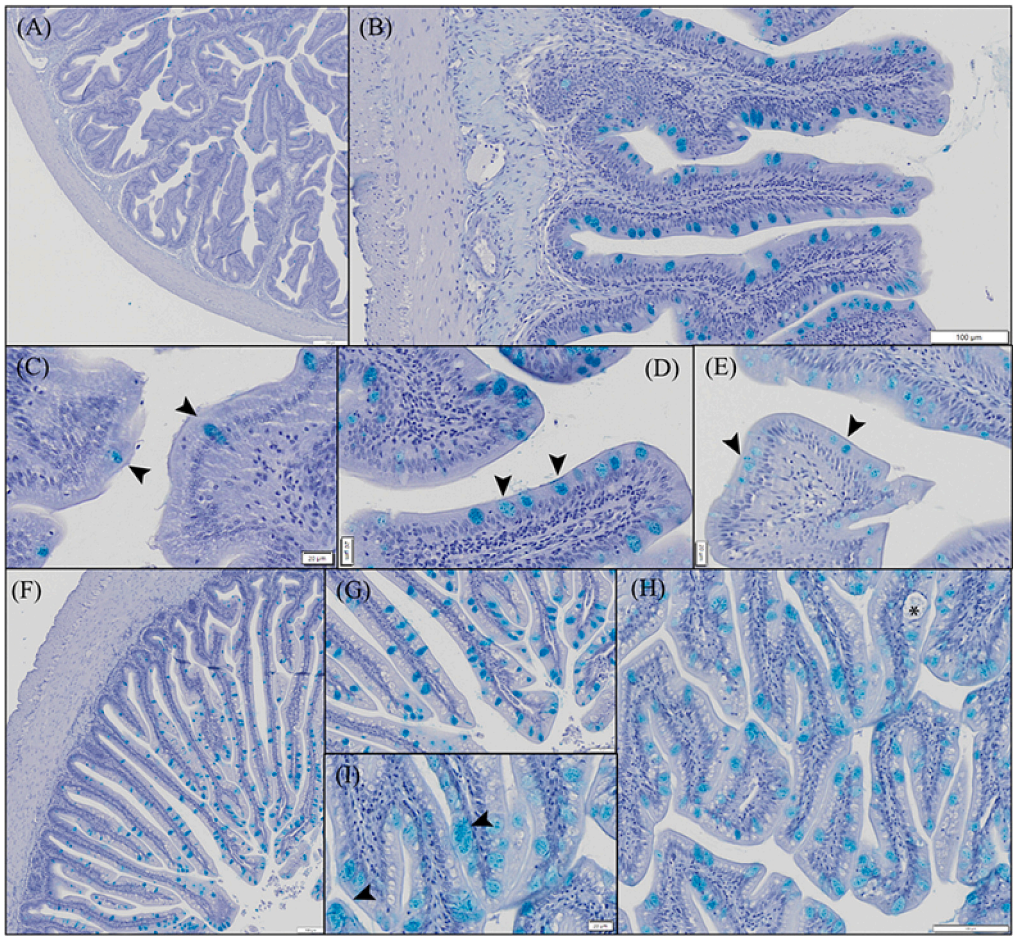

Fig. 4. Detailed micrographs of the different European sea bass (*D. labrax*) intestinal regions evaluated in mucus production studies at the end of the long-term feeding trial stained with Alcian Blue (pH = 2.5). (4A) Proximal gut goblet cells (➤) distribution pattern, observe the lower number of goblet cells distributed along the fold compared to posterior gut (4B) and rectum (4F), however they were similar in area (4C) to posterior gut goblet cells (4D, 4E), especially for the selected (GS) fish (4E). Scale bar 100 µm. Observe the variations on the distribution pattern of goblets cells along the fold for posterior gut (4B) and rectum (4F) section. Posterior gut presented similar density of goblet cells along the fold, with a slight increase in the mid-basal region compared to the apical region of the fold (4B), whereas rectum segment presents the contrary pattern (4F). Scale bar 100 µm. Rectum detailed micrograph of fish fed control diet (4G) and fish fed "future" diet (4H, 4I), observe the presence of larger goblet cells (➤) in fish fed "future" diet and the presence of hypertrophic vacuoles (*) compared to fish fed the control diet (4G). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fish fed the control diet, regardless of the genotype. Goblet cells area in the pre-ileorectal valve segment ranged between 55 and 95 µm^2, whereas for the post-ileorectal valve segment varied between 85 and 140 µm^2 (Fig. 3B, C). A similar size of goblet cells for GS fish in the proximal and distal gut regions was observed, whereas WT group had slight larger goblet cells in the posterior gut compared to proximal region (Fig. 3; Fig. 4A-D). A general trend to present larger goblet cells in the post-ileorectal valve region in WT fish compared to GS fish was noticed (Fig. 3C).

All fish evaluated presented an adequate proximal gut goblet cells distribution pattern, with lower density of goblet cells distributed along the fold compared to posterior gut or rectum regions (Fig. 3; Fig. 4A, B, F). As expected, posterior gut presented goblet cells scattered along the fold, with a slightly higher density on the mid-basal fold areas compared to apical area, whereas rectum region presented the opposite pattern (Fig. 4F). Rectum of fish fed future diet (Fig. 4H) presented larger goblet cells (Fig. 4G, I) and scattered hypertrophic vacuoles (*) compared to rectum of fish fed the control diet (Fig. 4G).

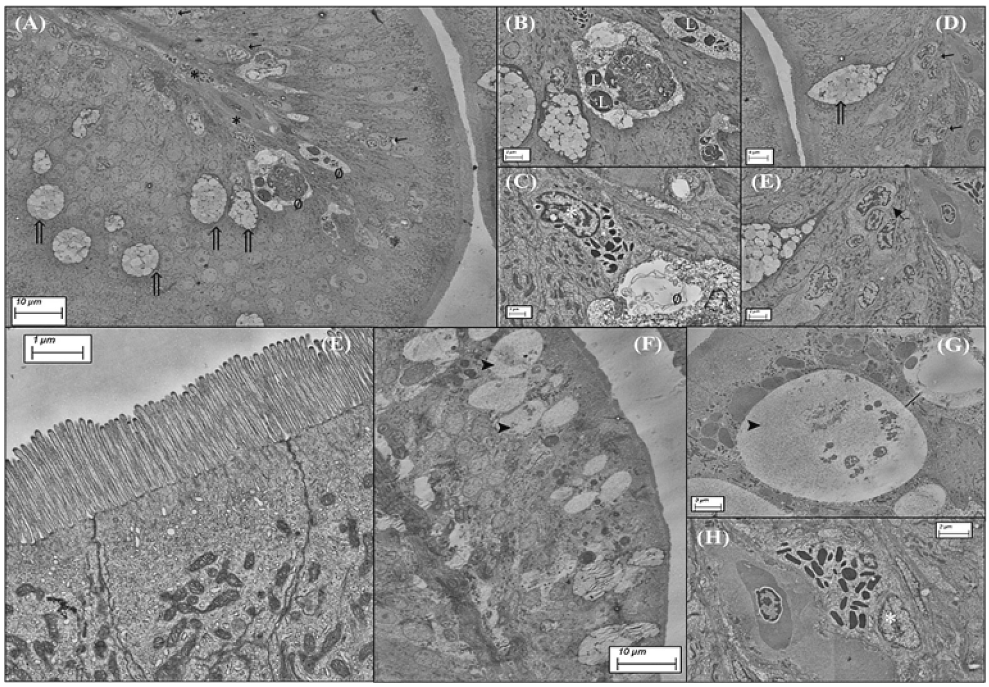

Fig. 5. Representative TEM micrographs of the intestinal regions of European sea bass (*D. labrax*) fed the experimental diets. (5A) Anterior gut structural pattern observed. Observe the conserved structure and the presence of goblet cells (⟹), granulocytes (*) intraepithelial lymphocytes (→) and phagocytes (∅). Scale bar 10 μm. (5B) Detailed micrograph of a phagocytic cell, observe the lipid vacuoles in the cytoplasm (L). Scale bar 2 μm. (5C) Detailed micrograph of a granulocyte (*) and a macrophage (∅) located near the basal mebrane. Scale bar 2 μm. (5D) Detailed micrograph of a goblet cell (⟹), tipically scattered among enterocytes and characterized by a clearly polarized shape with narrow base containing the nucleus and organelles and an expanded apical region containing the secretory granules. Scale bar 4 μm. (5E) Detailed micrograph of intraepithelial lymphocytes (→), characterized by a single nucleus with areas of condensed heterochromatin. Scale bar 2 μm. (5E) Pattern of posterior or TJs structure between fish genotypes not diets fed. Scale bar 1 μm. (5F) Pattern of rectum ultrastrcuture, observed the ultrastructure of the vacuoles (➤) located in the supranuclear region of the enterocytes. Scale bar 10 μm. (5G) Detailed micrograph of a supranuclear post-ileorectal vacuoles (➤), characterized by a heterodense content with the presence of scattered condensed material with higher electrondensity. Scale bar 3 μm. (5H) Detailed micrograph of a granulocyte (*) located in the post ileorectal valve segment. Scale bar 2 μm.

Table 4
Gene expression by intestinal section of European sea bass (*D. labrax*) along the long-term feeding trial.

			GS		WT		*P* value		
	dph		Control	Future	Control	Future	Genotype	Diet	G*D
Proximal gut	294	**il 1β**	1.59 ± 0.59	–	1.65 ± 0.65	–	ns		
		tnf-α	1.49 ± 0.81	–	1.13 ± 0.29	–	ns		
		il-10	2.65 ± 1.02	–	1.90 ± 0.48	–	ns		
		cd4	3.30 ± 1.35	–	5.54 ± 1.71	–	0.0309		
		cox2	1.01 ± 0.16	–	4.08 ± 1.00	–	0.0288		
		mhc I	2.26 ± 1.51	–	1.25 ± 0.87	–	ns		
		mhc II	1.07 ± 0.44	–	1.32 ± 0.34	–	ns		
	609	**il 1β**	1.06 ± 0.42	1.39 ± 0.55	1.31 ± 0.40	2.08 ± 0.75	ns	ns	ns
		tnf-α	1.14 ± 0.58	1.08 ± 0.36	1.04 ± 0.30	1.60 ± 0.96	ns	ns	ns
		il-10	1.01 ± 0.17	1.57 ± 0.65	1.24 ± 0.38	1.30 ± 0.55	ns	ns	ns
		cd4	1.22 ± 0.26	1.52 ± 0.51	1.70 ± 0.57	1.13 ± 0.63	ns	ns	ns
		cox2	3.08 ± 1.97	3.85 ± 2.23	3.14 ± 1.03	2.59 ± 1.11	ns	ns	ns
		mhc I	4.16 ± 1.32	4.81 ± 1.29	3.25 ± 1.46	6.24 ± 4.48	ns	ns	ns
		mhc II	1.64 ± 0.65	1.85 ± 0.54	1.92 ± 0.78	2.15 ± 0.98	ns	ns	ns
Distal gut	294	**il 1β**	1.03 ± 0.26	–	3.05 ± 2.02	–	0.0103		
		tnf-α	1.08 ± 0.43	–	2.09 ± 0.54	–	0.0047		
		il-10	1.10 ± 0.49	–	1.70 ± 0.83	–	ns		
		cd4	1.34 ± 0.34	–	2.31 ± 0.28	–	0.0003		
		cox2	1.27 ± 0.28	–	1.62 ± 0.38	–	0.0999		
		mhc I	1.15 ± 0.64	–	1.47 ± 0.98	–	ns		
		mhc II	1.01 ± 0.13	–	1.97 ± 0.38	–	0.0001		
	609	**il 1β**	1.28 ± 0.62	1.88 ± 0.58	7.54 ± 1.23	3.84 ± 2.04	0.0001	0.0042	0.0004
		tnf-α	1.58 ± 0.84	3.18 ± 0.89	2.69 ± 0.82	2.21 ± 0.94	ns	ns	0.0125
		il-10	3.74 ± 0.94	1.67 ± 0.67	1.21 ± 0.60	1.61 ± 0.80	0.0012	0.0387	0.0011
		cd4	1.25 ± 0.44	1.12 ± 0.42	1.03 ± 0.27	1.54 ± 0.31	ns	ns	ns
		cox2	1.77 ± 0.71	1.65 ± 0.62	1.72 ± 1.11	1.30 ± 0.77	ns	ns	ns
		mhc I	3.03 ± 1.33	2.69 ± 1.38	2.61 ± 1.61	5.51 ± 3.37	ns	ns	ns
		mhc II	1.60 ± 0.51	1.53 ± 0.58	1.32 ± 0.32	2.51 ± 1.50	ns	ns	ns

GS, genetically selected genotype; WT, wildtype European sea bass genotype. Control = control diet based in a 20% Fish meal/5% Fish oil; Future = alternative diet based in a 10% Fish meal/ 0% Fish oil. Data represent the mean ± SD. *t* = 294 dph, Student's t-test between families. *t* = 609 dph, two-way ANOVA, $p \leq 0.05$,. ns = not significant.

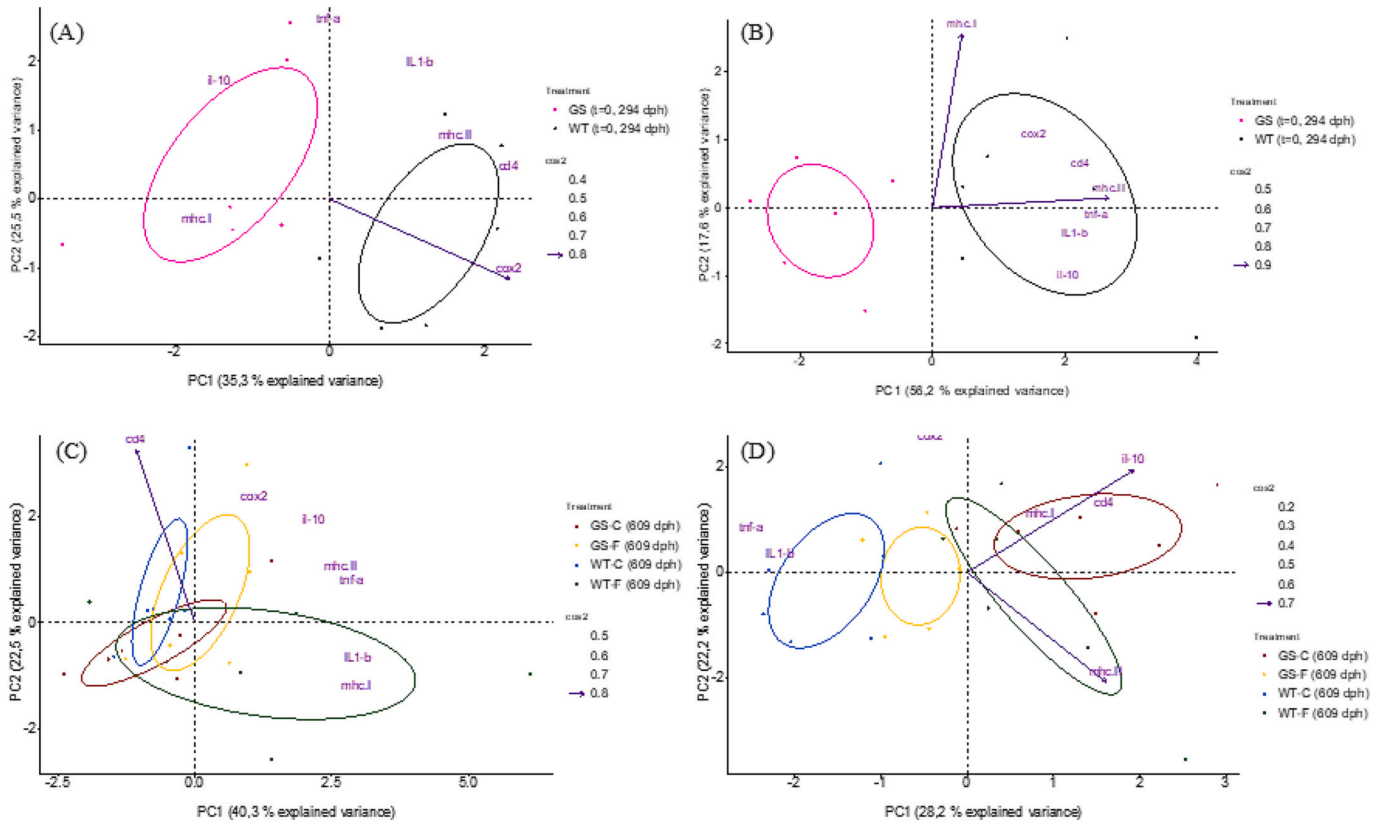

Fig. 6. Principal component analysis (PCA) representing the variability on the (A, C) proximal and (B, D) distal intestine relative gene expression in relation to fish genotype origin and dietary treatment fed, for initial sampling (294 dph) (A, B) and final sampling (609 dph) (C,D). Gene expression is represented as cos2 function by an intensity scale, and confidence ellipses are generated around mean group points. The points correspond to the replicates and are colored according to genotype and diet fed, being GS, genetically selected genotype; WT, wildtype European sea bass genotype. Control (A), control diet based in a 20% Fish meal/5% Fish oil; Future (B), alternative diet based in a 10% Fish meal/ 0% Fish oil. $T = 0$, 294 dph. Interleukine 1-β (*il-1β*); Interleukine 10 (*il-10*); tumor necrosis factor α (*tnfα*); cluster of differentiation 4 (*cd4*); cyclooxigenase 2 (*cox2*); major histocompatibility complex I (*mhcI*); major histocompatibility complex II (*mhcII*).

3.3. Ultrastructure study

For all the intestinal sections evaluated, the qualitative TEM analysis revealed a comparable morphological pattern for all fish with slight variations depending on genotype and diet supply during the long-term feeding trial. In this sense, the presence of infiltraded leucoytes was slightly higher in WT fish compared to GS fish (Fig. 5A-E) in accordance with the morphometric and histopathological findings of the present study. All analyzed fish presented normal microvillar morphology and intact and well-packaged enterocytes with similar membrane linning appearance, enterocyte cytoplasmic electron density and TJ structure (Fig. 5E), Goblet cells were scattered among enterocytes with a characteristic polarized shape, the apical region contating the secretory granules with different electron density (Fig. 5A; Fig. 5D). In the post-ileorectal valve segment we could identify vacuoles (Fig. 5F-G) located on the supranuclear region of enterocytes, with higher presence on the apical region of the fold compared to the basal. This vacuoles are typically characterized by a heterogenous content, with the presence of scattered condensed material with higher electrondensity. Fig. 5H shows the structural characteristic morphology of an infiltrated granulocyte.

3.4. Expression of GALT- related genes

At 294 dph (initial sampling), gene expression results revealed that fish genotype significantly affected the relative expression of *cd4* and *cox2* genes in the proximal gut and of *il-1β*, *tnf-α*, *cd4*, *cox2* and *mhc II* in the distal gut region of European sea bass, presenting WT genotype fish higher expression levels compared to fish from GS genotype (Table 4). At the end of the long-term feeding trial (609 dph), no significant differences attributed to neither genotype nor diet were observed for proximal gut gene expression for the studied genes. However, for the distal section (pre- and post ileoreactal valve regions), the relative gene expression of *il-1β* and *il-10* was significantly ($p < 0.05$) affected by both factors, genotype and diet (Table 4). In general, the data indicated that fish from WT genotype presented an upregulation ($p < 0.05$) of *il-1β* and a downregulation ($p < 0.05$) of *il-10* gene expression in comparison to fish from GS genotype when fed the control diet. However, when fed Future diet, fish from GS genotype showed higher expression of *il-1β* gene and lower expression of *il-10* than fish fed the control diet, resulting in a significant ($p < 0.05$) interaction between two factors (Table 4). Instead, the il1-b and 1 l-10 gene expression of WT fish fed the F diet showed an opposite trend.

No effect of diet and/or genotype was detected for *mhc-I, mhc-II, cox-2* and *cd4* transcript level gene expression in distal gut at the end of the long term feeding trial (Table 4).

At the initial sampling (294 dph) the principal component analysis (PCA), based on gene expression data, clearly separted the two genotypes. The first two PCA dimensions (PC1, PC2) together accounted for 60.8% and 70.8%, of the total variance in proximal and distal intestine, respectively (Fig. 6A,B). Specifically, the variations in the PC1 direction contributed to 35.3% and 56.2% of the total variation in proximal and distal intestinal portion, respectively.

At the end of the feeding trial (609 dph), the PCA analysis explained 62.8% and 50.4% of the variance found in proximal and distal intestine, respectively (Fig. 6C,D). In contrast to initial time, at proximal intestinal level, the experimental groups clustered together thus confirming the gene expression data obtained. For distal intestine, despite the PCA represented only the 50.4% of the total variance observed, WT-C and

Table 5
Alpha diversity measures of intestinal bacterial communities.

dph		GS		WT		P value		
		Control	Future	Control	Future	Genotype	Diet	G*D
294	**Chao 1**	571 ± 164	–	506 ± 115	–	0.449		
	Faith PD	9.03 ± 2.81	–	6.37 ± 0.98	–	0.053		
	Observed OTUs	504 ± 166	–	449 ± 113	–	0.523		
	Shannon	5.86 ± 0.89	–	5.75 ± 0.85	–	0.841		
	Simpson	0.94 ± 0.03	–	0.93 ± 0.03	–	0.892		
609	**Chao 1**	137 ± 64	167 ± 78	352 ± 261	423 ± 300	0.010	ns	ns
	Faith PD	2.66 ± 1.13	3.22 ± 1.12	6.20 ± 2.67	6.29 ± 2.37	0.001	ns	ns
	Observed OTUs	116 ± 51	136 ± 62	295 ± 237	368 ± 283	0.015	ns	ns
	Shannon	4.10 ± 0.54	4.16 ± 0.68	4.38 ± 1.20	4.93 ± 1.48	ns	ns	ns
	Simpson	0.87 ± 0.03	0.87 ± 0.05	0.86 ± 0.05	0.88 ± 0.06	ns	ns	ns

GS, Genetically selected genotype; WT, wildtype genotype. Control (A), control diet based in a 20% Fish meal/5% Fish oil; Future (B), alternative diet based in a 10% Fish meal/ 0% Fish oil. $T = 0$, 294 dph. Data represent the mean ± SD. Two-way ANOVA, $p \leq 0.05$. ns = not significant.

Table 6
Results of PERMANOVA multivariate analysis on both unweighted and weighted UniFrac distance matrix data.

PERMANOVA (Permutation N: 999)		
Unweighted		
	Pseudo-F	*p*
GS Control (609 dph) vs GS Future (609 dph)	1.15	0.298
GS Control (609 dph) vs GS (294 dph)	4.40	**0.005**
GS Control (609 dph) vs WT Control (609 dph)	2.03	**0.028**
GS Future (609 dph) vs GS (294 dph)	4.19	**0.005**
GS Future (609 dph) vs WT Future (609 dph)	2.11	**0.010**
GS (294 dph) vs WT (294 dph)	2.07	**0.008**
WT Control (609 dph) vs WT Future (609 dph)	0.97	0.465
WT Control (609 dph) vs WT (294 dph)	2.31	**0.003**
WT Future (609 dph) vs WT (294 dph)	2.59	**0.002**
Weighted		
	Pseudo-F	*p*
GS Control (609 dph) vs GS Future (609 dph)	3.22	0.079
GS Control (609 dph) vs GS (294 dph)	2.66	**0.045**
GS Control (609 dph) vs WT Control (609 dph)	3.87	0.067
GS Future (609 dph) vs GS (294 dph)	6.04	**0.011**
GS Future (609 dph) vs WT Future (609 dph)	2.75	0.115
GS (294 dph) vs WT (294 dph)	0.58	0.842
WT Control (609 dph) vs WT Future (609 dph)	3.00	0.114
WT Control (609 dph) vs WT (294 dph)	9.87	**0.007**
WT Future (609 dph) vs WT (294 dph)	2.03	0.121

GS-F were clearly separated from WT-F and GS-C along PC1. The GS-C group was located in the upper right area driven by higher expression of il-10 and mhcI, whereas WT-C clustered in opposite position on bidimensional graph driven by higher expression of pro-inflammatory citokines. This indicated that: (i) at younger ages (294 dph) fish genotype exert a clear effect on the relative gene expression of gut GALT-related genes when fed the same diet (acclimatation diet) for both intestinal regions and (ii) fish intestine region evaluated is determinant to define the effects of genotype and dietary treatment in terms of GALT-related gene expression in this fish species.

3.5. Microbiome analysis

All sequencing data were submitted to the European Nucleotide Archive (EBI ENA) public database, under the accession code PRJEB47388.

A total of 1,561,124 sequence reads remained after data processing from all microbiota samples [feed, intestinal samples from initial fish stage of development (294 dph, $T = 0$), and from the last development stage (609 dph, final sampling)], corresponding to an average read depth of 37,169 ± 10,222 per sample. Removal of those sequences assigned to mitochondria or chloroplast reduced the read depth to 31,663 ± 14,859.

To calculate the alpha diversity metrics, feed and mucosa samples were rarefied at a sequencing depth of 17,778 reads. Results of alpha diversity metrics on intestinal samples are reported in Table 5. There was no interaction between diet and genotype for any index considered. However, despite no differences in bacterial species richness and biodiversity was found in initial fish stage of development (294 dph, $T = 0$), at the end of feeding trial gut microbiota of GS European sea bass was characterized by lower species richness than WT group, regardless of the diet fed.

There was a significant genotype effect on all species richness indices considered (Chao 1, Faith-PD, and Observed OTUs) (Table 5). Comparison between gut microbial communities (beta-diversity) revealed an overall effect of genotype and diet, on presence/absence of specific taxa (unweighted UniFrac), but not on their relative abundance (weighted UniFrac) (Table 5). The permutational multivariate analysis applying Permanova test on Weighted UniFrac distances showed significant differences only between gut microbiota of initial (294 dph) and end point (609 dph) samples. The effect of both, genotype and diet, was significant only for unweighted Unifrac distances (Table 6).

To further evaluate differences in the bacterial composition between two genotypes, a PLS-DA was performed based on the relative abundances of most representative taxa (relative abundance >0.1%) at genus level. The PLS-DA clearly separated GS from WT fish along component 1 (21% explained variance) (Fig. 7). Differences between GS and WT fish at initial time were driven by 2 taxa (VIP > 1), belonging to the genera *Pseudomonas* and *Anaerococcus* found only in GS fish. The same analysis on final samples showed a great deal of compositional overlap between groups, though differences between genotypes were detected in both multivariate dispersion and centroid location (Fig. 8). Regardless of the diet, WT fish were widely dispersed indicating a larger inter-individual variability than GS fish characterized by well-defined clusters. In this case, filtering by VIP ≥ 1 identified 13 taxa, mainly associated to WT group fed future diet.

3.6. Microbiome profiles of feeds

The composition of the feed-associated microbiome was successfully outlined at the phylum, class, order, family, and genus. The microbiome profile considering all samples comprised 6 different phyla, 9 classes, 15 orders, 24 families, and 25 genera. However, by considering only the most abundant bacterial taxa, it mainly consisted of 3 phyla, 4 classes, 4 orders, 9 families, and 11 genera. Both phylum and genus level profiles of each feed are displayed in Fig. 9A and B, respectively. Table 7 reports the percentages of the most abundant taxa found in feed samples and relative statistics.

At phylum level feeds were characterized by higher percentage of Proteobacteria (86%), followed by Firmicutes (10%), and Fusobacteria

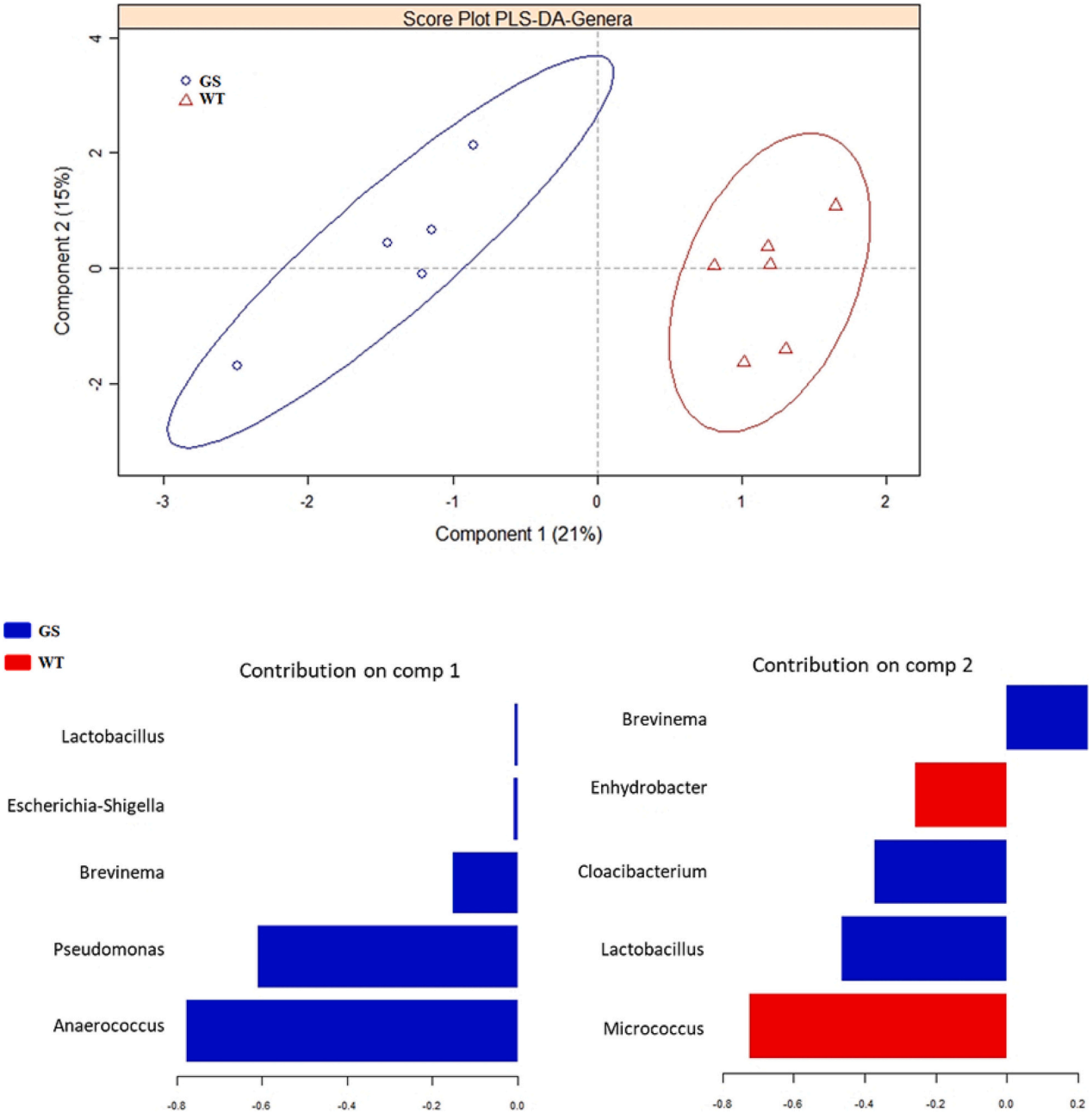

Fig. 7. Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundances of bacterial genera in the gut microbiota of initial fish stage of development (294 dph, T = 0). GS, Genetically selected genotype; WT, wildtype European sea bass genotype.

(3%) (Fig. 9A). The relative abundance of Firmicutes was higher in Future feed ($p < 0.05$). *Photobacterium*, *Lactobacillus*, and *Moritella* were the most abundant genera associated to feeds. Interestingly, among them, *Lactobacillus* were more abundant in Future feed (Fig. 9B, Table 7). However, except for these few taxa, two microbial profiles were similar. The permutational multivariate analysis made with PERMANOVA on both unweighted (pseudo-F: 0.618, $p = 0.694$) and weighted UniFrac distance data (pseudo-F: 1.311, $p = 0.387$), did not reveal any statistically significant differences between feed-associated microbial communities.

3.7. Profile and dietary modulation of gut bacterial communities

The entire microbial community profile of 36 intestinal mucosa samples was mainly composed of 9 phyla, 11 classes, 28 orders, 43 families, and 38 genera. By considering only the most representative taxa, the overall microbiota consisted of 5 phyla, 7 classes, 15 orders, 20 families, and 20 genera. The profiles of intestinal microbial communities for each feeding group are presented at phylum and family level (Fig. 10). In line with alpha diversity analysis results, there was an evident decrease of biodiversity, in terms of taxa found, in GS fish respect to WT fish samples at the end of feeding trial (609 dph). As shown in Venn diagram (Fig. 11A), a total of 21 and 32 different taxa were assigned to WT and GS fish at the initial sampling (294 dph), respectively. From them, 18 were shared between the two genotypes studied. However, the number of different bacterial taxa found in final samples was drastically reduced, especially in GS fish (Fig. 10B).

As expected Firmicutes and Proteobacteria were the most abundant phyla in the intestinal mucosa of sea bass (Fig. 10A). Together these phyla constituted >90% of fish gut microbiota regardless of the diet or

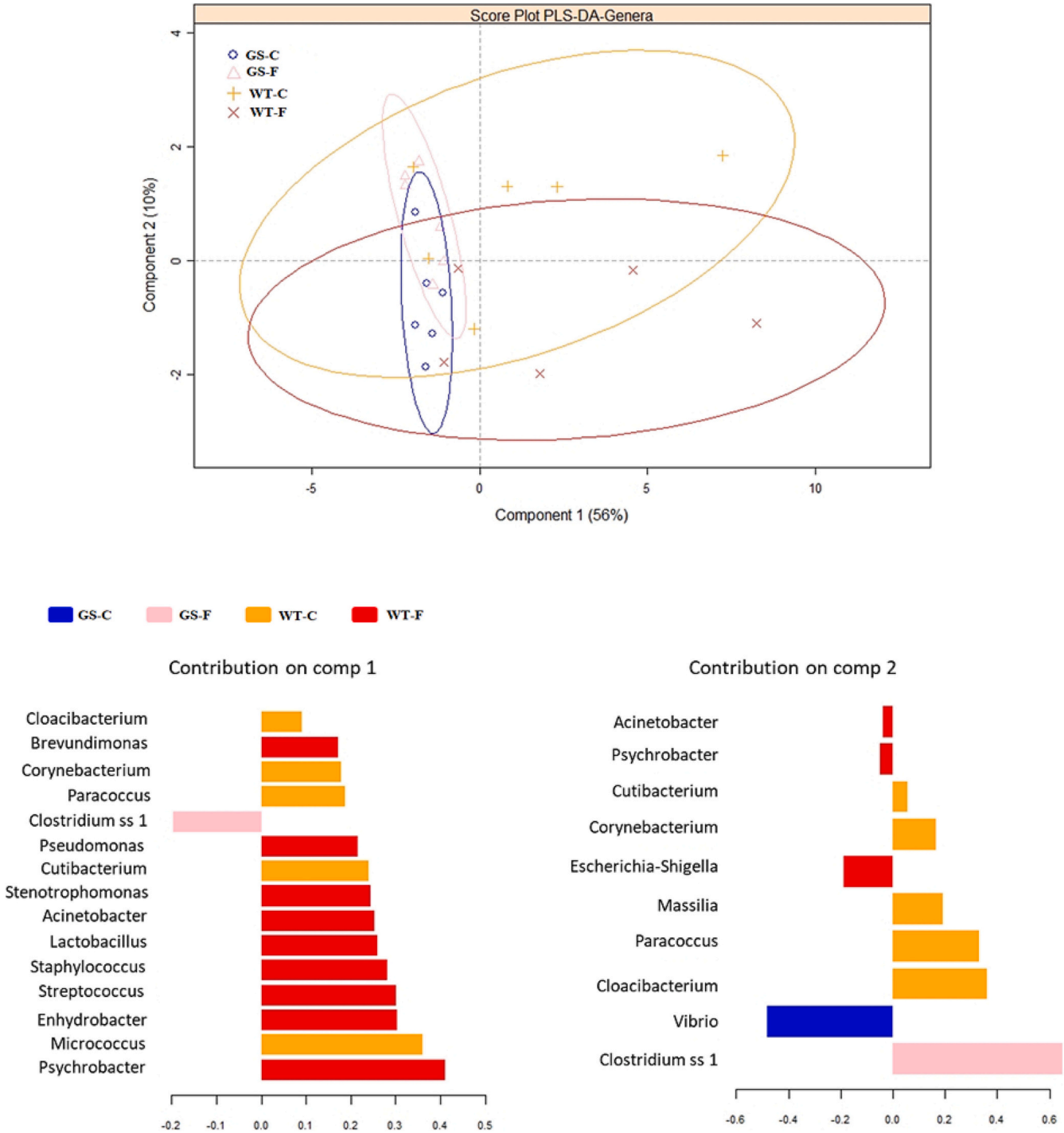

Fig. 8. Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundances of bacterial genera in the gut microbiota of final samples. GS, Genetically selected genotype; WT, wildtype European sea bass genotype. Control (A), control diet based in a 20% Fish meal/5% Fish oil; Future (B), alternative diet based in a 10% Fish meal/ 0% Fish oil.

genotype (Table 8).

The differences in gut microbial composition between two sea bass genotypes before starting the feeding trial (294 dph) were due to Spirochaetes phylum, mainly represented by genus *Brevinema*, and by genus *Anaerococcus* that were solely detected in GS genotype fish. In the same genotype, the genus *Pseudomonas* was higher abundant than in not WT fish (Table 8). Differential abundance analysis conducted on the end trial samples revealed an overall genotype effect on numerous taxa, without any diet or interaction effect (Table 8). At phylum level, Actinobacteria and Bacteroidetes, mainly represented by Actinobacteria and Bacteroidia class respectively, were more abundant in WT genotype regardless of the diet. Similarly, Bacilli and Alphaproteobacteria were enriched in WT sea bass. At family level, Lactobacillaceae, Streptococcaceae, Staphylococcaceae, Neisseriaceae, Caulobacteraceae were only found in intestine of wildtype fish, while Moraxellaceae family seemed to be mainly associate with this genotype (Fig. 10B, Table 8). Accordingly, *Lactobacillus*, *Streptococcus*, and *Staphylococcus* genera were practically undetectable in GS fish. Actually, the relative abundances of the most representative genera found were lower in GS fish than in WT genotype, irrespective to the diet (Table 8). Bacterial genera

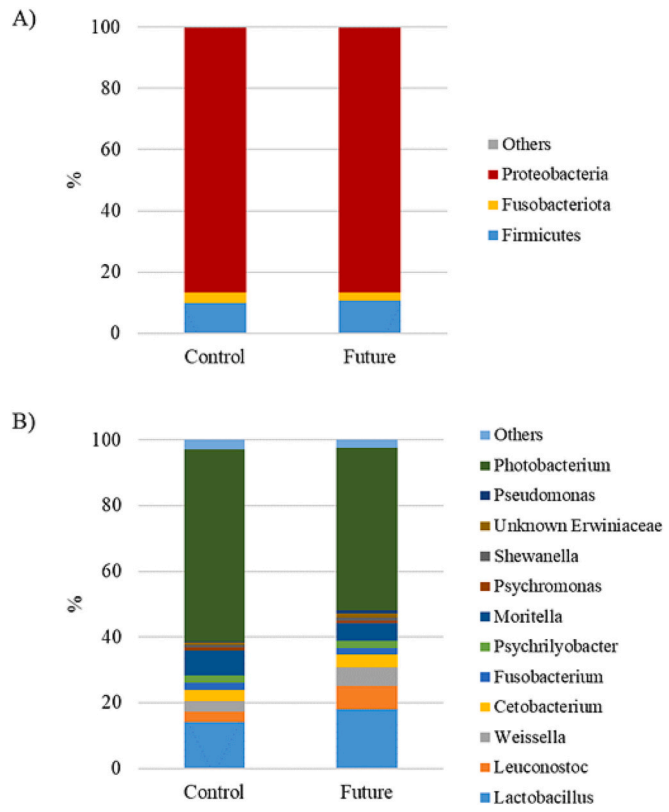

Fig. 9. Mean relative abundance (%) of the most prevalent bacteria in control and future feeds at phylum (A), and genus (B) taxonomic level ($N = 3$). Only bacteria with an overall abundance of 0.5% were reported. Bacteria with lower abundance were pooled and indicated as "others". Control = based in a 20% Fish meal/5% Fish oil; Future diet based in a 10% Fish meal/ 0% Fish oil.

Psychrobacter, Micrococcus, Enhydrobacter, Corynebacterium, Cutibacterium, Paracoccus, and *Stenotrophomonas* showed a significant genotype effect, resulting enriched in intestine of WT fish. An interaction effect diet x genotype was only found for *Clostridium* sensu stricto, being enriched in selected fish fed "future" diet F (Table 8). Finally, a positive correlation between *Paracoccus* abundance and il-1β gene expression was found in distal intestine (Pearson's $r = 0.93$; $p = 0.005$).

3.8. Comparison of predictive metabolic pathway abundances

The PICRUSt analysis was performed comparing WT and GS fish fed the future diet and between the same genotype fed different diets. Level 3 KEGG orthologue function prediction was used. Our analysis revealed 17 predicted metabolic pathways significantly different between two experimental groups (GS and WT) fed the future diet (Fig. 12A).

Metabolic inference from 16 rRNA gene sequencing data showed that GS fish fed future diet had an increased abundance of genes involved in sugar metabolism, transcription processes, bacterial motility proteins and chemotaxis. On the contrary, genes responsible of phenylalanine, sulfur, glutathione, and butanoate metabolism, replication and repair processes, benzoate and fatty acid degradation were enhanced in WT sea bass fed the same diet. Within the WT genotype, 6 metabolic pathways resulted influenced by diet (Fig. 12B), the PICRUSt analysis showed an increase in gene abundance for pentose and gluconate interconversions, pentose phosphate pathway and ascorbate and aldarate metabolism when fish were fed control diet. On the contrary, WT fish fed future diet (F) counted higher amount of sequences related to transfer RNA biogenesis and phenylalanine metabolism. In GS sea bass, a total of 10 metabolic pathways differed between two feeding groups (Fig. 12C). Fish fed control diet (C) were characterized by an increase in genes related to pyruvate metabolism, bacterial toxins, and flagellar assembly. Administration of the future diet to GS fish contributed, instead, to improve the metabolism of amino sugar and nucleotide sugar, galactose, glycerophospholipids, ascorbate, and aldarate.

Table 7
Mean relative abundance (%) ± SD (N = 3) of the most prevalent phyla, orders, classes, families, and genera found in tested feeds. Significant p values (< 0.05) are shown in bold.

Taxa	Control diet			Future diet			*p*-value
Phylum							
Firmicutes	9.98	±	0.24	10.71	±	0.19	**0.010**
Fusobacteria	3.45	±	0.60	2.74	±	0.09	0.108
Proteobacteria	86.38	±	0.74	86.45	±	0.19	0.886
Class							
Bacilli	9.54	±	0.24	10.44	±	0.12	**0.004**
Fusobacteriia	3.45	±	0.60	2.73	±	0.08	0.190
Alphaproteobacteria	54.38	±	6.92	66.74	±	1.80	0.036
Gammaproteobacteria	31.99	±	7.50	19.70	±	2.00	0.086
Order							
Lactobacillales	21.05	±	2.99	31.20	±	2.13	**0.003**
Fusobacteriales	7.78	±	2.25	8.24	±	0.21	0.190
Alteromonadales	9.25	±	0.61	6.92	±	0.94	**0.004**
Vibrionales	59.09	±	6.04	49.69	±	4.26	0.052
Family							
Lactobacillaceae	14.19	±	0.42	18.11	±	1.72	**0.005**
Leuconostocaceae	6.32	±	2.55	12.52	±	0.19	0.076
Fusobacteriaceae	7.78	±	2.25	8.25	±	0.23	0.696
Moritellaceae	7.54	±	0.46	5.25	±	1.07	**0.013**
Psychromonadaceae	0.91	±	0.11	0.76	±	0.09	0.104
Shewanellaceae	0.79	±	0.11	0.90	±	0.24	0.413
Erwiniaceae	0.84	±	0.11	1.42	±	0.05	**0.002**
Pseudomonadaceae	0.20	±	0.17	0.97	±	0.42	**0.020**
Vibrionaceae	59.10	±	6.06	49.76	±	4.19	0.077
Genus							
Lactobacillus	14.21	±	0.42	18.13	±	1.72	**0.006**
Leuconostoc	2.99	±	1.37	6.89	±	0.17	0.081
Weisella	3.34	±	1.27	5.64	±	0.02	0.113
Cetobacterium	3.26	±	0.79	3.90	±	0.28	0.305
Fusobacterium	2.41	±	0.63	1.96	±	0.05	0.359
Psychrilyobacter	2.12	±	0.85	2.40	±	0.01	0.117
Moritella	7.55	±	0.47	5.26	±	1.08	**0.013**
Psychromonas	0.91	±	0.11	0.75	±	0.09	0.105
Shewanella	0.79	±	0.11	0.90	±	0.23	0.412
Pseudomonas	0.20	±	0.17	0.97	±	0.42	**0.020**
Photobacterium	58.59	±	6.17	49.38	±	4.43	0.084

4. Discussion

An adequate and efficient response to the increasing demand of traditional fish derived raw materials as aquaculture production increases implies a successful integration in aquafeeds formulas of sustainable raw materials covering fish essential amino acids and n-3 LC-PUFA (EPA and DHA) nutritional requirements, particularly in marine fish species. Furthermore, to formulate novel aquafeeds especially addressed to fish genetically selected for high fish growth might be a key component of a successful and sustainable development of the aquaculture sector. In the present study we evaluated the effects of a novel diet (future diet) completely replacing FO by a blend of rapeseed, poultry, and algae oils and containing a low percentage of FM (10%) on two sea bass genotypes selected or not for multitrait and better growth performance. Regardless of the diet type, the GS genotype presented improved growth rate and feed utilization efficiency than WT fish after 300 days of feeding (Montero et al., 2023).

On one side, it has been widely demonstrated how European sea bass fed VOs-based diets, rich in n-6 PUFA, presented changes on gut fatty acid profile and enteroycte lipoproteins synthesis, altered gut associated immune system (GALT)-related gene expression and gut mucous cells morphological patterns, as well as produced changes in mucosal microbiota profiles, disturbing sea bass intestinal immune homeostasis (Montero et al., 2015; Torrecillas et al., 2015, 2017). Unfortunately, for

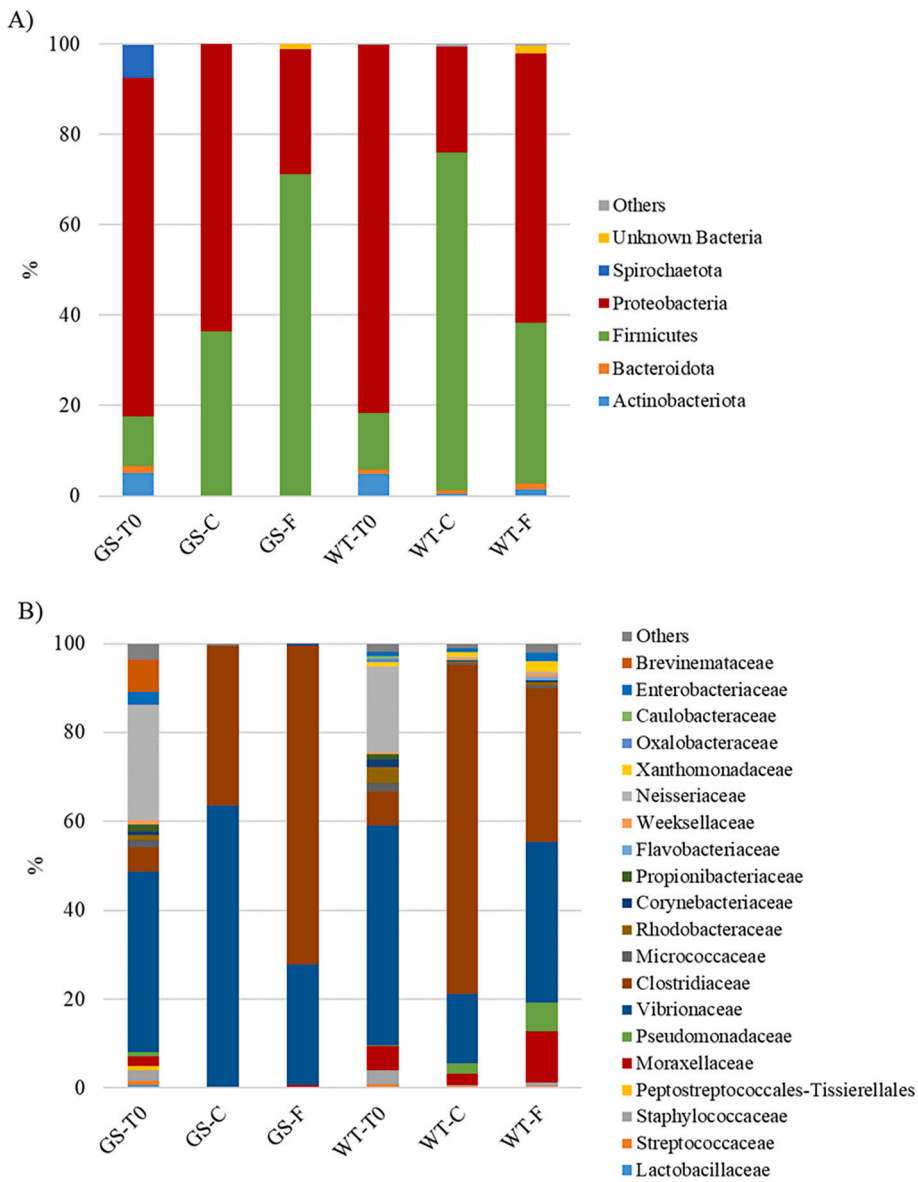

Fig. 10. Mean relative abundance (%) of the most prevalent bacteria in gut mucosa of sea bass at initial time (T0, 294 dph.) and at the end of feeding trial (609 dph) at phylum (A), and family (B) taxonomic level ($N = 6$). Only bacteria with an overall abundance of 0.5% were reported. Bacteria with lower abundance were pooled and indicated as "others". GS, Genetically selected genotype; WT, wildtype European sea bass genotype. Control (C), control diet based in a 20% Fish meal/5% Fish oil; Future (F), alternative diet based in a 10% Fish meal/ 0% Fish oil.

European sea bass the use of PO in combination or not with VO as FO replacer has been still scarcely investigated (Campos et al., 2019a, 2019b; Monteiro et al., 2018), especially addressing its effects on gut mucosal health or its associated microbiota populations. Nevertheless, the dietary inclusion of PO in European sea bass diets, as well as in other fish species, imbalances n-3/n-6 fatty acid ratio in tissues such as muscle or liver (Bowyer et al., 2012; Campos et al., 2019a; Carvalho et al., 2021; Monteiro et al., 2018; Xue et al., 2006) and this has been associated with the development of inflammatory diseases.

On the other side, genetic variability assessments for the capacity of fish to perform when fed on alternative ingredients-based diets are determinant to know whether genetic selection on growth can be made using new raw materials-based diets, but also for evaluate the ability of selected fish to modulate its gut microbiota and GALT in response to dietary shifts. Indeed, gut microbiota is influenced by intrinsic factors (e.g. genotype) and extrinsic factors (e.g. diet) on the basis of its own capacity to adapt its composition, diversity, function and metabolic activity when challenged (Egerton et al., 2018; Terova et al., 2021). Nowadays, we have plenty of information describing the potential induced changes in farmed fish microbiota when diet composition is altered, but in the case of the host genome background, the information is limited (Terova et al., 2021). Recently, in gilthead sea bream it has been detailed the importance of the host genetic background impact on gut microbiota, demonstrating how fast-growth selected gilhead sea bream has a more plastic microbiota capable of triggering a more effective response to face dietary changes, maintaining more stable the microbial community profile (Piazzon et al., 2020). Undoubtely, in the present study, the different pattern of response to new alternative feed formulations related to fish genotype was the result of the complex interactions between microbiota, mucus production, intestinal integrity and the underlying GALT functionality. Previous studies in Atlantic salmon indicate genotype-specific responses to dietary changes (such as vegetable oils) in the gut transcriptome and proteome, including possibly structural properties of the intestinal layer and defence against cellular stress (Morais et al., 2011).

A basal level of inflammation was detected in the proximal gut region of WT fish, which presented at level of *lamina propria*, a higher incidence of lymphocytic focuses and foamy-like phagocytic cells infiltrated in the mucosa than selected fish. Despite this morphological findings, no differences were observed neither in the morphometric characteristics of goblet cells nor in the expression of genes- related to GALT functioning in response to fish genotype or diet fed in proximal

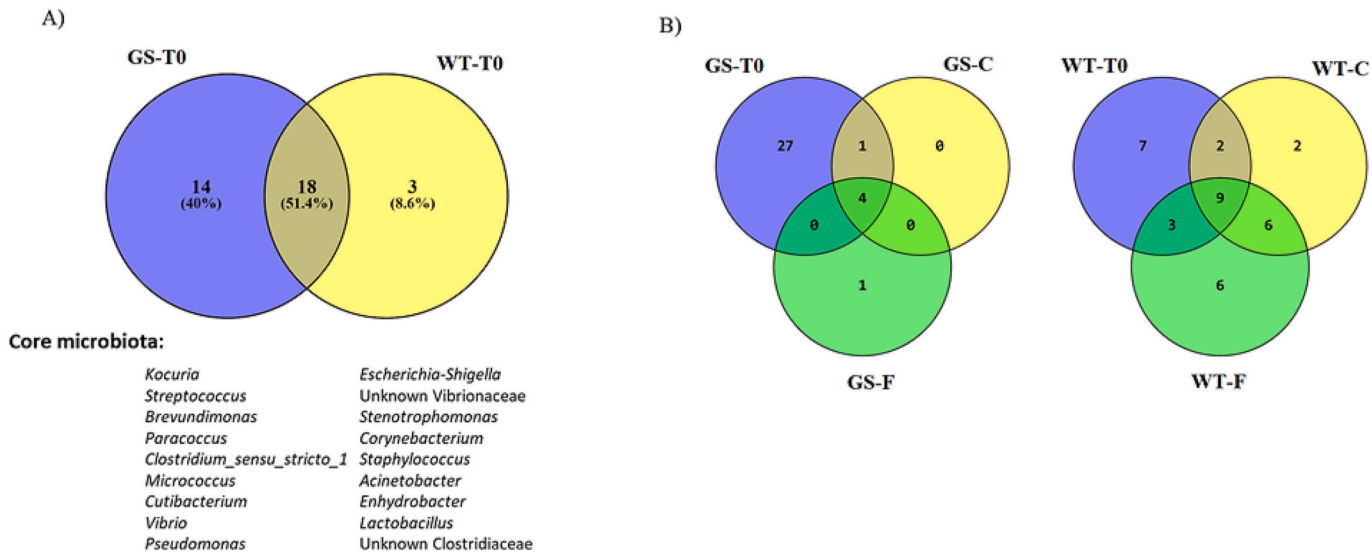

Fig. 11. Venn diagram depicting unique and shared taxa between samples of initial fish stage of development (294 dph, $T = 0$), and between samples of initial and final fish stage of development within each genotype (609 dph, B GS, Genetically selected genotype; WT, wildtype European sea bass genotype. Control (C), control diet based in a 20% Fish meal/5% Fish oil; Future (F), alternative diet based in a 10% Fish meal/ 0% Fish oil.

intestine. The lack of nutritional regulation in proximal intestine was consistent with the results observed in sea bass fed diets containing soybean oil (Torrecillas et al., 2015). At distal intestinal portion, WT fish presented a higher inflammatory status of the post-ileorectal valve section compared to GS fish. Futhermore, fish fed the future diet clearly showed supranuclear vacuoles with signs of hypertrophy, which were not observed in fish fed the control diet. Futhermore, WT fish presented larger goblet cells than GS fish in the distal intestinal section, and within the same genotype fish fed the future diet had in general larger goblet cells. The various diet compositions during last 35 years (6–7 generations) of the selection process and the multitrait genetic selection may have booth influenced GS fish capacity to better use feeds enriched in vegetal ingreedients with also limitation of gut inflamation as previously reported in rainbow trout selected fed with soybean meal (Venold et al., 2012).

Regarding GALT gene expression, a significant effect was found in distal intestine and interested three out of seven investigated genes. In particular, the expression of cytokines il-1β, tnf-α, and il-10 was different in distal intestine of our fish, showing an interaction effect diet x genotype.

The major responsiveness of GALT functioning related genes in posterior intestine agreed to the existence in European sea bass of a concentration gradient of GALT towards the anus (Abelli et al., 1997). Both il-1β and tnf-α are pro-inflammatory cytokines involved in the innate immune response, inflammation, apoptosis and cell proliferation (Sakai and Hikima, 2021). They are usually upregulated during infection and inflammation, and therefore commonly used as reference genes of intestinal inflammatory status. Regardless of the diet, WT fish showed higher expression of il-1β gene in distal intestine than GS genotype. However, the expression of il-1β gene was downregulated by "future" diet in WT fish intestine, whereas no diet effect was detected in selected fish. At present, several evidences of GALT variation induced by high dietary FM and/or FO replacement have been reported in fish, specifically when they are replaced by vegetable meals and oils (Montero et al., 2015; Torrecillas et al., 2017, 2015). Most of the alternative plant-derived feedstuff contain, indeed, a wide variety of antinutritional substances, such as saponins, tannins, phytic acid, and protease inhibitors, that can compromise intestinal health and its functionality (Francis et al., 2001; Krogdahl et al., 2010). Besides, unbalances of n-3/n-6 ratio due to the use of dietary VO has been reported to affect several parameters of the immune system in different aquaculture species inducing imbalances on fish immune response (Montero and Izquierdo, 2010). In these previous studies we usually assisted to an altered posterior gut-associated immune system (GALT)-related gene expression associated with an altered gut morphology. Here, the higher expression of il-1β in WT fish in comparison to selected genotype, revealed a condition of chronic inflammation in distal intestine of the first, regardless of the diet type. The decreased expression of il-1β observed in WT fish fed future diet was in line with our previous studies, which indicated that the substitution of FO by vegetable oils containing high levels of n-6 fatty acids may render GALT system less capable against infections (Montero et al., 2010; Torrecillas et al., 2015). In contrast, in selected fish, future diet administration caused an increase of tnf-α gene transcript and at the same time, a down regulation of anti-inflammatory cytokine il-10 in distal intestine. This could suggest an inflammatory condition, since the pro-inflammatory cytokine tnf-α is usually released by macrophages or activated T-cells in response to inflammation and infection, having a key role as a chemotactic agent for recruitment of leukocytes to the inflammation site (Ming et al., 1987) and maybe also related with the larger goblet cells and the hypertrophy of supranuclear vacuoles observed. Our results agree with those previously reported for other marine fish fed VO-based diets, such as gilthead sea bream and Senegalese sole (*Solea senegalensis*), in which an increase of tnf-α steady-stated levels was recorded in the head kidney and intestine, respectively (Montero et al., 2010, 2015). Similarly, FO substitution by soybean oil caused a down-regulation of il-10 relative expression in posterior gut combined with a decrease of the gut mucous barrier (Torrecillas et al., 2015). In fact, it has been demonstrated that il-10 helps the intestine to preserve the mucus barrier by maintaining mucin production in goblet cells (Hasnain et al., 2013).

If GALT plays a crucial role in providing the host with a protective barrier and immune defence against invading pathogens, on the other hand, the gut microbiota provides a physical presence that can directly prevent pathogen colonization by competing for attachment sites or nutrient resources. There is evidence that fish innate and adaptive immune system may influence and shape the gut microbiota composition which in turn influences host immune responses (López Nadal et al., 2020). A previous study in sea bass demonstrated that dietary levels of FM, FO or their combination did not affect digesta and gut mucosa species richness or diversity index, when level of FM was not higher than 20% (Torrecillas et al., 2017). Accordingly, in the present study, diet had lower influence upon intestinal microbial composition than genotype.

Table 8
Mean relative abundance (%) ± SD (N = 6) of the most prevalent phyla, orders, classes, families, and genera found in gut mucosa samples. Significant *p* values (< 0.05) are shown in bold. GS, Genetically selected genotype; WT, wildtype genotype. Control (C), control diet based in a 20% Fish meal/5% Fish oil; Future (F), alternative diet based in a 10% Fish meal/ 0% Fish oil.

	294 dph							609 dph														
Taxa	GS			WT			*Sig.*	GS-C			GS-F			WT-C			WT-F			*Sig.*		
																				Genotype	Diet	G*D
Phylum																						
Actinobacteria	5.13	±	2.12	4.97	±	3.88	0.756	0.03	±	0.03	0.03	±	0.03	0.57	±	0.64	1.38	±	2.59	**0.014**	0.776	0.656
Bacteroidetes	1.49	±	2.15	0.93	±	1.69	0.627	0.01	±	0.01	0.07	±	0.11	0.77	±	1.31	1.42	±	1.70	**0.009**	0.403	0.698
Firmicutes	10.95	±	8.36	12.41	±	19.36	0.315	36.34	±	37.09	71.06	±	32.15	74.64	±	34.87	35.58	±	40.81	0.817	0.996	0.204
Proteobacteria	75.01	±	18.16	81.64	±	22.40	0.171	63.61	±	37.12	27.80	±	31.94	23.46	±	34.36	59.54	±	40.54	0.703	0.849	0.214
Spirochaetes	7.19	±	10.51	0.00	±	0.00		0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00			
Class																						
Actinobacteria	5.05	±	2.13	4.97	±	3.88	0.792	0.03	±	0.05	0.03	±	0.03	0.58	±	0.66	1.40	±	2.63	**0.014**	0.768	0.650
Bacteroidia	1.49	±	2.15	0.93	±	1.69	0.626	0.01	±	0.01	0.07	±	0.11	0.79	±	1.35	1.45	±	1.72	**0.009**	0.399	0.689
Bacilli	4.33	±	2.31	4.40	±	4.78	0.831	0.03	±	0.03	0.02	±	0.03	1.01	±	1.70	1.51	±	2.29	**0.003**	0.837	0.515
Clostridia	6.56	±	8.75	8.00	±	14.80	0.648	36.31	±	37.10	71.74	±	32.50	73.98	±	35.34	34.31	±	40.32	0.915	0.951	0.181
Alphaproteobacteria	2.07	±	1.37	4.53	±	8.42	0.648	0.02	±	0.02	0.04	±	0.05	0.86	±	1.37	1.49	±	1.99	**0.006**	0.724	0.641
Gammaproteobacteria	73.09	±	18.16	77.16	±	23.54	0.660	63.60	±	37.13	28.10	±	32.42	22.78	±	34.24	59.83	±	42.47	0.698	0.898	0.212
Brevinematia	7.14	±	10.50	0.00	±	0.00		0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00			
Order																						
Micrococcales	2.52	±	1.56	1.96	±	1.38	0.523	0.03	±	0.05	0.01	±	0.02	0.28	±	0.35	0.56	±	0.98	**0.010**	0.857	0.528
Lactobacillales	1.95	±	1.34	0.96	±	0.93	0.235	0.00	±	0.00	0.01	±	0.01	0.62	±	1.26	0.67	±	1.27	**0.013**	0.885	0.825
Staphylococcales	2.39	±	1.13	3.23	±	3.99	0.889	0.02	±	0.02	0.01	±	0.01	0.23	±	0.46	0.62	±	1.11	**0.030**	0.658	0.321
Peptostreptococcales-Tissierellales	1.21	±	0.72	0.45	±	0.66	0.053	0.38	±	0.94	0.00	±	0.00	0.03	±	0.05	0.02	±	0.03	0.882	0.274	0.373
Enterobacterales	2.98	±	2.39	1.08	±	1.70	0.113	0.10	±	0.23	0.17	±	0.27	0.82	±	1.48	2.08	±	3.67	0.087	0.455	0.598
Pseudomonadales	3.00	±	1.33	5.50	±	4.56	0.343	0.22	±	0.45	0.70	±	1.15	4.81	±	9.04	17.89	±	26.10	**0.016**	0.189	0.279
Vibrionales	40.56	±	26.00	49.30	±	38.96	0.779	63.36	±	37.62	27.12	±	31.98	15.53	±	35.15	36.01	±	48.27	0.156	0.565	0.091
Clostridiales	5.35	±	8.95	7.54	±	14.48	0.645	35.84	±	36.67	71.74	±	32.49	73.85	±	35.45	34.43	±	40.40	0.908	0.935	0.177
Rhodobacterales	1.16	±	0.76	3.48	±	6.51	0.927	0.01	±	0.01	0.03	±	0.05	0.46	±	0.76	0.87	±	1.15	**0.011**	0.721	0.729
Corynebacteriales	0.96	±	0.41	1.87	±	3.01	0.522	0.01	±	0.01	0.02	±	0.02	0.18	±	0.26	0.28	±	0.57	0.051	0.947	0.694
Propionibacteriales	1.54	±	0.37	1.16	±	0.74	0.267	0.00	±	0.00	0.00	±	0.00	0.12	±	0.22	0.10	±	0.14	**0.026**	0.889	0.901
Flavobacteriales	1.19	±	1.85	0.40	±	0.75	0.420	0.01	±	0.01	0.07	±	0.11	0.58	±	0.86	1.45	±	1.75	**0.008**	0.290	0.565
Burkholderiales	26.48	±	7.78	20.16	±	15.86	0.354	0.00	±	0.00	0.03	±	0.05	0.58	±	0.83	0.84	±	1.01	**0.001**	0.602	0.854
Xanthomonadales	0.19	±	0.29	1.03	±	1.12	0.089	0.01	±	0.02	0.08	±	0.13	0.98	±	1.87	2.40	±	3.88	**0.036**	0.428	0.577
Brevinematales	7.17	±	10.54	0.00	±	0.00		0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00			
Family																						
Lactobacillaceae	0.73	±	0.77	0.10	±	0.08	0.077	0.00	±	0.00	0.00	±	0.00	0.14	±	0.24	0.15	±	0.29	**0.024**	0.993	0.868
Streptococcaceae	0.84	±	0.71	0.84	±	0.94	0.947	0.00	±	0.00	0.01	±	0.01	0.44	±	0.95	0.53	±	1.00	**0.016**	0.736	0.738
Staphylococcaceae	2.32	±	1.06	3.23	±	3.98	0.784	0.02	±	0.02	0.01	±	0.01	0.24	±	0.47	0.63	±	1.13	**0.031**	0.659	0.324
Peptostreptococcales-Tissierellales	1.14	±	0.73	0.45	±	0.66b	0.066	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.02	±	0.03	**0.032**	0.135	0.095
Moraxellaceae	2.05	±	1.08	5.43	±	4.62	0.152	0.17	±	0.35	0.63	±	1.11	2.47	±	4.10	11.53	±	14.21	**0.007**	0.092	0.185
Pseudomonadaceae	0.96	±	0.50	0.09	±	0.06	**2.00E-04**	0.04	±	0.10	0.07	±	0.10	2.44	±	5.17	6.46	±	12.10	0.063	0.549	0.559
Vibrionaceae	40.74	±	26.04	49.56	±	39.20	0.775	63.37	±	37.62	27.12	±	31.20	15.56	±	35.21	36.16	±	48.46	0.159	0.568	0.091
Clostridiaceae	5.37	±	9.00	7.56	±	14.50	0.645	35.84	±	36.67	71.74	±	32.50	74.11	±	35.33	34.64	±	40.54	0.894	0.939	0.177
Micrococcaceae	1.56	±	0.74	1.94	±	1.39	0.670	0.03	±	0.05	0.01	±	0.02	0.27	±	0.35	0.57	±	0.99	**0.010**	0.843	0.517
Rhodobacteraceae	1.17	±	0.77	3.50	±	6.57	0.927	0.01	±	0.01	0.03	±	0.05	0.47	±	0.78	0.88	±	1.50	**0.011**	0.722	0.729
Corynebacteriaceae	0.80	±	0.35	1.84	±	2.98	0.648	0.01	±	0.01	0.02	±	0.02	0.18	±	0.27	0.28	±	0.58	0.068	0.967	0.778
Propionibacteriaceae	1.50	±	0.40	1.16	±	0.74	0.313	0.00	±	0.00	0.00	±	0.00	0.12	±	0.22	0.10	±	0.14	0.026	0.887	0.900
Flavobacteriaceae	0.21	±	0.36	0.01	±	0.03	0.169	0.00	±	0.00	0.00	±	0.00	0.15	±	0.26	0.62	±	0.81	**0.004**	0.282	0.219
Weeksellaceae	0.98	±	1.53	0.40	±	0.76	0.782	0.01	±	0.01	0.07	±	0.10	0.43	±	0.62	0.84	±	1.11	**0.019**	0.415	0.864
Neisseriaceae	26.15	±	7.72	19.27	±	15.13	0.314	0.00	±	0.00	0.01	±	0.01	0.15	±	0.11	0.37	±	0.43	**3.00E-04**	0.360	0.720
Enterobacteriaceae	2.91	±	2.45	0.96	±	1.73	0.106	0.10	±	0.23	0.17	±	0.27	0.77	±	1.44	1.97	±	3.55	0.110	0.400	0.572
Xanthomonadaceae	0.20	±	0.30	1.03	±	1.13	0.089	0.01	±	0.02	0.08	±	0.13	1.00	±	1.92	2.41	±	3.89	**0.035**	0.431	0.579
Oxalobacteraceae	0.19	±	0.15	0.64	±	0.89	0.347	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00			
Caulobacteraceae	0.11	±	0.07	0.65	±	1.38	0.927	0.00	±	0.00	0.00	±	0.00	0.02	±	0.04	0.05	±	0.09	**0.042**	0.857	0.603

(*continued on next page*)

Table 8 (*continued*)

Taxa	294 dph GS	WT	*Sig.*	609 dph GS-C	GS-F	WT-C	WT-F	*Sig.* Genotype	Diet	G*D
Brevinemataceae	7.20 ± 10.60	0.00 ± 0.00		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00			
Genus										
Lactobacillus	0.75 ± 0.82	0.13 ± 0.12	0.107	0.00 ± 0.00	0.00 ± 0.00	0.14 ± 0.24	0.16 ± 0.31	**0.025**	0.979	0.858
Streptococcus	0.86 ± 0.72	0.87 ± 0.99	0.973	0.00 ± 0.00	0.01 ± 0.01	0.45 ± 0.97	0.55 ± 1.06	**0.017**	0.727	0.772
Staphylococcus	2.32 ± 1.04	3.44 ± 4.14	0.784	0.02 ± 0.02^{b}	0.01 ± 0.01	0.24 ± 0.48	0.67 ± 1.20	**0.031**	0.653	0.317
Psychrobacter	0.15 ± 0.30	0.04 ± 0.06	0.238	0.01 ± 0.02^{b}	0.02 ± 0.01	0.32 ± 0.37	1.64 ± 2.26	**0.005**	0.254	0.250
Pseudomonas	0.99 ± 0.54	0.12 ± 0.11	**0.001**	0.04 ± 0.10	0.07 ± 0.10	2.48 ± 5.28	6.65 ± 12.45	0.063	0.546	0.554
Vibrio	41.41 ± 26.05	47.31 ± 36.92	0.868	63.48 ± 37.53	27.07 ± 32.07	15.60 ± 35.23	36.40 ± 48.76	0.166	0.563	0.089
Micrococcus	1.19 ± 0.53	1.57 ± 1.48	0.772	0.03 ± 0.05	0.01 ± 0.01	0.26 ± 0.36	0.19 ± 0.19	**0.004**	0.667	0.842
Clostridium_sensu_stricto_1	2.98 ± 5.06	5.12 ± 8.82	0.782	13.67 ± 20.16^{b}	39.28 ± 17.33^{a}	18.70 ± 21.25^{ab}	3.75 ± 3.24^{b}	0.055	0.356	**0.006**
Acinetobacter	0.39 ± 0.24	2.58 ± 4.85	0.927	0.16 ± 0.35	0.58 ± 1.12	1.94 ± 3.71	9.30 ± 14.92	0.055	0.182	0.284
Enhydrobacter	1.49 ± 0.91	3.21 ± 3.95	0.476	0.01 ± 0.01	0.03 ± 0.06	0.24 ± 0.50	0.97 ± 1.80	**0.027**	0.257	0.349
Corynebacterium	0.80 ± 0.40	1.57 ± 2.24	0.927	0.00 ± 0.01^{b}	0.02 ± 0.02	0.15 ± 0.24	0.19 ± 0.36	**0.046**	0.971	0.600
Cutibacterium	1.53 ± 0.43	1.25 ± 0.69	0.381	0.00 ± 0.00^{b}	0.00 ± 0.00	0.12 ± 0.23	0.11 ± 0.14	**0.026**	0.900	0.906
Cloacibacterium	0.86 ± 1.25	0.20 ± 0.45	0.370	0.00 ± 0.00	0.02 ± 0.04	0.21 ± 0.35	0.05 ± 0.10	0.065	0.683	0.264
Paracoccus	0.98 ± 0.43	3.62 ± 6.85	0.927	0.01 ± 0.01	0.02 ± 0.05	0.40 ± 0.67	0.19 ± 0.32	**0.023**	0.552	0.372
Escherichia-Shigella	2.84 ± 2.43	0.96 ± 1.78	0.106	0.06 ± 0.13	0.02 ± 0.03	0.03 ± 0.06	0.15 ± 0.27	0.463	0.420	0.200
Stenotrophomonas	0.12 ± 0.16	1.02 ± 1.20	0.110	0.01 ± 0.02	0.08 ± 0.13	1.01 ± 1.95	2.46 ± 4.01	**0.045**	0.462	0.614
Anaerococcus	0.89 ± 0.45	0.00 ± 0.00		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00			
Massilia	0.16 ± 0.17	0.66 ± 0.92	0.234	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00			
Brevundimonas	0.08 ± 0.09	0.68 ± 1.44	0.645	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.10			
Brevimena	7.27 ± 10.69	0.00 ± 0.00		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00			

Regardless of the diet, wildtype fish showed higher species richness than selected genotype. Actually, it is known that, in addition to diet alterations, host genetics has a role in shaping the gut microbiota (Bonder et al., 2016). Similar to our findings, rainbow trout selected for growth on a plant-based diet had lower gut microbial richness than common commercial genotype (Blaufuss et al., 2020). Therefore, it could be a direct consequence of selective breeding for growth. In contrast, in sea bream, no significant differences were found in richness and diversity indices, when comparing the intestinal resident bacterial diversity of three groups of families, regardless of the diet, but there was an effect of the diet on intestinal microbial community composition depending on genetic background of the fish (Piazzon et al., 2020).

In the present study weighted and unweighted UniFrac showed a significant divergence between pre and end of trial samples, but only unweighted UniFrac, based on presence and absence of specific OTUs, displayed a significant effect of genotype and diet on microbial community profiles. Accordingly to our data, significant effects of genotype and diet were observed on rainbow trout gut bacterial communities (Blaufuss et al., 2020; Chapagain et al., 2020). The PLS-DA made on initial samples clearly separated the two genotypes. Differences between selected and wildtype fish were mainly driven by *Pseudomonas* and *Anaerococcus* genera, which were found only in genetically selected sea bass. When the same analysis was performed on final samples, a great deal of compositional overlap between groups was revealed, although differences between genotypes were still detected. The gut microbiota of genetically selected fish shared a reduced individual variability, indicating an enhanced capacity to cope with changes in diet composition.

As suggested by Piazzon and colleagues (Piazzon et al., 2020), the selection for growth could be indirectly linked to plasticity to different diets and disease resistance. In agreement with our results, sea bream fast-growth families showed fewer changes at the level of bacterial composition (Piazzon et al., 2020), demonstrating also the capacity to reshape their intestines to adapt to the diet with no impact on their growth performances (Perera et al., 2019).

As already reported by literature, the gut autochthonous microbiota of sea bass was dominated by Proteobacteria, Firmicutes, and Actinobacteria regardless of the diet or genotype (Carda-Diéguez et al., 2014; Gatesoupe et al., 2016; Rimoldi et al., 2020).

In line with alpha diversity results, the microbiota taxonomical analysis at the genus level showed that the most abundant taxa were exclusively found in non-selected fish, irrespective to the diet. A significant association with wildtype sea bass, regardless of the diet, was found for *Lactobacillus*, *Streptococcus, Staphylococcus*, *Psychrobacter*, *Pseudomonas*, *Micrococcus*, *Enhydrobacter*, *Corynebacterium*, *Cutibacterium*, and *Paracoccus* genera as well as for Moraxellaceae family. *Micrococcus*, *Corynebacterium*, and *Cutibacterium* are members of Actinobacteria class.

Bacteria belonging to Moraxellaceae family, such as *Acinetobacter* and *Psychrobacter*, are considered in human biomarkers of Crohn's disease (El Mouzan et al., 2018; Sekido et al., 2020). It was in line with our gene expression data indicating a chronic inflammation status in wildtype sea bass not related to diet. Futhermore, *Paracoccus*, disclosed a significant positive correlation with il-1β gene expression in distal intestine. Similarly, in sea bream, *Paracoccus yeei* was positively correlated with proinflammatory cytokine tnf-α expression in head kidney (Naya-Català et al., 2021. The potential opportunistic pathogen *Clostridium_sensu_stricto_1* (*Clostridium* ss1) was instead associate to selected genotype. *Clostridium ss1* genus constitutes a large cluster of *Clostridium* species that includes both commensal and pathogenic species. What it is known is that the members of *Clostridium* ss1 respond differently to fermentation substrates, but exhibit a constant capacity to synthesise butyrate (Wang et al., 2019). Accordingly with our results, in Nile Tilapia (*Oreochromis niloticus*) this bacterial genus was negatively correlated with most intestinal metabolites, particularly with change in DHA (Wu et al., 2021).

PICRUSt analysis was used in this study to enumerate differential

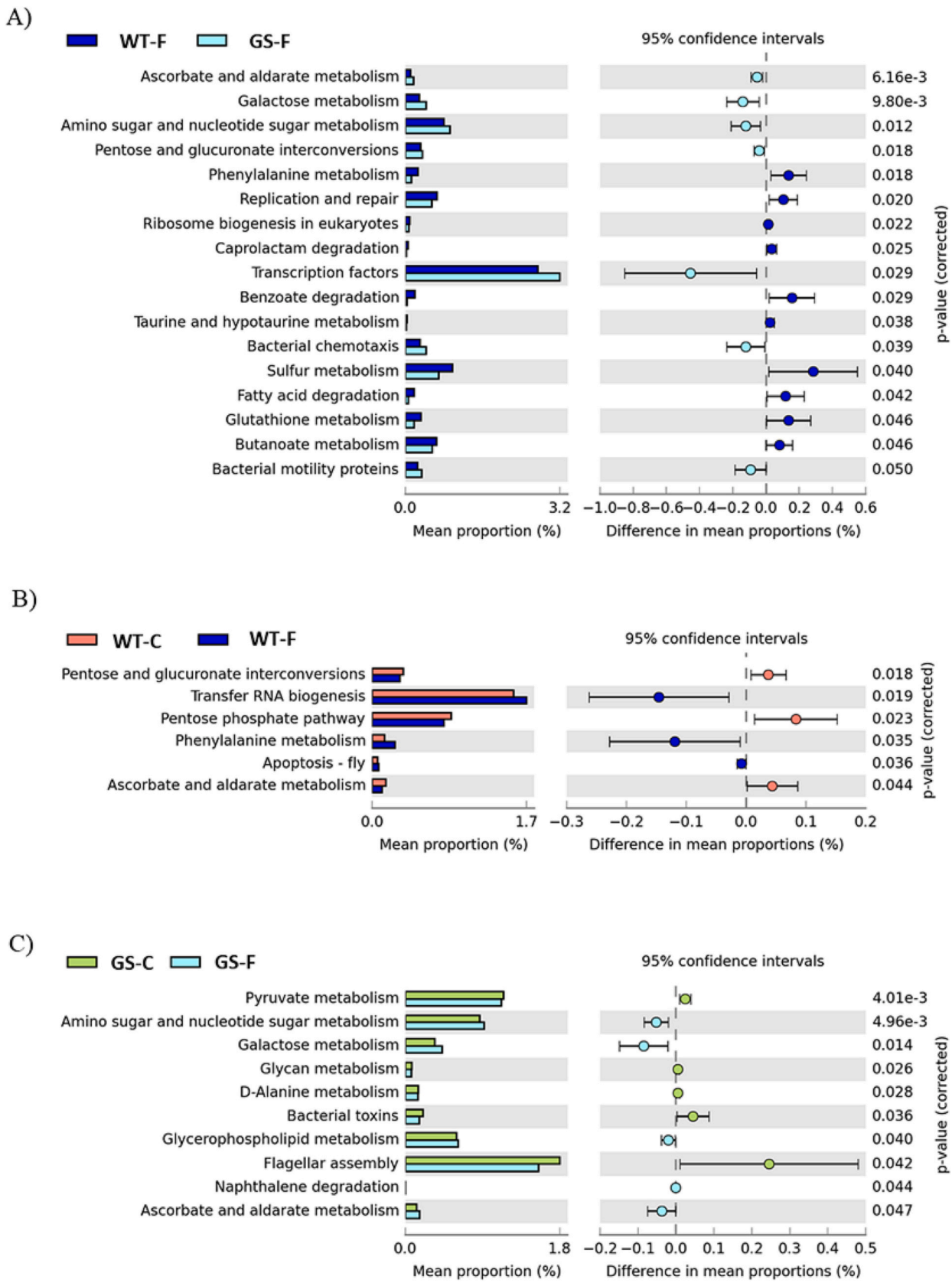

Fig. 12. PICRUSt analysis results of predicted functional pathways of gut mucosal microbiota at the end of the long term-feeding trial (609 dph). GS, Genetically selected genotype; WT, wildtype European sea bass genotype. Control (C), control diet based in a 20% Fish meal/5% Fish oil; Future (F), alternative diet based in a 10% Fish meal/ 0% Fish oil.

functional capabilities of microbial communities in two fish genotypes. Compared to control diet, administration of "future" diet in selected fish decreased bacterial functional pathways related flagellar assembly and bacterial toxin, and increased amino sugar, nucleotide sugar, and galactose metabolism. Galactose metabolism plays a central role in biofilm formation by several bacterial species (Chai et al., 2012; Hobley et al., 2015). Sugar nucleotides, such as UDP-glucose and UDP-galactose, are indeed essential for exopolysaccharide biosynthesis within the biofilm matrix to provide three-dimensional architectural integrity and functionality to the biofilm community. Further matrix components facilitate interactions between bacteria and host cell, and these host-bacterial interactions are quite interesting since they provide pharmacological targets that can potentially be exploited to limit mucosal adherence of pathogens, inappropriate stimulation of inflammatory immune responses and consequent development of chronic intestinal inflammation. Other pathways, related to infection and inferred to be downregulated in selected fish fed "future" diet, could indicate that these animals harbor fewer bacteria potentially harmful (pathogens) for host.

In WT fish fed "future" diet, microbial genes for transfer RNA biogenesis, phenylalanine metabolism, and apoptosis were over-represented. Whereas pentose phosphate pathway and ascorbate metabolism, both involved in oxidative stress, were upregulated when fish were fed with control diet.

A total of 17 predicted metabolic pathways differed between selected and wildtype sea bass fed the future diet. Microbial pathways involved in in sugar metabolism, transcription factor, and bacterial chemotaxis were more abundant in selected genotype. Phenylalanine, sulfur, glutathione, and butanoate metabolism, replication and repair processes, benzoate and fatty acid degradation were, instead, upregulated in non-selected fish. Benzoate degradation pathway is correlated to Moraxellaceae family, which was enriched in WT genotype. The increase of sugar metabolism observed in selected genotype could be reasonably correlated to the higher presence of *Clostridium* and to selection for growth. The presence of certain groups of bacteria, such as Clostridiales, has been related to improvements in feed efficiency and growth performance in animal husbandry including fish (Geraylou et al., 2012; McCormack et al., 2017; Rimoldi et al., 2018b). Similarly, Piazzon et al. (Piazzon et al., 2020) found higher abundance of *Clostridiaceae* in the fast-growth families of sea bream.

5. Conclusions

In summary, the multiple traits genetic selection for growth and for limiting ventral fat deposition during 6–7 fish generations (35 years) had a great influence on the gut microbiota composition and species richness, and on the basal inflammatory intestinal status of European sea bass. The core gut microbiota of selected fish was less variable, but more flexible with dietary changes than microbiota of wildtype fish, better adapting its function while maintaining its composition. In genetically selected fish, the core gut microbiota showed more changes in the inferred pathway analysis with an increase of carbohydrate metabolism pathway. A significant genotype effect was found for specific bacterial taxa, such as *Paracoccus* genus and genera belonging to Moraxellaceae family that were enriched in wildtype fish, regardless of the diet. Interestingly, the relative abundance of *Paracoccus* genus was positively correlated with higher proinflammatory cytokine il-1β expression found in distal intestine of wildtype sea bass.

Altogether, our data greatly complete the results reported in the just published paper of our group (Montero et al., 2023) on the same individuals of European sea bass, demonstrating that investment in research for breeding and feed development lead to improvements in fish production efficiency, promoting animal health and welfare and ultimately can have a great positive impact to the industry.

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CRediT authorship contribution statement

S. Torrecillas: Conceptualization, Methodology, Data curation, Writing – original draft. **S. Rimoldi:** Methodology, Data curation, Writing – original draft. **D. Montero:** Conceptualization, Funding acquisition. **A. Serradell:** Methodology, Data curation. **F. Acosta:** Methodology, Data curation. **F. Allal:** Methodology, Data curation. **A. Bajek:** Data curation. **G. Terova:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare no competing interests.

Data availability

All sequencing raw data were submitted and are freely available on the European Nucleotide Archive (EBI ENA) public database, under the accession code PRJEB47388.

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