UNDERSTANDING THE INTERPLAY BETWEEN DIET, FEED INGREDIENTS AND GUT MICROBIOTA FOR SUSTAINABLE AQUACULTURE

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UNDERSTANDING THE INTERPLAY BETWEEN DIET, FEED INGREDIENTS AND GUT MICROBIOTA FOR SUSTAINABLE AQUACULTURE

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EDITORIAL
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Editorial: Understanding the Interplay Between Diet, Feed Ingredients and Gut Microbiota for Sustainable Aquaculture

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Keywords: fish nutrition, microbiome, aquaculture, fish feed, host-microbe associations, fish health

Editorial on the Research Topic

Understanding the Interplay Between Diet, Feed Ingredients and Gut Microbiota for Sustainable Aquaculture

Aquaculture is one of the fastest-growing food production sectors providing more than half of the fish supply worldwide and thus providing a healthy food source for human consumption. To achieve such growth, aquaculture must overcome several challenges to increase its productivity within our planetary boundaries. These challenges are mainly related to the source and sustainability of its feed ingredients. Traditionally, fish meal and oil have been the main aquafeed ingredients, originating from wild fisheries catch. However, due to the finite nature of these ingredients, materials of plant-origin, such as soybean meal, wheat or corn, as well as novel ingredients such as insect meal, algal meal or microbial proteins, have become an alternative, more sustainable solution, reducing fish-based ingredients in aquafeeds to <20% (Turchini et al., 2019; Agboola et al., 2021; Cottrell et al., 2021). To what extent this shift in the nutritional composition will impact the long-term physiology and health of fish, and especially carnivorous fish, is still a subject of extensive research.

Traditionally, research on fish nutrition focused on the phenotypic (i.e., growth, body length), and physiological (i.e., digestion) parameters as a benchmark to evaluate the quality of feed ingredients and diets. However, during the past years, given the vital importance of gut commensal microbes on digestion and health, more studies are addressing the dietary effects on gut microbiota composition in aquatic animals, in addition to the physiological and phenotypic parameters; this may offer complementary information, especially with regard to dietary studies on alternative or novel feed ingredients (Clements et al., 2014; Zhou et al., 2018). Such information will shed light on how alterations in gut microbiota profiles due to different feed ingredient types and diets can be reflected in fish intestinal function, feed efficiency, growth performance and health status (Zhou et al., 2018). Therefore, this Research Topic aimed to gather information on the interaction between dietary formulations and feed ingredients, and the gut microbiota, while evaluating the impact on fish performance, feed utilization, and overall fish health. The Research Topic includes 10 research articles with a general premise on the impact of different dietary ingredients, including pre-and probiotics, on fish performance, health and (gut) microbiota composition. Moreover, the impact of the rearing environment vs. feed was assessed in two of the Research Topic articles.

During the past decades, research on fish meal and oil replacement has been intensive in order to make it possible to switch to alternative feed ingredients. Novel ingredients such as

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microbial meals, insect meals and terrestrial animal by-products have been constantly evaluated on whether they satisfy the demand of the aquafeed industry, with promising applications. Besides growth performance, the impacts of such ingredients on gut health and microbiota composition are of major importance in assessing their value as aquafeed ingredients. In this Research Topic, Tran et al. evaluated the effects of feeding dietary defatted black soldier fly larvae meal on gut health, microbiota and oxidative status of pikeperch. Enhancement of antioxidant activity in the liver, and increase of the microbial richness and diversity in the gut were reported, in agreement with previous studies (Bruni et al., 2018; Huyben et al., 2019), although enrichment of chitin-degrading microbes was not observed (Ringø et al., 2012). Moreover, Solé-Jiménez et al. investigated the impact of microbial and processed animal proteins as the main protein source for gilthead seabream on histology, short-chain fatty acid concentration and microbiota composition in the gut. Total fish meal replacement by this ingredient mixture altered the microbial diversity in the gut along with the short-chain fatty acid concentration. The use of predictive tools to infer microbial functionality indicated that taxa related to inflammatory response increased in abundance when fish meal was replaced. Both studies by Tran et al. and Solé-Jiménez et al. highlighted the connection between the gut health status and microbial markers. Finally, Singh et al. evaluated the use of filamentous fungi as novel alternative ingredients for rainbow trout, as well as the impact of feeding duration and feed processing on the gut microbiota composition and fish performance. The authors reported an increase in lactic acid bacteria in the gut of rainbow trout with fungi addition, which can be a marker for improved gut health.

Nutrient digestion and utilization are among the most important functions of the gut microbiota, affecting also metabolism and growth rate (Llewellyn et al., 2014; Lindsay et al., 2020). Alterations in nutrient composition can alter the microbiota composition; however, the relationship with fish performance is not still well-established. Pelusio et al. evaluated the impact of dietary lipid level and the interaction with different environmental temperatures on the gut microbiota of gilthead seabream, showing that only high temperature affects the microbiota composition. Higher temperatures are known to affect microbiota diversity (Huyben et al., 2018; Kokou et al., 2018); however, an effect was observed only within a few genera, with *Lactobacillus* prevailing at dietary lipid levels of 16%, and *Streptococcus* and *Bacillus* at dietary lipid levels of 21%. Alterations in the type of dietary lipids can also result in gut microbiota differences (Huyben et al., 2020), while such effects can relate to the host's age. Nikouli et al. evaluated the impact of total fish oil replacement by plant oils for Atlantic Salmon post-larvae. The authors reported that the major factor affecting the gut microbiota was the age or developmental stage of the host, with fish from both diets showing a similar microbiota profile. Such findings come in agreement with other studies in early life stages in fish (Xiao et al., 2021), suggesting the importance of comparing similar age groups when examining dietary effects on the gut microbiota.

Besides the main dietary ingredients, prebiotics, probiotics as well as immune-stimulating feed additives are considered important modulators of various biological processes such as digestion, immune stimulation or antioxidative properties through their effects on the gut microbiota (Kiron, 2012; Dawood et al., 2019). Naya-Català et al. evaluated the interaction between fish performance and gut microbiota in gilthead seabream, when egg white hydrolysate was used on plant-based diets. The authors reported that the addition of such peptides improved the gut and liver health when fish were fed plant-based diets, while a change in the gut microbiota was also reported, with proprionate-producing bacteria increasing in abundance. These results suggest that feed additives can potentially restore several negative effects of sub-optimal feed composition *via* modulation of the gut microbiota. In general, the levels of such additives have to be well-investigated as a negative effect on the growth performance, gut health and microbiota can also be present when included in the diet at high doses. As such, Liu et al. supplemented different levels of histamine in the diets of grouper and reported negative effects when this additive is supplemented in levels >0.2%. On the other hand, Moroni et al. reported that addition of a nisin-producing probiotic such as the *Lactococcus lactis* strain, improved the growth intake and immune-related gene expression in gilthead seabream, which also coincided with alteration in the gut microbiota composition. Those positive effects were reported, despite the fact that the probiotic was not able to colonize the gut; this is also supported by other studies (Balcázar et al., 2007; Hoseinifar et al., 2018; Maas et al., 2021a,b), which suggest that probiotic colonization is not necessary to achieve beneficial effects on the gut health and microbiota composition.

Although the feed can be an important factor shaping the gut microbiota, the surrounding environment and environmental factors can also greatly affect the microbiota composition, and thus interfering with results observed by the diet (Giatsis et al., 2015; Xiao et al., 2021). This is more prevalent during early life stages, as Minich et al. reported in yellowtail kingfish, while the microbiota composition can also be differently affected when comparing different tissues. Gills were reported to be more influenced by the surrounding environment than the skin, while the trends in the microbiota diversity are different between external mucosal surfaces like the skin and the gills, and internal surfaces, like the gut, with the latter being also more stable over time. Moreover, in the study by Lorgen-Ritchie et al., the impact of smoltification—the transition from freshwater to seawater—on the gut microbiota of Atlantic salmon was reported, following the same cohort through this process. The transition to seawater had a significant impact on gut microbiota diversity and composition, while there were also distinguishable stage-specific core taxa. Such findings highlight the importance of the rearing environment and life stage on the gut microbiota, which should be considered when studying the effects of diet or feed on the gut microbiota composition.

To conclude, the studies included in this Research topic highlighted the importance of diet, the rearing environment, and

the feeding trials' duration on the gut microbiota composition. By compiling these ten articles, we hope that researchers and aquaculture professionals in the field of fish nutrition will find this information interesting, especially when developing new feed formulations or testing different feed ingredients and additives. Future studies should connect gut microbiota changes and performance measurements, like growth, gene expression or plasma metabolites. This will enable us to understand how microbiota changes relate to specific phenotypes and provide several valuable biomarkers for fish health and performance. Moreover, exploring the functional properties of the gut microbiota and connecting them to metabolic and physiological changes in the fish-hosts is the next step to improving our understanding of fish-microbe interactions.

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FK prepared the manuscript. SG and VK reviewed the manuscript. All authors approved for publication.

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ORIGINAL RESEARCH
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The Effects of Nisin-Producing *Lactococcus lactis* Strain Used as Probiotic on Gilthead Sea Bream (*Sparus aurata*) Growth, Gut Microbiota, and Transcriptional Response

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The present research tested the effects of dietary nisin-producing *Lactococcus lactis* on growth performance, feed utilization, intestinal morphology, transcriptional response, and microbiota in gilthead sea bream (*Sparus aurata*). A feeding trial was conducted with fish weighting 70–90 g. Fish were tagged with passive, integrated transponders and distributed in nine 500 L tanks with 40 fish each. Fish were fed for 12 weeks with either a control (diet A) or experimental diets (diets B and C) in triplicate (3 tanks/diet). Extruded pellets of diets B and C were supplemented with a low (2×10^9 CFU/kg) and a high (5×10^9 CFU/kg) dose of probiotic, respectively. No significant differences were found between groups for the feed conversion ratio or specific growth rates. However, the final body weight of fish fed diet C was significantly higher than the control group with intermediate values for fish fed diet B. Histological analysis conducted using a semi-quantitative scoring system showed that probiotic did not alter the morphology of the intestine and did not trigger inflammation. With regard to the transcriptomic response, a customized PCR array layout was designed to simultaneously profile a panel of 44 selected genes. Significant differences in the expression of key genes involved in innate and acquired immunity were detected between fish fed probiotic and control diets. To analyze the microbiota associated to the feeds and the gut autochthonous microbial communities, we used the Illumina MiSeq platform for sequencing the 16S rRNA gene and a metagenomics pipeline based on VSEARCH and RDP databases. The analysis of gut microbiota revealed a lack of colonization of the probiotic in the host's intestinal mucosa. However, probiotic did modulate the fish gut microbiota, confirming that colonization is not always necessary to induce host modification. In fact, diets B and C were enriched with Actinomycetales, as compared to diet A, which instead showed a

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higher percentage of *Pseudomonas*, *Sphyngomonas*, and *Lactobacillus* genera. These results were confirmed by the clear separation of gut bacterial community of fish fed with the probiotic from the bacterial community of control fish group in the beta-diversity and PLS-DA (supervised partial least-squares discriminant analysis) analyses.

Keywords: aquaculture, gilthead sea bream, probiotic, *Lactococcus lactis*, gut microbiota, transcriptomic

INTRODUCTION

The definition of "Probiotics" has changed many times during this century. However, according to (Food and Agriculture Organization of the United [FAO] and World Health Organisation [WHO], 2001) probiotics are "live microorganisms that confer a health benefit on the host when administered in adequate amounts." The most commonly used probiotics are bacteria belonging to *Lactobacillus*, *Bifidobacterium*, *Bacillus,* and *Enterococcus* genera (European Medicines Agency [EMA], and European Food Safety Authority [EFSA], 2017; EFSA FEEDAP [EFSA Panel on Additives and Products or Substances used in Animal Feed] et al., 2018), but some fungal genera have also been reported as novel probiotics.

In the last 25–30 years, the use of probiotics in animal production has increased (Chaucheyras-Durand and Durand, 2010; Ezema, 2013). Indeed, several publications have reported numerous beneficial effects associated with the supplementation of live yeast or bacteria (mostly *Lactobacillus*) in the diet of terrestrial animals, including amelioration of resistance to pathogens, improvement in growth parameters (in swine and poultry), increase in productivity and quality of eggs in laying hens, and enhancement of milk production in cattle (Gallazzi et al., 2008; Shabani et al., 2012; Puphan et al., 2015; Uyeno et al., 2015; De Cesare et al., 2017; Wang et al., 2017; Dowarah et al., 2018; Forte et al., 2018).

In aquaculture, a great number of bacterial species are currently used as probiotics (for a review, please see Newaj-Fyzul et al., 2014). These microorganisms can be administered as multi-species (multi-strain) or single-species (single-strain) (Food and Agriculture Organization of the United [FAO], 2016) and provided either as a suspension in water, or added to the feed. However, use in feed is considered the best option; therefore, this approach is employed most frequently (Nayak, 2010; Jahangiri and Esteban, 2018). In the European Union (EU), probiotic strains, must obtain a market authorization by the EFSA (European Safety Food Authority)[1], which grants a QPS (Qualified Presumption of Safety) status. The QPS is based on reasonable evidence. No microorganism belonging to a QPS status group needs to undergo a full safety assessment, but microorganisms that pose a safety concern to humans, animals, or environment are not considered suitable for QPS status and must undergo a full safety assessment. The QPS assessment requires: (1) the identity of the strain to be conclusively established, and (2) absence of resistance to antibiotics (for bacteria) or antimycotics (for yeasts) used in human and veterinary medicine (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2020).

The increase in the use of probiotics in aquaculture is mostly related to the need to decrease or even avoid the use of antibiotics, increasing at the same time the sustainability of the aquaculture industry. The negative effects of antibiotics overuse include the accumulation of residue in the aquatic environment, particularly in the marine sediments where antibiotics can persist for months, favoring the selection of multi-antibiotic-resistant bacterial strains. Indeed, there is an increasing risk that antibiotic-resistant bacteria, initially derived from food-producing animals, could render the latest generation of antibiotics virtually ineffective for humans (Cabello, 2006; World Health Organisation [WHO] et al., 2006). Another negative outcome of antibiotics being used as growth promoters in cultured fish is the reduction of biodiversity and quantity of indigenous gut microbiota, which can impair fish immune responses (Borch et al., 2015).

For these reasons, the use of antibiotics as growth promoters in animal production has been fully banned in the EU since 2006 (Casewell et al., 2003; European Parliament and the Council of the European Union, 2003, 2019; European Medicines Agency [EMA], and European Food Safety Authority [EFSA], 2017) and many research efforts have been undertaken to replace them with probiotics for animal health management (Ezema, 2013).

Several studies have demonstrated that probiotics can reduce pathogenic bacteria due to direct competition-colonizing dynamics, through which microorganisms can partition spatial niche habitats in the intestinal mucosa (Balcázar et al., 2007b; Sugimura et al., 2011). Probiotics can also produce inhibitory molecules, such as bacteriocins, siderophores, enzymes, and hydrogen peroxide, or inhibit pathogenic bacteria by decreasing the intestinal pH through the release of organic acids (Ringø, 2008; Zhou X. et al., 2010; Ustyugova et al., 2012; Perez et al., 2014; Dahiya et al., 2020).

In addition, probiotics enhance the host immune system by generating systemic and/or local responses (Balcázar et al., 2006b; Salinas et al., 2008) that include activation of various antioxidant pathways and an increase in several innate immune parameters, such as phagocytosis, lysozyme levels, respiratory burst peroxidase and antiprotease activity, cytokine production, and white blood cell count (Nayak, 2010; Lazado and Caipang, 2014; Newaj-Fyzul et al., 2014; Simó-Mirabet et al., 2017).

In cultured fish, probiotics improve fish growth and feed conversion rates, too, due to an increase in feed digestibility and absorption of nutrients (Dimitroglou et al., 2011; Martínez Cruz et al., 2012). These effects stem from the capacity of probiotics to secrete enzymes, such as proteases, amylases, and lipases that hydrolyze molecules, which the fish intestine cannot

[1]https://www.efsa.europa.eu/en

otherwise digest (Balcázar et al., 2006b; Abd El-Rhman et al., 2009). Furthermore, the use of probiotics can restore the eubiotic state of the intestinal microbiota after antibiotic treatment or a pathogenic insult or can help maintain gut microbiota homeostasis, even in larval stages, when vaccination is difficult (Abdelhamid et al., 2009; Borch et al., 2015).

Hence, positive effects of different probiotics have been reported in several fish species, such as Nile tilapia (*Oreochromis niloticus*) (Ridha and Azad, 2012), common carp (*Cyprinus carpio*) (Feng et al., 2019), African catfish (*Clarias gariepinus*) (Al-Dohail et al., 2009), olive flounder (*Paralichthys olivaceus*) (Heo et al., 2013), Asian sea bass (*Lates calcarifer*) (Ringø, 2008; Lin et al., 2017), red drum (*Sciaenops ocellatus*) (Zhou Q.C. et al., 2010), European sea bass (*Dicentrarchus labrax*) (Carnevali et al., 2006; Mahdhi, 2012), common dentex (*Dentex dentex*) (Hidalgo et al., 2006), gilthead sea bream (*Sparus aurata*) (Suzer et al., 2008; Varela et al., 2010), rainbow trout (*Oncorhynchus mykiss*) (Merrifield et al., 2010), and abalone (*Haliotis midae*) (Macey and Coyne, 2005), and in crustaceans, such as white shrimp (*Litopenaeus vannamei*) (Lin et al., 2004).

According to the above findings, the aim of the present research was to evaluate the effects of the lactic acid bacteria *Lactococcus lactis* subsp. *lactis* SL242, used as feed additive, on growth performance, feed utilization, intestinal morphology, transcriptional response, and microbiota in gilthead sea bream (*Sparus aurata*).

The probiotic strain *L. lactis* subsp. *lactis* SL242 was selected due to important characteristics of *Lc. lactis* in general and SL242 in particular. *Lc. lactis* are mesophilic lactic acid bacteria that are present in the intestinal microbiota of fish (Tarnecki et al., 2017; Ringø et al., 2020) and can adapt to the water temperature of many reared fish species. Lactococci are proteolytic bacteria (Samaržija et al., 2001) that are potentially useful for improving the digestion of proteins contained in fish feed. The proteolytic system of lactococci includes a cell wall-associated proteinase and an extracellular peptidase (Samaržija et al., 2001). Furthermore, SL242 produces the antibiotic nisin A (Malvisi et al., 2016), which can inhibit or kill vegetative cells and bacterial spores (European Safety Food Authority [EFSA], 2005). Due to its antibacterial activity, nisin is of great interest in aquaculture. Nisin-susceptible bacterial species are found among *Bacillus*, *Clostridium*, *Listeria*, *Staphylococcus*, *Streptococcus*, and *Vibrio* genera (European Safety Food Authority [EFSA], 2005; Malvisi et al., 2016; Hamid et al., 2020), including known aquatic pathogens, such as *V. parahaemolyticus*, and *V. alginotlyticus* (Hamid et al., 2020). *Lc. lactis* probiotics have also shown inhibitory action against *Yersinia rukeri* and *Aeromonas salmonicida*, which can affect fish growth (Balcázar et al., 2007a, 2006b). Furthermore, *Lc. lactis* probiotic has been effective against *Aeromonas hydrophila* in *Oreochromis niloticus* (Zhou X. et al., 2010).

MATERIALS AND METHODS

Ethics Statement

Procedures for fish manipulation and tissue collection were carried out according to the Spanish (Royal Decree RD53/2013) and the current EU legislation (2010/63/EU) for handling of experimental fish. All procedures were approved by the Ethics and Animal Welfare Committees of Institute of Aquaculture Torre de la Sal (IATS-CSIC, Castellón, Spain) (Permit number 824/2019) and "Generalitat Valenciana" (permit number 2019/VSC/PEA/0197).

Animals

On June 2019, juveniles of gilthead sea bream were purchased from a Mediterranean hatchery (Piscimar, Burriana, Spain) and adapted for more than 2 months to the indoor experimental facilities of IATS-CSIC, under natural photoperiod and temperature conditions (40°5′N; 0°10′E). Seawater was pumped ashore (open system); oxygen content of water effluents was always above 85% saturation, and unionized ammonia remained below 0.02 mg/L. During the acclimation and experimental period, water temperature increased from 20–22°C in June to 28°C in August, decreasing thereafter from 24–25°C in mid-September to 13–16°C in December.

Diets

Extruded pellets of a control (diet A) and two experimental diets (diets B and C) were manufactured by VRM Srl Naturalleva (Verona, Italy), mimicking commercial fish feed formulations with traditional vegetable proteins and oils as the main replacers of fishmeal and fish oil, respectively (**Table 1**). The mash of each diet was extruded using a single-screw extruder (X-165, Wenger United States). To ensure product stability, the probiotic was homogenized with the dietary oil and included by vacuum coating (La Meccanica vacuum coater, Italy) during the post-extrusion process. During the vacuum process, only dry basal extruded pellets of diets B and C were supplemented with 2.5 and 6.2 g/100 kg of *L. lactis* subsp. *lactis* SL242, corresponding to a probiotic dosage of 2×10^9 CFU/kg (low dose) and 5×10^9 CFU/Kg (high dose), respectively. Sacco S.r.l [Cadorago (Co), Italy] provided the probiotic strain.

The two doses were chosen on the basis of our experience and literature data (Villamil et al., 2002; Adel et al., 2017) in order to verify the most effective one. They are also in line with dosages that could be used commercially in a cost-effective manner.

The final feeds were stored in a refrigerated room (6–7°C) for the entire duration of the feeding trial. A preliminary stability study of SL242 in the feed supplemented with probiotic was conducted for 12 weeks (the duration of the experiment), at 6°C. At the end of this period, the average loss of viability determined by plate count resulted about 50%, consistent with our expectations. Although further improvement may be warranted for a commercial probiotic product, at this stage of the process, the observed stability is considered acceptable.

Feeding Trial

In September 2019, fish weighing 70–90 g were randomly distributed in nine 500 L tanks to establish triplicate groups of 40 fish each (initial rearing density, 6.6–6.7 kg/m^3). All fish were tagged with PIT (passive integrated transponders) (ID-100A 1.25 Nano Transponder, Trovan) in the dorsal skeletal muscle. Fish were individually weighed and measured at initial, intermediate,

TABLE 1 | Ingredients and chemical composition (%) of control diet (Diet A) used in the trial.

Ingredients	Diet A
Fishmeal	10.1
Corn gluten	24.3
Guar germ meal	10.0
Soybean meal	13.1
Soya protein concentrate	13.6
Wheat	10.8
Fish oil	7.5
Rapeseed oil	3.5
Camelina oil	3.5
Lactic bacteria	0.0
Lysine	0.9
DL-methionine	0.4
Monoammonium phosphate	1.2
Taurine	0.4
Vitamins[a] and Minerals[b]	0.7
Proximate composition (%)	
Gross energy (MJ/kg)	18.92
Digestible energy, DE (MJ/kg)	17.26
Crude fat	18.0
Crude protein	43.8
Digestible protein, DP	38.8
DP/DE (mg/kJ or g/MJ)	22.5
Fiber	2.6
Nitrogen free extract	24.6
Starch	8.7
Non-starch polysaccharides	18.5

Diet B and C were formulated with the addition of probiotic (5×10^6 CFU/g feed).
[a]Vitamin premix (IU or mg/kg diet): DL-α tocopherol acetate 60 IU; sodium menadione bisulfate 5 mg; retinyl acetate 15,000 IU; DL-cholecalciferol 3,000 IU; thiamine 15 mg; riboflavin 30 mg; pyridoxine 15 mg; vitamin B_{12} 0.05 mg; nicotinic acid 175 mg; folic acid 500 mg; inositol 1,000 mg; biotin 2.5 mg; calcium pantothenate 50 mg.
[b]Mineral premix (g or mg/kg of diet) bi-calcium phosphate 500 g, calcium carbonate 215 g, sodium salt 40 g, potassium chloride 90 g, magnesium chloride 124 g, magnesium carbonate 124 g, iron sulfate 20 g, zinc sulfate 4 g, copper sulfate 3 g, potassium iodide 4 mg, cobalt sulfate 20 mg, manganese sulfate 3 g, sodium fluoride 1 g.

and final sampling points (every 4 weeks), by using a FR-200 Fish Reader W (Trovan, Madrid, Spain) for data capture and pre-processing.

The trial lasted 12 weeks (October 2019–December 2019). Fish were hand-fed once daily (12 a.m.), 5–6 days per week to visual satiety with either control or experimental diets for the entire duration of the trial. Feed intake and mortalities (<1%) were recorded daily and normal fish behavior was assessed routinely by camera monitoring.

Sample Collection

At the end of the feeding trial, four fish per replicate (12 fish/diet) were anesthetized with 0.1 g/L of tricaine-methasulfonate (MS-222, Sigma-Aldrich) and then sacrificed by severing the spinal cord. The intestine (excluding the pyloric ceca) of each fish was dissected out, weighed, and measured aseptically to calculate the intestine weight index (IWI) and intestine length index (ILI). Then, anterior (AI) and posterior (PI) intestine tissue portions (∼0.4 cm) were put either into RNAlater, or in 10% neutral buffered formalin for subsequent molecular (AI) and histological (AI, PI) analyses. The remaining part of AI was opened and washed with sterile Hank's balanced salt solution before collecting the autochthonous intestinal bacteria by scraping intestinal mucosa with the blunt end of a clean scalpel. Then, mucus samples were transferred to a sterile Eppendorf tube and stored in ice until subsequent (within 2 h) DNA extraction for microbiota analysis.

To characterize feed-associated bacterial communities, two samples of 200 mg each from each feed were taken at the end of the trial and used for bacterial DNA extraction and sequencing.

Histological Analysis

Fixed samples of AI and PI were dehydrated in ethanol solutions with gradually increasing concentrations and then, embedded in paraffin. Sections of 5 μm were obtained with a microtome (Leica RM2245) and stained with hematoxylin and eosin (H&E), following standard histological protocols. The sections were examined under a stereomicroscope Eurotek Tecno NB50T (Orma Srl, Milan, Italy) and photographed with a digital camera Eurotek CMOS MDH5 (Orma Srl, Milan, Italy). Based on previous studies (Knudsen et al., 2007; Uran et al., 2008; Urán et al., 2009; Khojasteh, 2012), the semi-quantitative scoring system focused on five different gut morphological parameters (mucosal folds, connective tissue, *lamina propria* of simple folds, and supranuclear vacuoles). Histological alterations of each morphological parameter were classified using a score value ranging from one (normal condition) to five (severe alteration). The final values, obtained by the sum of score values for each parameter, were then used to classify the severity of the morphological damage by using a class-based scoring system: Class I (values $\leq$ 10)—normal tissue structure with slight histological alterations; Class II (values 11–15)—moderate histological alterations; and Class III (values $>$ 15)—severe histological alterations of the organ.

Gene Expression Analysis

Total RNA from AI was extracted using a MagMax-96 total RNA isolation kit (Life Technologies, Carlsbad, CA, United States). The RNA yield was higher than 3.5 μg with absorbance measures (A260/280) of 1.9–2.1. cDNA was synthesized with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, United States), using random decamers and 500 ng of total RNA in a final volume of 100 μL. Reverse transcription (RT) reactions were incubated 10 min at 25°C and 2 h at 37°C. Negative control reactions were run without the enzyme. As reported previously (Estensoro et al., 2016), a customized PCR array layout was designed to simultaneously profile a panel of 44 selected genes, including markers of epithelial integrity (11), nutrient transport (4), mucins (3), cytokines (9), immunoglobulins (2), cell markers and chemokines (7), and pattern recognition receptors (8) (**Table 2**). qPCR reactions were performed using an iCycler IQ Real-Time Detection System (Bio-Rad, Hercules, CA, United States). Diluted RT

TABLE 2 | PCR-array layout for intestine gene expression profiling.

Function	Gene	Symbol	GenBank
Epithelial integrity	Proliferating cell nuclear antigen	*pcna*	KF857335
	Transcription factor HES-1-B	*hes1-b*	KF857344
	Krueppel-like factor 4	*klf4*	KF857346
	Claudin-12	*cldn12*	KF861992
	Claudin-15	*cldn15*	KF861993
	Cadherin-1	*cdh1*	KF861995
	Cadherin-17	*cdh17*	KF861996
	Tight junction protein ZO-1	*tjp1*	KF861994
	Desmoplakin	*dsp*	KF861999
	Gap junction Cx32.2 protein	*cx32.2*	KF862000
	Coxsackievirus and adenovirus receptor homolog	*cxadr*	KF861998
Nutrient transport	Intestinal-type alkaline phosphatase	*alpi*	KF857309
	Liver type fatty acid-binding protein	*fabp1*	KF857311
	Intestinal fatty acid-binding protein	*fabp2*	KF857310
	Ileal fatty acid-binding protein	*fabp6*	KF857312
Mucus production	Mucin 2	*muc2*	JQ277710
	Mucin 13	*muc13*	JQ277713
	Intestinal mucin	*i-muc*	JQ277712
Cytokines	Tumor necrosis factor-alpha	*tnfα*	AJ413189
	Interleukin 1 beta	*il1β*	AJ419178
	Interleukin 6	*il6*	EU244588
	Interleukin 7	*il7*	JX976618
	Interleukin 8	*il8*	JX976619
	Interleukin 10	*il10*	JX976621
	Interleukin 12 subunit beta	*il12*	JX976624
	Interleukin 15	*il15*	JX976625
	Interleukin 34	*il34*	JX976629
Immunoglobulins	Immunoglobulin M	*igm*	JQ811851
	Immunoglobulin T	*igt*	KX599201
Cell markers and chemokines	CD4	*cd4-1*	AM489485
	CD8 beta	*cd8b*	KX231275
	C-C chemokine receptor type 3	*ccr3*	KF857317
	C-C chemokine receptor type 9	*ccr9*	KF857318
	C-C chemokine receptor type 11	*ccr11*	KF857319
	C-C chemokine CK8/C-C motif chemokine 20	*ck8/cl20*	GU181393
	Macrophage colony-stimulating factor 1 receptor 1	*csf1r1*	AM050293
Pattern recognition receptors (PRR)	Galectin 1	*lgals1*	KF862003
	Galectin 8	*lgals8*	KF862004
	Toll-like receptor 2	*tlr2*	KF857323
	Toll-like receptor 5	*tlr5*	KF857324
	Toll-like receptor 9	*tlr9*	AY751797
	C-type lectin domain family 10 member A	*clec10a*	KF857329
	Macrophage mannose receptor 1	*mrc1*	KF857326
	Fucolectin	*fcl*	KF857331

reactions (×6) were used for qPCR assays in a 25 μL volume in combination with a SYBR Green Master Mix (Bio-Rad, Hercules, CA, United States) and specific primers at a final concentration of 0.9 μM (**Supplementary Table 1**). The program used for PCR amplification included an initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation for 15 s at 95°C and annealing/extension for 60 s at 60°C. All the pipetting operations were executed by means of an EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany) to improve data reproducibility. The efficiency of PCRs (>92%) was checked, and the specificity of reactions was verified by analyzing the melting curves (ramping rates of 0.5°C/10 s over a temperature range of 55–95°C), and linearity of serial dilutions of RT reactions ($r^2 > 0.98$). Fluorescence

data acquired during the extension phase were normalized by the delta-delta CT method (Livak and Schmittgen, 2001), using beta-actin as housekeeping gene due to its stability in different experimental conditions (average CT between experimental groups varied less than 0.2).

Bacterial DNA Extraction

The bacterial DNA was extracted from feeds (2 samples/feed) and from intestinal samples (7–10 fish/dietary group). Intestinal mucus samples (200 μl) were treated with 250 μg/ml of lysozyme (Sigma) for 15 min at 37°C. Then, DNA was extracted using the High Pure PCR Template Preparation Kit (Roche) following the manufacturer's instructions. DNA concentration, quality, and purity were measured using a NanoDrop 2000c (Thermo Fisher Scientific) and agarose gel electrophoresis (1% w/v in Tris-EDTA buffer). Samples were stored at −20°C until sequencing. The same procedure was used to extract DNA from the control and experimental feeds (previously ground to a fine powder) to evaluate the concentration of the probiotic supplement.

Illumina MiSeq Sequencing and Bioinformatic Analysis

The V3-V4 region of the 16S rRNA gene (reference nucleotide interval 341–805 nt) was sequenced using the Illumina MiSeq system (2 × 300 paired-end run) at the Genomics Unit from the Madrid Science Park Foundation (FPCM, Spain). The details on the PCR and sequencing of amplicons have been described elsewhere (Piazzon et al., 2019). Raw sequence data were uploaded to the NCBI (National Center for Biotechnology Information) and Sequence Read Archive (SRA) under NCBI BIOPROJECT ID: PRJNA679278; NCBI BIOSAMPLE ID: SAMN16828235-61; and SRA ACCESSION: SRR13081673-99. Raw forward and reverse reads were quality filtered using FastQC[2], and pre-processed using Prinseq (Rahlwes et al., 2019). Terminal N bases were trimmed at both ends and sequences with >5% of total N bases were discarded. Reads that were <150 bp long with a Phred quality score <28 in both of the sequence ends and with a Phred average quality score <26 were excluded. Then, forward and reverse reads were merged using fastq-join (Aronesty, 2013).

Bacterial taxonomy was assigned using the Ribosomal Database Project (RDP) release 11 as a reference database (Cole et al., 2014). Reads were aligned with a custom-made pipeline using VSEARCH and BLAST (Altschul et al., 1990; Rognes et al., 2016). Alignment was performed establishing high stringency filters (≥90% sequence identity, ≥90% query coverage). Taxonomic assignment results were filtered and data were summarized in an Operational Taxonomic Units (OTUs) table. Sample depths were normalized by total sum scaling and then made proportional to the total sequencing depth, following previously described recommendations (McKnight et al., 2019). Species richness estimates and alpha diversity indexes were calculated using the R package Phyloseq (Mcmurdie and Holmes, 2013). Rarefaction curves were obtained by plotting the number of observed taxonomic assignations in an OTU table against the number of sequences in each sample using the R package phyloseq.

[2]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Inferred Metagenome and Pathway Analysis

Piphillin was used to normalize the amplicon data by 16S rRNA gene copy number and to infer the metagenomics content (Iwai et al., 2016). This analysis was performed with the OTUs significantly driving the separation by probiotic in the PLS-DA analysis (described in the section "Statistics"). For the analysis, a sequence identity cut-off of 97% was implemented, and the inferred metagenomics functions were assigned using the Kyoto Encyclopedia of Genes and Genomes database (KEGG, Oct 2018 Release). Raw KEGG pathway output from Piphillin was analyzed with the R Bioconductor package DESeq2 using default parameters, after flooring fractional counts to the nearest integer (Love et al., 2014; Bledsoe et al., 2016; Piazzon et al., 2019). Comparisons were also performed between different diets to evaluate possible pathway differences across diets.

Statistics

Data on growth and gene expression were analyzed by one-way ANOVA using SigmaPlot v14 (Systat Software Inc., San Jose, CA, United States). Normality of the data was verified by Shapiro-Wilk test, and Dunn's *post hoc* test was used for multiple comparisons between groups. For analysis of qualitative histological data, we conducted the non-parametric Kruskall-Wallis test, followed by Dunn's test for the multiple comparisons. GraphPad Prism8 (GraphPad Software, Inc., La Jolla, CA, United States) was used for both analyses. Microbiota species richness, alpha diversity indexes, and phylum abundance between experimental groups were determined by Kruskal-Wallis test followed by Dunn's *post hoc* test. Beta diversity was tested with permutational multivariate analysis of variance (PERMANOVA), using the non-parametric method *adonis* from the R package Vegan with 10,000 random permutations. To further study microbiota differences between dietary groups, supervised partial least-squares discriminant analysis (PLS-DA) and hierarchical clustering of samples were sequentially applied using EZinfo v3.0 (Umetrics, Umea, Sweden) and *hclust* function (gplots R package), respectively. Hotelling's T^2 statistic was calculated by employing the multivariate software package, whereby points above the 95% confidence limit for T^2 were considered as outliers and discarded. Values of normalized counts of OTUs present in 3 or more samples were included in the analyses, and the significant contribution to the group separation was determined by the minimum variable importance in the projection (VIP) values (Wold et al., 2001; Li et al., 2012), which renders an accurate clustering using the average linkage method and Euclidean distance feasible. The quality of the PLS-DA model was evaluated by the parameters R2Y (cum) and Q2 (cum), which indicate the fit and prediction ability, respectively. To assess whether the supervised model was being overfitted, a validation test consisting

on 600 random permutations was performed using SIMCA-P + (v11.0, Umetrics).

RESULTS

Growth Performance

Data on growth performance, feed intake, and feed conversion ratio (FCR) are reported in **Table 3**. All fish grew efficiently during the first 30 days of the trial (FCR = 1.27–1.28), reaching an overall FCR of 1.55–1.60 at the end of trial. The decrease in the length of the day and temperature from October to December should be noted.

No statistically significant differences were found between groups for the condition factor and specific growth rates (SGR), although the highest SGR tended to be achieved in fish fed diet C (high dose of probiotic). Indeed, the final body weight of these animals was higher than in the control group (diet A) ($P < 0.05$) with intermediate values for fish fed diet B (low dose of probiotic). Thus, total weight gain varied from 97% in fish fed diet A to 106% in fish fed diet C.

Histological and Biometric Scoring

Histological analysis of gilthead sea bream intestine was performed according to the aforementioned morphological criteria. The intestinal scoring data are reported in **Table 4**. The AI (**Figures 1A–C**) and PI (**Figures 1D–F**) portions were not affected by probiotic administration. Although the mucosal folds of the PI were significantly different ($P < 0.05$) between groups fed diets A and B, the total scores, calculated for each group, fall within an evaluation of Class I. In particular, the simple and complex folds appeared thin and regularly branched, *lamina propria* and connective tissue appeared normally proportioned and supranuclear vacuoles were numerous and well-distributed. Regarding the index of intestine length (ILI) (**Table 4**), diet B showed a significantly lower ILI than the control group (diet A) ($P < 0.05$), but no differences were observed between the other groups. No differences in the intestine weight index (IWI) were observed between groups.

Gene Expression Profiling

All genes included in the PCR-array were found at detectable levels with the highest expression level for markers of nutrient transport (*alpi, fabp1, and fabp2*), epithelial integrity (*cx32.2*), mucus production (*muc2, muc13*) and pattern recognition receptors (*fcl*) (**Supplementary Table 2**). Regarding the probiotic effect, statistically significant changes were found in the expression patterns of 5 out of 44 genes ($P < 0.05$) (**Figure 2**). In particular, expression of interleukin 10 (*il10*), interleukin (*il12),* and toll-like receptor 2 (*tlr2*) was upregulated in fish fed diet C (high probiotic dose) with intermediate values (not statistically different from the control group) in fish fed diet B (low probiotic dose). In contrast, the highest values of toll-like receptor 5 (*tlr5)* and galectin-8 (*lgals8)* were seen in fish fed diet B, whereas intermediate values were found in fish fed diet C. The probiotic treatment altered other markers (desmoplakin, *dsp*; interleukin 34, *il34*; C-C chemokine receptor 3, *ccr3*; and macrophage mannose receptor 1, *mrc1*) to a lesser extent, with an overall enhancement of gene expression that was especially evident in fish fed diet C ($P < 0.1$).

TABLE 3 | Growth performance of gilthead sea bream (*Sparus aurata*).

Diet	Mean body weight (g)		WG[1] (%)	SGR[2] (%)	Feed intake	CF[3]		FCR[4]
	Initial	Final			(g dry feed/fish)			
Period T0-T1, 24/09/2019–24/10/2019								
A	82.67 ± 0.86	130.53 ± 1.35	57.9 ± 0.4	1.52 ± 0.01	61.82 ± 0.59^{ab}	2.89 ± 0.02	A	1.27 ± 0.01
B	83.45 ± 0.74	130.01 ± 1.20	55.8 ± 0.8	1.48 ± 0.02	59.58 ± 0.73^{a}	2.84 ± 0.02	B	1.28 ± 0.01
C	83.28 ± 0.83	132.08 ± 1.32	58.6 ± 0.8	1.54 ± 0.02	60.61 ± 0.75^{b}	2.86 ± 0.01	C	1.27 ± 0.01
Period T1-T2, 25/10/2019–15/11/2019								
A	130.53 ± 1.35	149.43 ± 1.56	14.5 ± 0.6	0.66 ± 0.03	38.78 ± 2.17	2.76 ± 0.02	A	1.80 ± 0.02
B	130.01 ± 1.20	150.08 ± 1.40	15.4 ± 0.4	0.68 ± 0.01	36.99 ± 1.19	2.74 ± 0.01	B	1.84 ± 0.04
C	132.08 ± 1.32	152.99 ± 1.66	15.8 ± 0.8	0.70 ± 0.02	33.93 ± 2.06	2.73 ± 0.01	C	1.86 ± 0.06
Period T2-T3, 15/11/2019–18/12/2019								
A	149.43 ± 1.56	163.04 ± 2.02^{a}	9.1 ± 0.6	0.28 ± 0.02	35.71 ± 0.69	2.78 ± 0.02	A	2.40 ± 0.05
B	150.08 ± 1.40	166.30 ± 1.90^{ab}	10.8 ± 0.3	0.31 ± 0.03	33.74 ± 1.37	2.73 ± 0.03	B	2.31 ± 0.03
C	152.99 ± 1.66	171.24 ± 2.07^{b}	11.9 ± 1.8	0.36 ± 0.02	32.69 ± 1.46	2.81 ± 0.01	C	2.02 ± 0.25
Overall, 24/09/2019–18/12/2019								
A	82.67 ± 0.86	163.04 ± 2.02^{a}	97.2 ± 1.4	0.80 ± 0.01	135.85 ± 3.29	2.78 ± 0.02	A	1.57 ± 0.05
B	83.45 ± 0.74	166.30 ± 1.90^{ab}	99.3 ± 0.7	0.81 ± 0.01	129.62 ± 2.71	2.73 ± 0.03	B	1.60 ± 0.03
C	83.28 ± 0.83	171.24 ± 2.07^{b}	105.6 ± 2.5	0.85 ± 0.02	126.44 ± 6.86	2.81 ± 0.01	C	1.55 ± 0.02

Data are reported as mean ± SEM, different superscript letters indicate significant differences ($P < 0.05$) between diet groups in the same sub-column.
[1]Weight gain, WG = (100 × body weigh increase)/initial body weight.
[2]Specific growth rate, SGR = 100 × (ln final body weight–ln initial body weight)/days.
[3]Condition factor, CF = 100 × (body weight/standard length).
[4]Feed conversion ratio, FCR = dry feed intake/wet weight gain [total feed supplied (g DM, dry matter)/WG (g)].

TABLE 4 | Histological scoring (for anterior and posterior intestine) and biometric measurement [intestinal length index (ILI) and intestinal weight index (IWI)] of gilthead sea bream (*Sparus aurata*) juveniles fed the control (A) and experimental (B and C) diets.

Diet	Mucosal folds	Connective tissue	Lamina propria of simple folds	Supranuclear vacuoles	Total score	ILI[1] (cm)	IWI[2] (g)
			Anterior intestine			Biometric measurement	
A	1.1 ± 0.1	1.7 ± 0.06	1.7 ± 0.06	1.5 ± 0.04	6.1 ± 0.2	97.21 ± 7.62[a]	2.43 ± 0.06
B	1.0 ± 0.04	1.5 ± 0.2	1.5 ± 0.1	2.2 ± 0.5	6.3 ± 0.9	75.73 ± 6.74[b]	2.38 ± 0.12
C	1.1 ± 0.04	1.7 ± 0.1	1.5 ± 0.2	2.0 ± 0.4	6.2 ± 0.7	86.43 ± 8.02[ab]	2.40 ± 0.17
			Posterior intestine				
A	1.2 ± 0.2[a]	1.6 ± 0.09	1.9 ± 0.2	2.2 ± 0.3	6.9 ± 0.8		
B	1.8 ± 0.3[b]	2.0 ± 0.2	1.7 ± 0.1	2.1 ± 0.2	7.6 ± 0.8		
C	1.3 ± 0.07[ab]	1.8 ± 0.04	1.7 ± 0.08	2.0 ± 0.07	6.8 ± 0.07		

Data are reported as mean ± SEM of 12 fish per diet. Different superscript letters indicate significant differences (Dunn's pot-hoc test, $P < 0.05$) between dietary groups in the same sub-column.
[1]Intestinal length index, ILI = 100 × (intestine length/standard length).
[2]Intestinal weigth index, IWI = 100 × (intestine weight/fish weight).

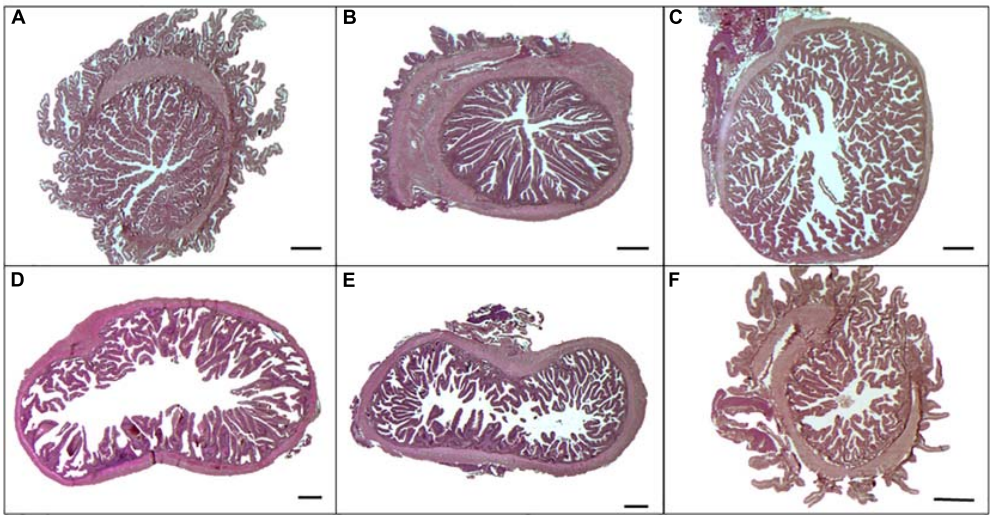

FIGURE 1 | Light microscope images obtained from anterior **(A–C)** and posterior **(D–F)** intestine of gilthead sea bream juveniles (*Sparus aurata*) fed with diets A, B, and C, stained with hematoxylin and eosin (H&E). Scale bar = 500 μm.

Characterization of Feed-Associated Bacterial Communities

At the end of the trial, the normalized counts of *L. lactis* subsp. *lactis* resulted 8–11 in diet A (<0.0001% total bacterial counts); 30,204 in diet B (2.5% total counts); and 61,828 (5.4% total counts) in diet C (**Figure 3**). By excluding Cyanobacteria/Chloroplast (>90% total counts), Firmicutes and Proteobacteria proved to be the most highly represented bacterial phyla in the three feeds, whereas the rest of the bacterial population consisted of Bacteriodetes and Fusobacteria phyla (**Supplementary Figure 1A**). However, the percentage of Firmicutes varied considerably between feeds, with higher values in feed B (4.2%) and C (7.8%) than in the control feed, in which Firmicutes represented only 2% of the total counts. Thus, by recalculating the relative bacterial abundances after excluding Cyanobacteria/Chloroplast, the percentage of Firmicutes rose from 34% in the control diet A to 70% in diet B and 79% in diet C (**Supplementary Figure 1B**). Then, by specifically analyzing the relative abundance of the probiotic *L. lactis subsp. lactis* in comparison to the most representative genera within the phylum Firmicutes, the percentage of *L. lactis* subsp. *lactis* was close to 0% in the control diet, whereas in B and C diets, it was significantly higher, reaching values of 64 and 71%, respectively (**Supplementary Figure 1C**).

Alpha Diversity and Gut Microbiota Composition

Illumina sequencing of AI-adherent bacteria yielded 3,677,860 high-quality and merged reads, with an average value of 136,217 reads per sample (**Supplementary Table 3**). When annotated, the

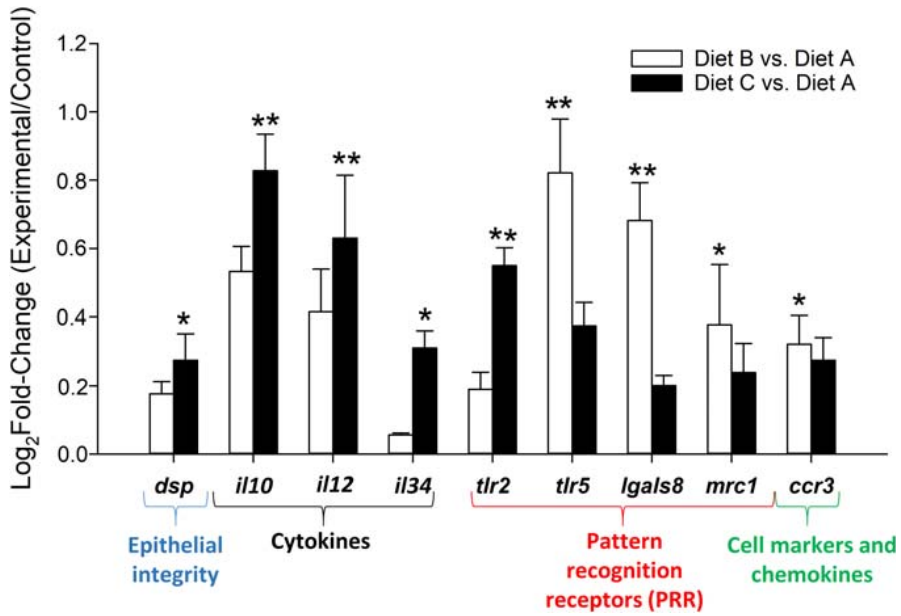

FIGURE 2 | Fold change of differentially expressed genes (Dunn's *post hoc* test; $^{**}P < 0.05$; $^{*}P < 0.1$) in the anterior intestine of fish fed experimental diets (diets B and C) relative to the control diet (A). Data are the mean + SEM of 9–12 fish per diet. White columns (fish fed diet B). Black columns fish fed diet C.

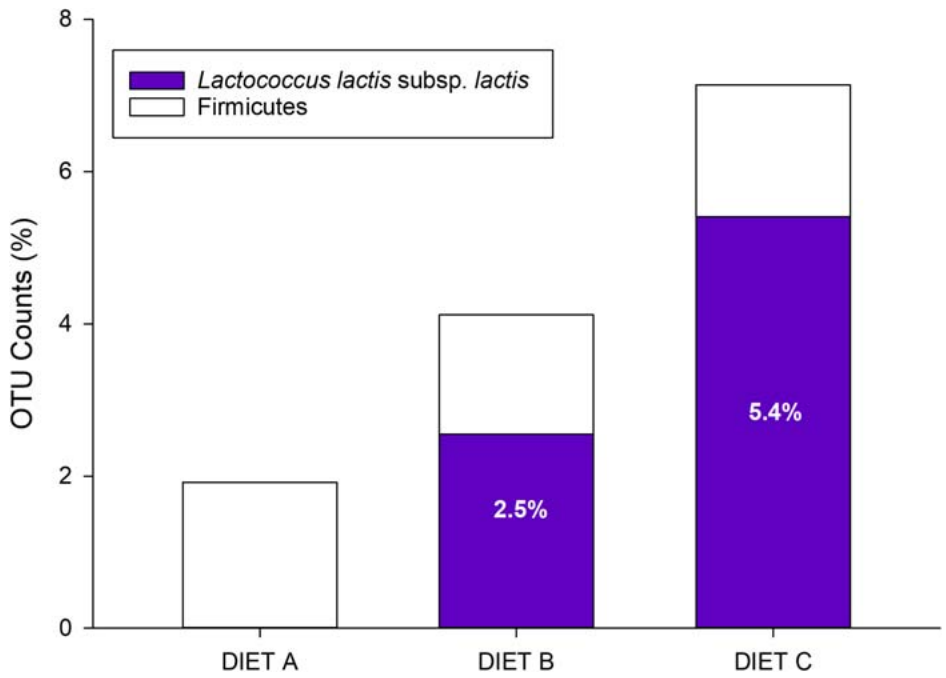

FIGURE 3 | Relative abundance of Firmicutes phylum and *Lactococcus lactis* subsp. *lactis* in control (Diet A) and experimental diets (Diets B and C).

reads were assigned to 1,313 OTUs at 97% identity threshold. Rarefaction analysis showed curves that approximated saturation (horizontal asymptote); thus, a good coverage of the bacterial community was achieved and the number of sequences for analysis was considered appropriate (**Supplementary Figure 2**). Indeed, up to 85% of the OTUs were classified at the level of species and more than 90% at the level of genus (94.1%), family (96%), order (97%), class (97.2%), and phylum (99%).

As shown in **Table 5**, the richness estimator (ACE) indicated a higher OTU richness in fish fed diet B than in fish fed diet A or diet C. At the same time, alpha diversity estimators (Shannon and Simpson) disclosed a reduced evenness in fish fed diet C, which

TABLE 5 | Species richness estimate (ACE) and diversity indexes (Shannon and Simpson) of the adherent microbial communities in the anterior intestine of fish fed diet A (10), diet B (10), and diet C (7).

	Diet			K-W test
	A	B	C	*P*-value
ACE	205.17 ± 16.76^{b}	294.98 ± 32.04^{a}	162.08 ± 23.39^{b}	0.006
Shannon	2.14 ± 0.12^{a}	2.4 ± 0.13^{a}	1.58 ± 0.2^{b}	0.006
Simpson	0.82 ± 0.02^{a}	0.85 ± 0.02^{a}	0.65 ± 0.08^{b}	0.02

Different superscript letters indicate significant differences be dietary groups [Kruskal-Wallis (K-W) test, Dunn's pot-hoc test, $P < 0.05$].

indicates that abundant OTUs predominated over the others in this group of fish.

Changes in bacterial composition were also found at the phylum level (**Figure 4**). Proteobacteria was the most abundant phylum in the three groups, ranging from 55.9% in fish fed diet C to 55.7% in fish fed diet A, and 50.1% in the diet B fed group. The second-most abundant phylum was Firmicutes, representing the 26.6% of the OTU counts in fish fed diet A, decreasing progressively with the probiotic supplementation in fish fed diet B (26.2%) and diet C (5.6%). The same trend was shown by the phylum Bacteroidetes, ranging from 2.7% in fish fed diet A to 1.3% in fish fed diet B and 0.1% in fish fed diet C. The phylum Actinobacteria increased from 9.3% in fish fed diet A to 16.3% in fish fed diet B but decreased to its minimum level in group C (3.2%). Finally, Spirochetes appeared in a significant proportion (32%) only in fish fed with diet C, being practically absent in the other groups (<3%).

Beta Diversity, Discriminant Analysis, and Inferred Pathways

No significant differences in beta diversity were found when experimental groups were computed independently (PERMANOVA, $P = 0.34$, $F = 1.031$, $R^2 = 0.04$). In contrast, when B and C groups were computed together, beta diversity became statistically significant (PERMANOVA, $P = 0.032$, $F = 1.8789$, $R^2 = 0.099$). Taking this analysis further, a PLS-DA model was constructed with a 99% to the total variance explained (**Figure 5**). During the statistical processing to construct the model, two fish from the Diet A group and one fish from the Diet C group appeared as outliers and were excluded from the model. This approach displayed a clear separation of control fish and fish fed probiotic diets (B + C group) along component 1 (84.52%) with a higher individual variability within fish fed diet B than in those fed diet C. This PLS-DA model was successfully validated with a permutation test (pCV ANOVA = 0.015) discarding the possibility of over-fitting of the supervised model (**Supplementary Figure 3**).

Differences between control fish and the probiotic-fed merged groups were driven by 81 OTUs (VIP > 1), mainly belonging to the phyla Proteobacteria, Spirochetes, and Firmicutes. A detailed list of the VIPs can be found in **Supplementary Table 3**. The inferred metagenomic analysis using DESeq2 disclosed nine differentially abundant pathways across groups (**Figure 6**). Pathways related to protein digestion and absorption, as well as renin secretion were over-represented in the probiotic fed fish groups, whereas the control group showed a relative preponderance of pathways related to shigellosis, proteasome and autophagy.

DISCUSSION

In aquaculture the use of probiotics is significantly increasing and a growing number of studies are demonstrating their positive effects in the most economically important fish species (Merrifield et al., 2010; Varela et al., 2010; Mahdhi, 2012; Ridha and Azad, 2012; Chauhan and Singh, 2019).

As mentioned previously, one of the most interesting effects of probiotics is the increase in the animals' growth performance (Sun et al., 2012; Nguyen et al., 2017; Won et al., 2020). In the present study, gilthead sea bream fed diets C and B, supplemented with high and low doses of *L. lactis* subsp. *lactis*, respectively, reached a higher final biomass than control fish fed with diet A, and differences in biomass gain were statistically significant between groups C and A. Although differences between fish groups arose at the December sampling, most of the weight gain was attained during September–October, as this period still corresponds to the active fish feeding behavior at IATS-CSIC latitude. This result highlights, albeit slightly, the beneficial action of the probiotic, suggesting a more efficient digestion and utilization of nutrients in gilthead sea bream fed probiotics. Indeed, although no significant differences were detected in FCR and SGR between dietary groups, the lowest FCR (1.60 ± 0.03) and the highest SGR (0.85 ± 0.02) were registered in fish fed diet C. Similar results were obtained in gilthead sea bream by Suzer et al. (2008) and Varela et al. (2010), using *Lactobacillus* spp. and *Shewanella putrefaciens* Pdp11, respectively. Positive results in fish growth performance, using *L. lactis* as probiotic, were also obtained in other cultured fish species, such as common carp, European sea bass, tilapia, and olive flounder (Balcázar et al., 2006a; Carnevali et al., 2006; Heo et al., 2013; Xia et al., 2018; Feng et al., 2019).

Histological analysis was conducted using a semi-quantitative scoring system. The parameters taken into account for the AI and PI morphological evaluation were related to the mucosal folds that represent the intestinal absorptive surface area, and to the associated connective tissue (Dimitroglou et al., 2011; Khojasteh, 2012; Puphan et al., 2015). Our results confirmed that probiotic did not alter the morphology of the gut and did not trigger intestinal inflammation. Indeed, no structural modifications were detected in fish fed with diets supplemented with probiotic (diets B and C), in comparison to the control group fed diet A. In line with our results, other studies have shown that probiotics improve gut morphology, leading to an increase in intestinal absorption capacity (Batista et al., 2016; Won et al., 2020). In contrast, Cerezuela et al. (2012; 2013) reported several negative effects related to the administration of probiotics in gilthead sea bream. In particular, those authors showed that both *Tetraselmis chuii* and *Bacillus subtilis* induced intestinal inflammation with numerous signs of edema in the

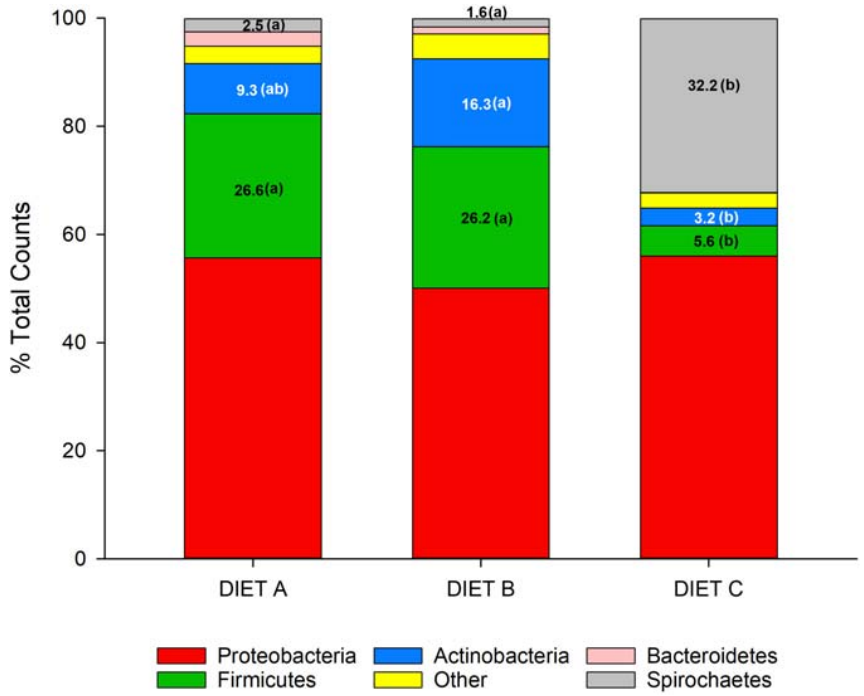

FIGURE 4 | Stacked bar chart representing the relative abundance (%) of bacterial phyla in fish fed control (A) and experimental (B and C) diets. The Kruskal–Wallis test (Dunn's *post hoc* test, $P < 0.05$) showed significant differences between groups for the phyla Firmicutes, Actinobacteria and Spirochetes. The differences are indicated by different letters in parenthesis that correspond to pairwise comparisons within each phylum between groups.

mucosal folds. Therefore, more in-depth histological analyses are needed to better understand the effects of different probiotic strains on the adsorptive surface area in fish intestine and, in particular, on the villi length and density.

Numerous studies that have investigated the effects of probiotics on the piscine immune system have reported an enhanced immune response, thus improving survival rates and resistance to a pathogenic attack (Nayak, 2010; Lazado and Caipang, 2014). Different probiotic strains stimulate the immune system in fish, but the effect appears to be species-specific. *L. lactis* supplementation increased the concentration of several pro- and anti-inflammatory cytokines (Tnfα, Il1β, Il6, Il12, Il10 and Tgfβ) in common carp serum (Feng et al., 2019) and upregulated the expression of *tnfα, ifnγ, hsp70,* and *il1*β genes in the intestine of tilapia (Xia et al., 2018; Won et al., 2020). Conversely, *L. lactis* did not induce any differences in the abundance of cytokines and pattern recognition receptors (PRRs) transcripts in intestine or head kidney of trout (Pérez-Sánchez et al., 2011). In gilthead sea bream, the anti-inflammatory action of a *Bacillus*-based probiotic induced decreased expression of *lgals8* and *cd4* transcripts in anterior intestine, lower amounts of circulating IgM and cortisol, a lower respiratory burst activity of blood leukocytes, and lower numbers of eosinophilic granulocytes (in particular, mast cells) in the intestinal submucosa (Simó-Mirabet et al., 2017). Herein, significant differences in the expression of key genes involved in innate and acquired immunity (interleukins and PRRs) were detected between fish fed probiotic and control diets. Among the mechanisms induced by probiotics, it has been postulated that the activation of immunity derives from the interaction of the host with the probiotic microbial associated molecular patterns (MAMPs) (Yang et al., 2014). The direct effect of MAMPs was recently demonstrated by feeding grouper (*Epinephelus coioides*) with MAMPs isolated from the probiotic *Bacillus pumilus* SE5. Indeed, an activation of intestinal immunity via up-regulation of TLR signaling pathways was observed (Yang et al., 2019). Thus, the observed activation of the immune system in the present study is likely taking place by direct induction of gilthead sea bream PRRs by components on the cell wall of the probiotic, such as peptidoglycan or lipoteichoic acid, which are in fact TLR2 agonists (Dammermann et al., 2013).

The density, composition and function of intestinal microbiota of fish, including gilthead sea bream, are shaped by numerous factors, such as diet, sex, developmental stage, and rearing conditions (Piazzon et al., 2017, 2019; Rimoldi et al., 2020), as well as multiple endogenous host-microbe interactions, such as the host's genetic background (Piazzon et al., 2020), and possible intestinal disorders or intestinal diseases (Bakke-Mckellep et al., 2007; Green et al., 2013). Furthermore, microbiota vary taxonomically and functionally in different sections of the GIT of fish (Kokou et al., 2020). There is also a distinction between the allochthonous, i.e., free-living, transient microbiota associated with the digesta (feces), and autochthonous communities that colonize the mucosal surface of the digestive tract and make up the core community (Merrifield et al., 2010; Ringø et al., 2016; Nguyen et al., 2017; Egerton et al., 2018).

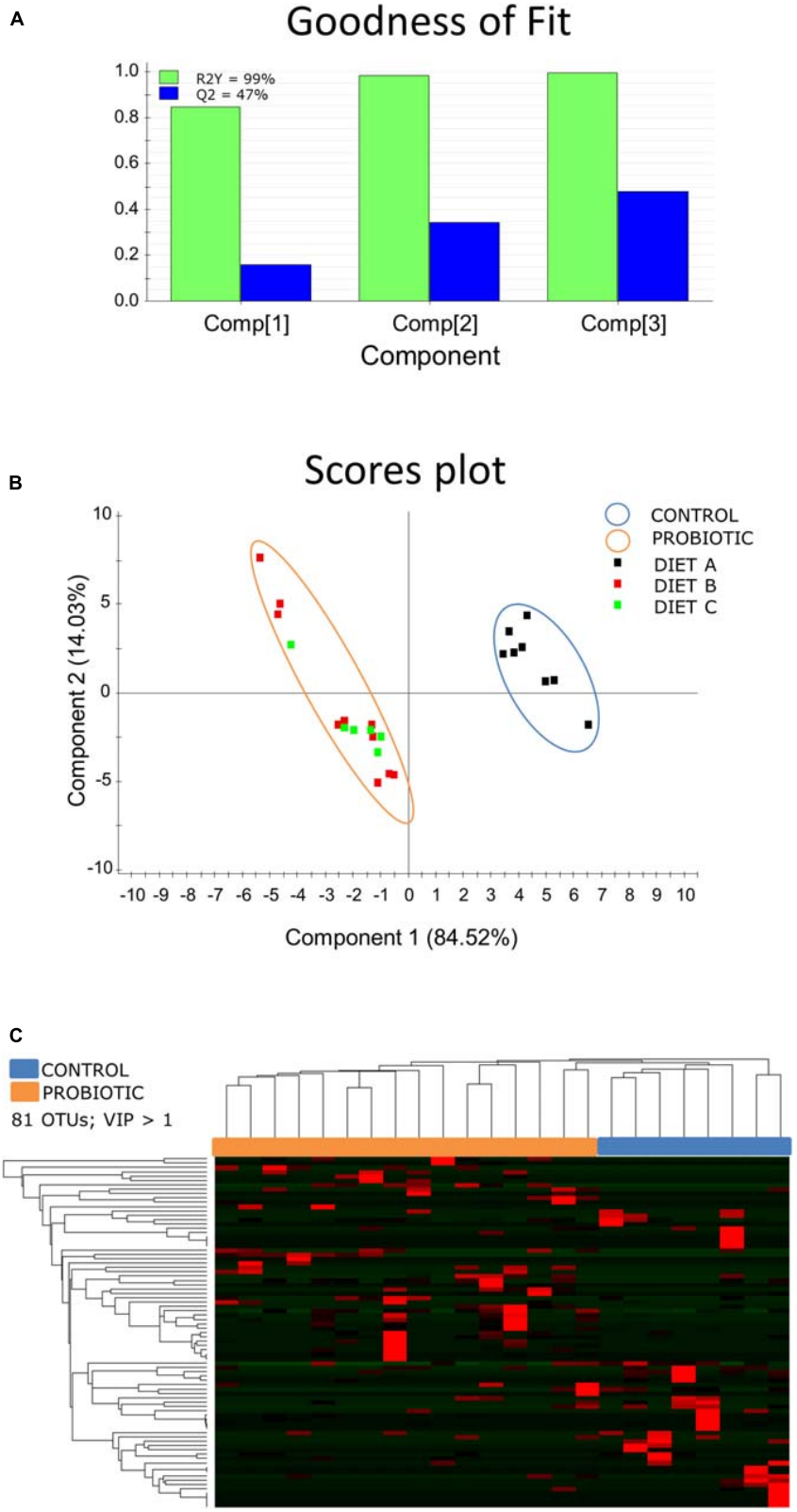

FIGURE 5 | (A) Graphical representation of the goodness-of-fit of the PLS-DA model. **(B)** Two-dimensional PLS-DA score plot representing the distribution of the samples between the first two components in the model. **(C)** Heatmap showing the abundance distribution (*z*-score) of the OTUs identified to be driving the separation between fish fed probiotic diets (B + C; orange) and fish fed diet A (blue).

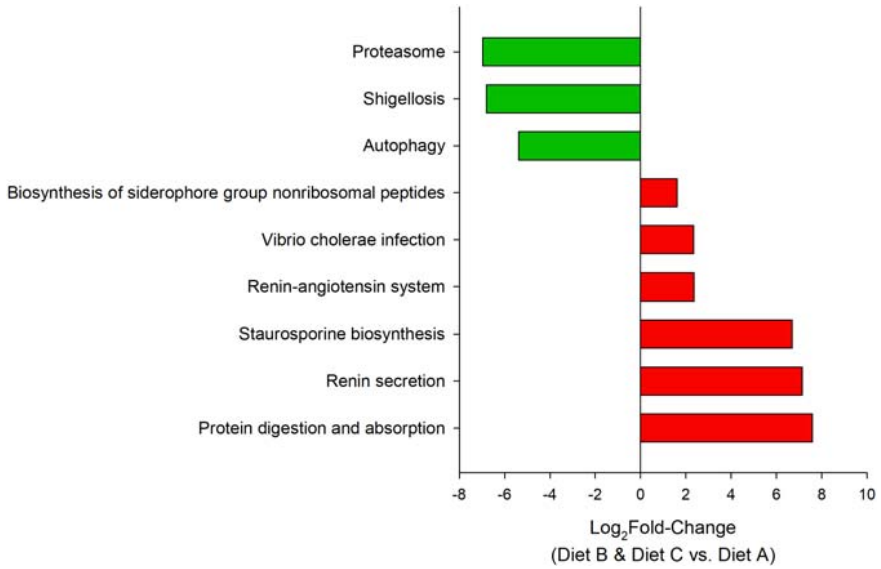

FIGURE 6 | Bar plots depicting the changes in metabolic capacities in the comparison between fish fed probiotic diets (B + C group) and fish fed the control diet A. Bars show the $\log_2$-fold change in the metabolic pathway.

Taxonomically, gut bacteria are classified according to phyla, classes, orders, families, genera, and species. The "core" intestinal microbiota, which can often persist in spite of changing factors is constituted by Proteobacteria, Firmicutes, and Actinobacteria phyla in both freshwater and marine fish species (Silva et al., 2011; Kormas et al., 2014; Ghanbari et al., 2015; Piazzon et al., 2019). These taxa are largely considered important players in nutritional provisioning, immune defense, and metabolic homeostasis (Estruch et al., 2015; Givens et al., 2015; Rimoldi et al., 2019; Terova et al., 2019).

Accordingly, in the present experiment, gilthead sea bream were fed with three different feeds and at the end of the experiment, the microbiota of these feeds was analyzed. Data revealed that Firmicutes and Proteobacteria were the bacterial phyla represented most, followed in descending order by Bacteriodetes and Fusobacteria. Then, by analyzing specifically the relative abundance of the probiotic *L. lactis* subsp. *lactis* compared to the most representative genera of Firmicutes phylum, we found that the percentage of *L. lactis* subsp. *lactis* was close to 0% in diet A (control), whereas in diets B and C, it was definitely high, reaching values of 64 and 71%, respectively. This results is in agreement with the supplementation of a low and a high dose of probiotic to diets B and C, respectively.

With regard to the gut microbiota, gilthead sea bream fed diet C showed a significant increase in bacteria belonging to the Spirochetes phylum, which were practically absent in the gut of fish fed diets B and A (<3%). In the same fish group, a decrease in Actinobacteria, Bacteroidetes, and Firmicutes phyla was recorded. The Firmicutes phylum is composed of more than 200 different genera, such as *Lactobacillus*, *Bacillus*, *Enterococcus*, *Ruminococcus, and Clostridium.* Lactic acid bacteria (LAB) include, among others, *Streptococcus* sp., *Lactobacillus* sp., *Leuconostoc* sp. and *Carnobacterium* sp., which are considered as beneficial microorganisms that contribute to an healthy status of the fish intestine (Kim et al., 2012; Terova et al., 2019). It is known that commensal Firmicutes and Bacteroidetes are the major producers of short chain fatty acids, such as butyrate, acetate, and propionate that are the end products of fiber fermentations.

While is difficult to assess from genomic data alone the physiological effect on the host of the microbiota changes we found, it is worth noting that Firmicutes/Bacteroidetes ratio in the gut has been directly related to lean body mass in both human and animals (Magne et al., 2020). Indeed, the ratio of Firmicutes vs. Bacteroidetes was increased in obese individuals as compared to lean ones. Actually, gilthead sea bream fed with diets containing probiotic showed a higher Firmicutes/Bacteroidetes ratio than control fish and this could be correlated to their better growth performances. Likewise in mice, the reduced amount of Bacteroidetes was a direct consequence of probiotic supplementation (Grazul et al., 2016). In addition, gilthead sea bream fed diet C, showing the best FCR and SGR values, had the highest percentage of Spirochetes. In swine, the Spirochaetaceae bacterial family was shown to correlate positively with the host weight (Unno et al., 2015). The gut microbiome of the feeding group C was also characterized by a Proteobacteria/Firmicutes ratio five times higher than in the other groups. This result is not surprising because *Lc. lactis* subsp. *lactis* SL242 produces the antibiotic nisin, displaying strong activity against Gram-positive bacteria (Li et al., 2018), and a vast majority of Firmicutes are Gram-positive.

The analysis of gut-adherent (autochthonous) microbiota did not reveal significant differences between fish groups in relation to *L. lactis*, suggesting a lack of colonization of the probiotic in the host's intestinal mucosa. This was not a surprising result since it is known that probiotics generally do not colonize the digestive tract i.e., they do not become established permanently or for a long-term (weeks, months, or years) in the intestinal tract (Marco, 2019). Thus, the ingested bacteria can be beneficial

while they are in the gut, but they do not have a lasting effect and continued probiotic consumption is needed for sustained impact. Thus, instead of colonizing, the new bacteria may temporarily complement resident microbial communities, forming part of a transient (allochthonous) microbiome in fish without displacing the native gut microbiota, but instead altering digestive tract function by producing active metabolites that modulate the activity of the gut microbiota, or by stimulating the intestinal epithelium directly (Marco, 2019). Hence, in the present trial, although the probiotic did not colonize the host's intestinal mucosa, it did modulate the fish gut microbiota, confirming that colonization is not always necessary to induce host modification. Indeed, diets B and C were enriched with Actinomycetales, as compared to diet A, which instead showed a higher percentage of *Pseudomonas*, *Sphyngomonas*, and *Lactobacillus* genera. These results were confirmed by the clear separation of bacterial community of fish fed with the probiotic from the bacterial community of control fish group (diet A) in the beta-diversity and PLS-DA analyses. Furthermore, the KEGG pathway analysis underlined such differences, highlighting several pathways potentially affected by the diet. Particularly interesting were those related to protein absorption and digestion.

In the present study, the analysis of gut microbial communities revealed significant differences between fish groups in term of species richness and diversity. Among alpha diversity indices, fish fed with diet B showed the highest level of richness estimator ACE and biodiversity, in comparison to the other two fish groups. In contrast, dietary group C, although achieving the best growth performances, showed the lowest gut bacterial diversity.

A reduction in bacterial diversity is usually considered an adverse outcome, since this could lead to less competition for opportunistic or invading pathogens due to a functionally unbalanced ecosystem (Cerezuela et al., 2013; Li et al., 2014; Rimoldi et al., 2020). However, while an increase in intestinal microbial biodiversity following prebiotics (dietary compounds that induce the growth or activity of gut microbiota) administration has been frequently described, the data currently available on the effects of probiotics in fish are more controversial. For instance, in line with our results, the species richness and diversity indexes decreased in gilthead sea bream in response to dietary administration of the probiotic *Bacillus subtilis*, either alone or in combination with prebiotics or microalgae (Cerezuela et al., 2012, 2013). In contrast, in line with what we found in fish fed diet B, lactic acid bacteria supplementation was associated with an increase in bacterial diversity in the intestinal mucus of Atlantic salmon (Gupta et al., 2019). In addition, probiotics, such as lactic acid bacteria, are known to produce several antimicrobial compounds capable of suppressing the growth of other microorganisms, which can alter the gut microbiota in terms of both composition and biodiversity (Collado et al., 2007).

CONCLUSION

According to analysis of gut-adherent (autochthonous) microbiota, the probiotic *L. lactis* subsp. *lactis* did not colonize in the host's intestinal mucosa. However, the probiotic did modulate the fish gut microbiota, confirming that colonization is not always necessary to induce host modification. Indeed, gut microbiota of fish fed diets B (low dose of probiotic) and C (high dose) were clearly separated from the bacterial community of control fish in the beta-diversity and PLS-DA analyses. Furthermore, the KEGG pathway analysis underlined such differences, highlighting several pathways potentially affected by the diet. Particularly interesting were those related to protein absorption and digestion.

With regard to fish growth performance, there were no significant differences between groups for the FCR and SGR. The only difference was the final body weight of fish fed diet C (high dose of probiotics) that resulted higher than the control group.

Dietary probiotic administration did not alter the morphology of the intestine and did not trigger inflammation.

Researches such as these highlight the interaction between fish diet and their microbiota and suggest that manipulating diet to tune the gut microbiome may be a promising intervention, together with well-designed probiotics.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (accession: SAMN16828235–SAMN16828261 and PRJNA679278).

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics and Animal Welfare Committees of Institute of Aquaculture Torre de la Sal (IATS-CSIC, Castellón, Spain) (Permit number 824/2019) and "Generalitat Valenciana" (Permit number 2019/VSC/PEA/0197).

AUTHOR CONTRIBUTIONS

FM, FN-C, and MP: experimental investigation, methodology, data curation, formal analysis, and writing—review and editing. SR: methodology, formal analysis, and writing—review and editing. JC-G: methodology, data curation, formal analysis, and writing—review and editing. AG: conceptualization and writing—review and editing. IM: writing—review and editing. FB: experimental investigation and methodology. JP-S: conceptualization, experimental investigation, data curation, and writing—review and editing. GT: conceptualization, data curation, and writing—review and editing. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.659519/full#supplementary-material

Supplementary Figure 1 | (A) Relative abundance of phyla in control (Diet A) and experimental diets (Diets B and C). **(B)** Relative abundance of phyla excluding Cyanobacteria/Chloroplast in control and experimental diets. **(C)** Relative abundance of Firmicutes genera, including *Lactococcus lactis* subsp. *lactis* in control and experimental diets.

Supplementary Figure 2 | Rarefaction curves obtained from the sequencing data of the different groups included in this study (Diets A, B, C).

Supplementary Figure 3 | Validation plot of the PLS-DA model between control (Diet A) and experimental (Diets B and C) groups, consisting of 600 random permutations.

Supplementary Table 1 | Primers for qPCR gene expression. Colors in letters of column Gene show different gene functions included in the array as follows: red: Epithelial integrity; dark blue: Nutrient transport; green: Mucus production; Orange: Cytokines; purple: Immunoglobulins; black: Cell markers and chemokines; light blue: Pattern recognition receptors.

Supplementary Table 2 | Relative gene expression of anterior intestine in fish fed control **(A)** and experimental **(B,C)** diets. Data are the mean ± SEM of 9–12 fish. All data values were referred to the expression level of *hes1-b* of control fish with an arbitrary assigned value of 1. [1]*P*-value results from ANOVA. Bold font indicates statistically significant differences between groups ($P < 0.05$).

Supplementary Table 3 | Sequencing and taxonomy assignment results.

Supplementary Table 4 | OTUs driving the separation between fish fed probiotic diets (B + C) and fish fed control diet A in the PLS-DA model (VIP > 1).

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Impacts of the Marine Hatchery Built Environment, Water and Feed on Mucosal Microbiome Colonization Across Ontogeny in Yellowtail Kingfish, *Seriola lalandi*

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The fish gut microbiome is impacted by a number of biological and environmental factors including fish feed formulations. Unlike mammals, vertical microbiome transmission is largely absent in fish and thus little is known about how the gut microbiome is initially colonized during hatchery rearing nor the stability throughout growout stages. Here we investigate how various microbial-rich surfaces from the built environment "BE" and feed influence the development of the mucosal microbiome (gill, skin, and digesta) of an economically important marine fish, yellowtail kingfish, *Seriola lalandi*, over time. For the first experiment, we sampled gill and skin microbiomes from 36 fish reared in three tank conditions, and demonstrate that the gill is more influenced by the surrounding environment than the skin. In a second experiment, fish mucous (gill, skin, and digesta), the BE (tank side, water, inlet pipe, airstones, and air diffusers) and feed were sampled from indoor reared fish at three ages (43, 137, and 430 dph; n = 12 per age). At 430 dph, 20 additional fish were sampled from an outdoor ocean net pen. A total of 304 samples were processed for 16S rRNA gene sequencing. Gill and skin alpha diversity increased while gut diversity decreased with age. Diversity was much lower in fish from the ocean net pen compared to indoor fish. The gill and skin are most influenced by the BE early in development, with aeration equipment having more impact in later ages, while the gut "allochthonous" microbiome becomes increasingly differentiated from the environment over time. Feed had a relatively low impact on driving microbial communities. Our findings suggest that *S. lalandi* mucosal microbiomes are differentially influenced by the BE with a high turnover and rapid

succession occurring in the gill and skin while the gut microbiome is more stable. We demonstrate how individual components of a hatchery system, especially aeration equipment, may contribute directly to microbiome development in a marine fish. In addition, results demonstrate how early life (larval) exposure to biofouling in the rearing environment may influence fish microbiome development which is important for animal health and aquaculture production.

Keywords: microbiome, built environment, yellowtail kingfish, *Seriola lalandi*, aquaculture, fisheries, ontogeny, mariculture

INTRODUCTION

Aquaculture, which is the farming of aquatic organisms including algae, invertebrates, and vertebrates, has been one of the fastest growing agriculture sectors (8.8% annual growth between 1980 and 2010) for the past 40 years (The State of World Fisheries and Aquaculture, 2020). Demand for seafood has continually grown with global fish production in 2018 at around 179 million metric tons (MMT), of which 82 MMT comes from aquaculture (The State of World Fisheries and Aquaculture, 2020). While 86.5% of total finfish production occurs in inland freshwater systems, with the majority in Asia (The State of World Fisheries and Aquaculture, 2020), marine culture has the highest growth potential with 2% of oceans being suitable for fish farming (Oyinlola et al., 2018). For marine aquaculture growth, Australia, Argentina, India, Mexico, and the United States have the greatest potential based on suitable habitat (Gentry et al., 2017). Freshwater finfish production has primarily been driven by carp, catfish, and tilapia, while marine fish production is dominated by Atlantic salmon which has a freshwater hatchery stage. Despite the recognized opportunities for marine finfish aquaculture production, very few marine fish species have been successful compared to freshwater fish, due in part to the inability to spawn and produce quality fingerlings in captivity. This has led to the common practice of catching wild juveniles and their transfer to captive rearing environments. In recent years, however, certain high value marine species, including the yellowtail kingfish (YTK) *Seriola lalandi*, have been successfully reared in the lab (Welch et al., 2010). The *Seriola* genus, within the family Carangidae, contains several species of yellowtail (Purcell et al., 2015; Oyinlola et al., 2018) that are globally distributed across broad temperature range (Poortenaar et al., 2001). *S. lalandi*, is reared in temperate waters across the Pacific Ocean (Nakada, 2002; Food and Agriculture Organization of the United Nations, 2008; Orellana et al., 2014) in Japan (Nakada, 2002), Australia (Nakada, 2002; Hutson et al., 2007), New Zealand (Orellana et al., 2014; Symonds et al., 2014), Chile (Orellana et al., 2014), and North America (The State of World Fisheries and Aquaculture, 2020).

Fish, unlike mammals, are not thought to inherit their microbiome vertically. Understanding the factors which influence microbiome development in fish is an important first step in mitigating disease and promoting health. One of the primary challenges in marine fish hatcheries is poor survival rate which is often attributed to a combination of disease and nutrition (Sepúlveda et al., 2017). Even in the wild, the survival rate for fish larvae is 44× higher for freshwater fish (5.3%) as compared to marine (0.12%; Houde, 1994). Wild marine fish, particularly temperate coastal pelagics like *Seriola spp.* (Ben-Aderet, 2017), are exposed to wide ranges in environmental variables such as temperature, oxygen, and nutrients both diurnally with vertical migration for feeding and temporally with changing seasons. The mucosal microbiome of coastal pelagics is highly differentiated across body sites, primarily in the gill, skin, digesta, and gut tissue with the microbiome on external sites (gill and skin) most influenced by these changing environmental variables (Minich et al., 2020a). In mammals, both phylogeny and diet influence gut microbiome development (Groussin et al., 2017), whereas fish microbiomes are influenced more by environmental variables including habitat, trophic level, phylogeny, and diet (Sullam et al., 2012; Egerton et al., 2018). Diet also varies widely by development stage particularly in the larval to fry stages (Infante et al., 2000). While mammals have a significant proportion of their gut microbiome colonized or inherited vertically from the mother during birth (Mändar and Mikelsaar, 1996; Dominguez-Bello et al., 2010; Korpela et al., 2018), the initial establishment of the gut microbiome in fish is less understood. Even fewer studies have sought to identify the source colonizers of gill and skin communities.

Microbial colonization throughout development of the fish is a function of both exposure and host selection. At the earliest stage, bacteria which form biofilms on the outside of the egg eventually can colonize both external and internal mucosal sites of freshly hatched larvae upon ingestion of the yolk sac (Hansen and Olafsen, 1999). Marine fish differ from freshwater fish in that they must drink vast quantities of water to maintain osmoregulation, which in turn provides a large source of potential microbes for gut colonization (Hansen and Olafsen, 1999). The first live feeds the larvae consume, which in hatchery settings are often artemia and rotifers, also contribute to the gut microbiome development (Ringø, 1999; Egerton et al., 2018; Wang et al., 2018). In larval YTK, *S. lalandi*, gut microbiome composition and density changes most when transitioning from a live rotifer feed to pellet based feeds around 30 days post hatch (Walburn et al., 2019) with many of the gut microbes having anti-microbial functionality (Ramírez et al., 2019). In a study assessing gut enteritis in farmed *S. lalandi* from seapens, gill, and skin microbiomes correlated with disease state suggesting these communities were either responding to overall health decline or contributing to stress (Legrand et al., 2017). Skin and gut microbiomes of captively

reared *S. lalandi* were also influenced by diet and temperature (Horlick et al., 2020). For a freshwater hatchery, the tank side and tank water were shown to significantly influence the skin and gut microbiomes of Atlantic salmon (Minich et al., 2020b). Despite the array of studies evaluating impacts of various husbandry methods on microbiome composition of mucosal sites (gill, skin, and gut), there is a lack of information for how microbiomes on surfaces in the built environment (BE) directly contribute to marine fish.

To evaluate how the collective hatchery microbiome influences the mucosal microbiome of a marine fish, we investigated the economically important YTK *S. lalandi*. This study sought to answer three primary questions: (1) Are body sites differentially influenced by the BE or feed microbiome?, (2) What surfaces within a hatchery environment contribute to the mucosal microbiome of the fish?, and (3) Does the BE and feed microbiome source contribution vary across age and development of the fish? To answer these questions, we sampled the mucosal microbiomes of 92 fish across three broad development stages (fry, pre-stocking juvenile, and mature adult). Specifically, we used 16S rRNA amplicon sequencing of microbial communities from the fish (gill, skin, and digesta "allochthonous") together with various hatchery surfaces including tank water, tank side, inlet water pipe, air stones, and air diffusers along with feed used in all stages of production. To our knowledge this is the first study to quantify and compare the relationship of the BE microbiome with the fish microbiome across multiple age classes of a marine fish.

MATERIALS AND METHODS

Sampling Design

All sampling events occurred in June of 2018 in Port Stephens Australia at the Department of Primary Industries New South Wales. Two broad sampling regimes were carried out (**Supplementary Table 1**). A total of 92 "YTK" were sampled in Port Stephens, Australia. In the first experiment, gill and skin swabs were sampled from a total of 36 living fish across three different indoor rearing condition tanks (12 fish per tank) along with corresponding BE samples including tank water, the tank side, inlet pipes, and air diffusers. These fish were all siblings and 130 days post hatch "dph." Fish were reared in either a flow through system "FT," a traditional moving bed bioreactor "MBBR" Recirculating Aquaculture Systems "RAS," or a modified BioGill RAS. Fish were reared at a max of 25 kg/m^3 fed at a maximum of 0.5 kg food/day/m^3 and reared in 10 m^3 tanks. Additional details can be found in the white paper (Enabling land-based production of juvenile YTK in NSW). Fish were non-lethally sampled during routine biometric measurements where individuals were weighed and measured. Prior to taking the weight and length, the skin and gill of each fish was swabbed using a cotton swab [Puritan] and placed directly into a 2 ml PowerSoil tube. For these three tank conditions, "BE" samples were taken from the tank water, swab of tank side (biofilm), swab of air diffuser, swab of air stone, and swab of inlet water pipe. For the two RAS tanks, an additional inlet water sample was taken which represents cleaned water (post filtration). Comparisons were made to determine if there was a relationship between the external fish mucosal sites and the BE and if so how that varied across the water filtration or rearing system.

For the second experiment, fish were sampled cross sectionally at different ages including 43 dph (indoor), 137 dph (indoor), and 430 dph (indoor and outdoor). Fish at 430 dph included fish sampled from an ocean net pen along with fish which were transferred from an ocean net pen back to an indoor system. For the age comparison cohort, three body sites were sampled including the gill, skin, and digesta "allochthonous" samples along with corresponding BE samples described in experiment 1. The BE "built environment" samples included tank water, inlet pipe, airstone, air diffuser, and tank side. Specifically 12 fish were similarly non-lethally sampled from three different age classes: 43, 137, and 430 dph from indoor tanks. The 430 dph fish from the indoor tank were initially reared indoor until 245 dph following methods described by Stewart Fielder et al. (2011) and then transferred to ocean netpens where they were grown for 106 days. At 351 dph, they were then transported back to the indoor system where they were held until sampled at 430 dph. An additional 20 fish at 430 dph from the seapen were harvested for another experiment and opportunistically sampled. All fish were measured for length and mass with condition factor calculated. A total of 92 fish were sampled across the two experiments. For the entire experiment, 304 samples were processed for DNA extraction including 19 controls, 45 "BE" samples, 92 gill swabs, 92 skin swabs, and 56 digesta swabs (**Supplementary Table 1**).

Microbiome Sample Preparation and Processing

After swabbing the BE and fish mucosal sites, individual swab heads were broken off into a 2 ml PowerSoil tube and then stored at −20°C for 2 weeks until DNA extraction to preserve microbiome integrity (Song et al., 2016). All molecular processing was done according to the standard Earth Microbiome Project protocols (Thompson et al., 2017; earthmicrobiome.org). Batches of samples were extracted in groups of 48 using the Mobio PowerSoil kit (Cat# 12888–50). Lysis in single tubes were used to minimize noise from well-to-well contamination (Minich et al., 2019; Walker, 2019). A serial dilution (titration) of a positive control, *Escherichia coli* isolate (n = 12), along with negative control blanks (n = 7) were included to estimate the limit of detection of the assay (Minich et al., 2018b). By using the Katharoseq method, we empirically calculated the read count used to exclude samples (Minich et al., 2018b). For library preparation, DNA samples of equal volume (0.2 μl) were processed using the EMP 16S rRNA 515F (Parada)/806R (Apprill) primers (Caporaso et al., 2011; Apprill et al., 2015; Parada et al., 2016; Walters et al., 2016) with 12 bp golay barcodes at a miniaturized PCR reaction volume of 5 μl reactions in triplicate (Minich et al., 2018a). After PCR, equal volumes of each library (2 μl) were pooled and processed through the MinElute PCR purification kit (Qiagen Cat# 28004) followed by a 1× Ampure cleanup. The final library was sequenced using a MiSeq 2 × 250 bp kit (Caporaso et al., 2012).

Microbiome Analysis

Sequences were uploaded, demultiplexed, and processed in Qiita (Gonzalez et al., 2018), using the Qiime2 commands (Bolyen et al., 2019; Estaki et al., 2020). Specifically, sequences from the first read were trimmed to 150 bp following the EMP protocol, and processed through the deblur pipeline and SEPP (Janssen et al., 2018) to generate Amplicon Sequence Variants "ASVs" (Amir et al., 2017). ASVs were rarified to 5,000 reads per sample. General Alpha and Beta diversity measures (Whittaker et al., 2001; Reese and Dunn, 2018) were generated in Qiita. Microbial Alpha diversity comparisons (Reese and Dunn, 2018) were calculated for richness, Shannon diversity (Shannon, 1948), and Faith's Phylogenetic Diversity (Faith, 1992). For statistical analysis, grouped comparisons (>2 groups) were compared using Kruskal–Wallis test (Kruskal and Allen Wallis, 1952) with Benjamini Hochberg FDR 0.05 (Benjamini and Hochberg, 1995). To compare the age of fish with alpha diversity metrics, both linear regression and Spearman correlation (Spearman, 1904) were used using PRISM 9.0 (La Jolla, CA, United States). Beta diversity measures were calculated using both Unweighted UniFrac and Weighted normalized UniFrac (Hamady et al., 2010; Lozupone et al., 2011). Categorical group comparisons of beta diversity were calculated using PERMANOVA tests (Anderson, 2001, 2017). Lastly, to quantify the effects or sources of microbes from the BE onto the fish mucus, we applied the microbial source tracking software SourceTracker2 (version 2.0.1; Knights et al., 2011). Prior to SourceTracker2 analysis, ASVs which had less than 100 total counts across the dataset were removed to reduce sparsity and improve performance of the microbial source tracking.

RESULTS

Microbiome Sequence Data

Both negative and positive controls were used to determine the overall limit of detection to exclude or include samples. Serial dilutions of positive controls indicated a sample exclusion criterion of 2,406 reads (**Supplementary Figure 1**). To be conservative, we choose to rarefy at 5,000 reads which yielded a total of 246 samples (out of the original 304 samples) and 17,348 unique ASVs. After removing controls, a total of 236 samples were retained resulting in 17,161 ASVs. This includes two primary datasets: the tank rearing comparison of fish at 130 dph (gill, skin, and BE × three tank types) and the age comparison of fish sampled at 43, 137, and 430 dph (gill, skin, digesta, and BE). Overall, sample success was very high (**Supplementary Table 1**).

Impact of Rearing System (FT vs RAS) on Fish Mucosal Microbiome (at 130 dph)

To first assess how the rearing condition influences the microbiome of the BE and external mucosal sites of the fish (gill and skin), 12 YTK (130 dph) fish and various tank controls were sampled from three unique rearing systems. Microbial diversity in the gill varied across tank systems for richness (**Figure 1A**: $P = 0.0376$, KW = 6.563), Shannon (**Figure 1B**: $P = 0.0008$, KW = 14.26), and Faith's Phylogenetic diversity (**Figure 1C**: $P = 0.0273$, KW = 7.199) with FT grown fish having slightly higher microbial diversity compared to RAS reared. Skin samples did not differ in microbial diversity based on rearing type. In the BE, water generally was highest in microbial diversity, while both air stones and air diffusers had the lowest diversity across all sample types. When comparing the water communities of the FT and RAS tanks, the richness and phylogenetic diversity trended higher in RAS (**Figures 1A–C**). Interestingly, the inlet pipe biofilms were highly variable across the FT and RAS systems with the FT tank having a very high microbial diversity compared to RAS systems. The tank side biofilms were generally higher in microbial diversity in the RAS tanks as compared to the FT tank. When comparing beta diversity, the largest compositional differences were due to the feed vs all other sample types, with most feed pellet communities highly differentiated from the BE and fish mucus with the exception of live rotifer feeds. Many chloroplasts ASVs were present in the pellet feeds, likely from plant ingredients, which likely drove this separation. Upon chloroplast removal, read counts for feed samples drop to levels which would largely exclude them from analysis thus suggesting that feed samples have very low proportions of microbes. The second largest driver in microbial community composition was the fish body sites for both Weighted and Unweighted UniFrac (**Figures 1D,E**). For individual body sites, the tank systems also had a moderate impact with gill samples being more differentiated across tank systems (**Table 1**). Specifically, for gill samples, the tank rearing system had an impact on the microbial community for both Unweighted Unifrac distance (**Table 1**, PERMANOVA, $P = 0.001$, and $F = 2.72$) and Weighted normalized Unifrac distances (**Table 1**, PERMANOVA, $P = 0.001$, and $F = 11.01$). Pairwise comparisons of Unweighted Unifrac distances revealed that gill microbiomes of RAS reared fish were also differentiated but in general less differentiated as compared to the FT reared fish (**Figure 1E** and **Table 1**). Pairwise comparisons of Weighted normalized Unifrac distances revealed the same pattern, with fish reared in different RAS systems having a differentiated community but more even more differentiated when compared to fish reared in FT systems (**Figure 1D** and **Table 1**). Skin microbial communities were only influenced by the rearing method when comparing Unweighted Unifrac (**Table 1**) but not with Weighted normalized Unifrac. When comparing YTK from the same age and genetic cohort reared in three different conditions, gill microbial communities were more influenced by the environmental conditions than the skin, while microbial communities of the BE were highly variable across tank systems.

Impact of Age on Fish Mucosal Microbiome

After quantifying the variation which existed across tank systems at a single age of fish, we next wanted to evaluate the extent by which mucosal microbiomes (gill, skin, and gut) varied with fish age. Specifically, we sought to investigate factors governing the randomness vs. deterministic mechanisms for microbial

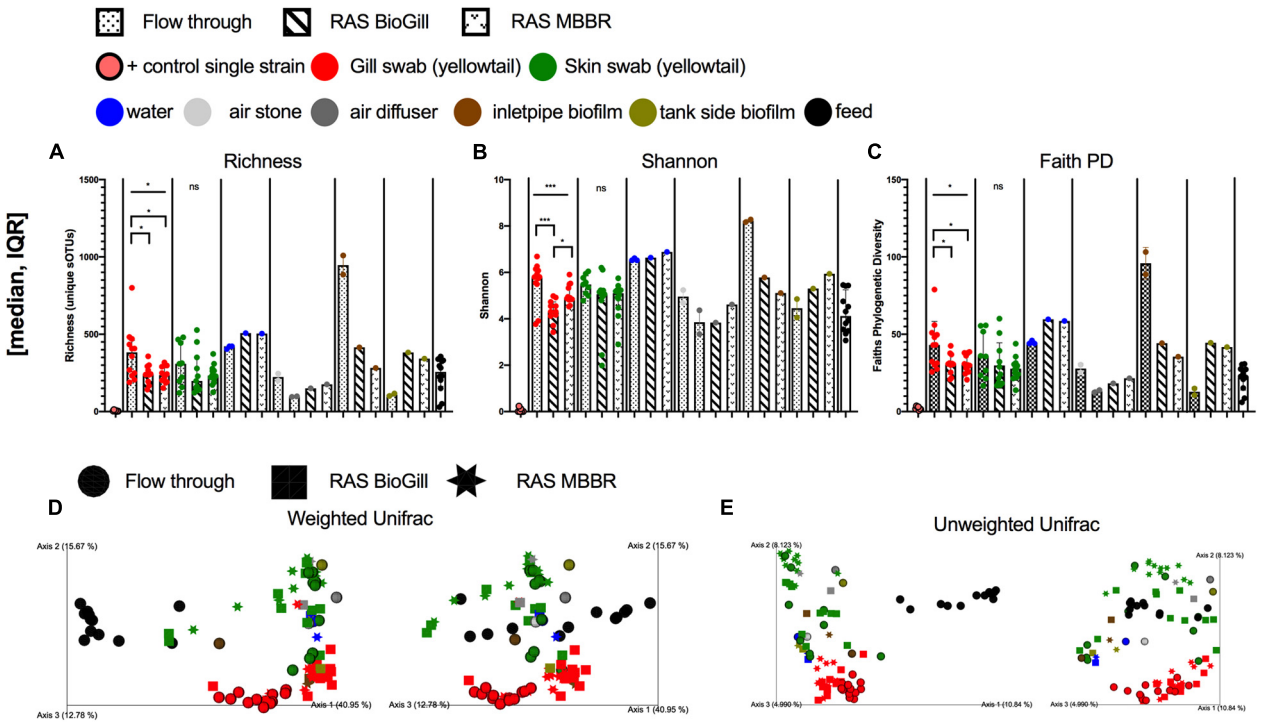

FIGURE 1 | Microbial diversity of the hatchery built environment along with fish gill and skin mucus at 130 days post hatch across three rearing tanks (flow through, RAS BioGill, and RAS MBBR). Alpha diversity as measured by **(A)** richness, **(B)** Shannon, and **(C)** Faith's phylogenetic diversity. Gill and skin (group comparison calculated with Kruskal–Wallis test, Benjamini Hochberg FDR 0.05). Beta diversity calculated using **(D)** Weighted normalized UniFrac and **(E)** Unweighted UniFrac distance. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$).

TABLE 1 | Multivariate statistical comparison of impacts of rearing system across gill and skin (PERMANOVA, 999 permutations).

Unweighted Unifrac		**YTK_tank_system**		
Body_site		***n***	***P***	***F***
Gill	FT vs RAS BioGill vs RAS MBBR	35	0.001	2.72
	FT vs RAS BioGill	23	0.001	2.82
	FT vs RAS MBBR	24	0.001	3.29
	RAS BioGill vs RAS MBBR	23	0.001	1.95
Skin	FT vs RAS BioGill vs RAS MBBR	32	0.002	1.73
	FT vs RAS BioGill	20	0.565	0.94
	FT vs RAS MBBR	21	0.001	2.21
	RAS BioGill vs RAS MBBR	23	0.002	2.08
Weighted normalized Unifrac		**YTK_tank_system**		
Body_site		***n***	***P***	***F***
Gill	FT vs RAS BioGill vs RAS MBBR	35	0.001	11.01
	FT vs RAS BioGill	20	0.001	17.43
	FT vs RAS MBBR	21	0.001	11.55
	RAS BioGill vs RAS MBBR	23	0.018	3.18
Skin	FT vs RAS BioGill vs RAS MBBR	32	0.182	1.60
	FT vs RAS BioGill	20	0.256	1.62
	FT vs RAS MBBR	21	0.038	2.87
	RAS BioGill vs RAS MBBR	23	0.413	0.83

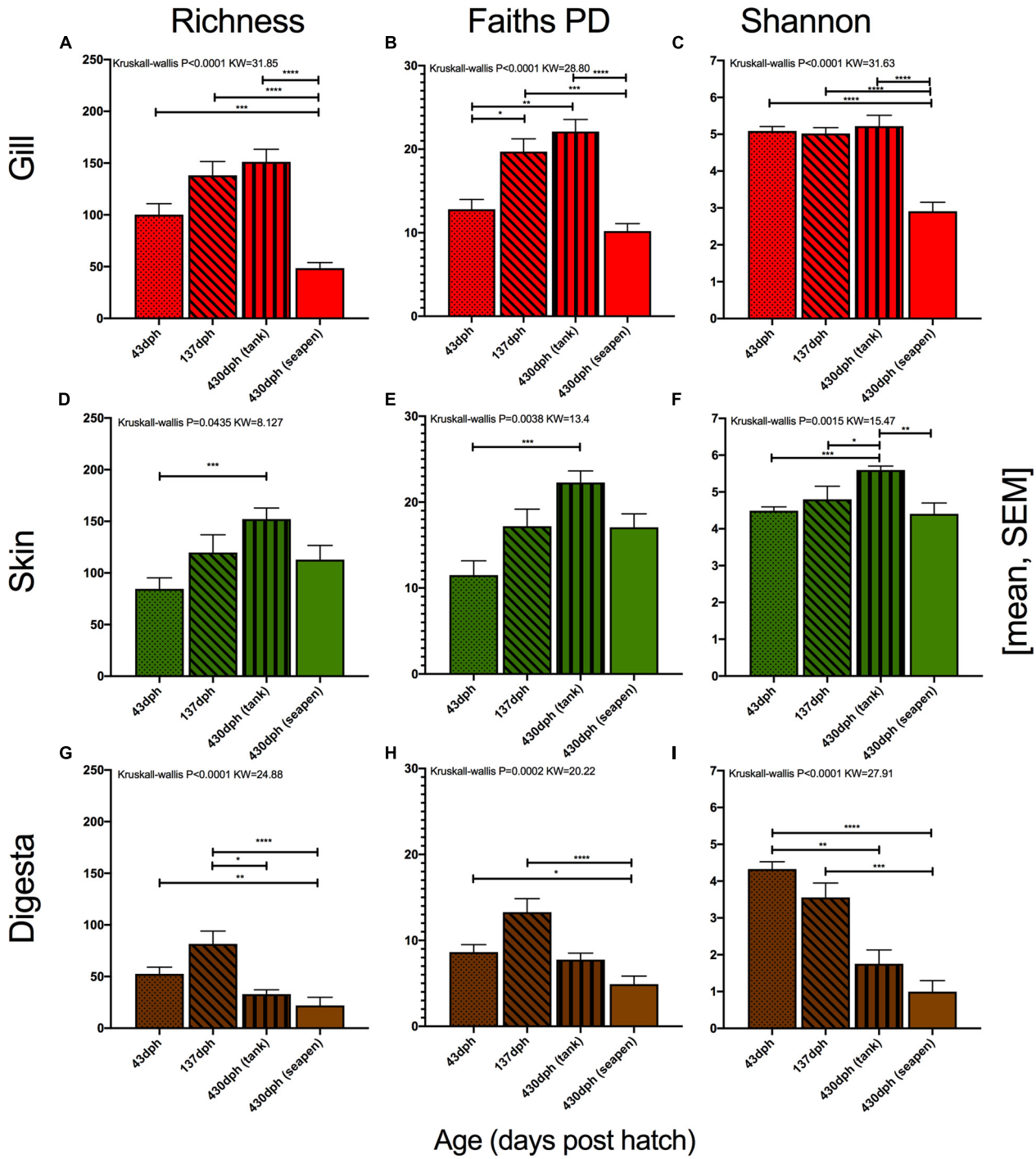

FIGURE 2 | Alpha diversity measures: richness, Faith's Phylogenetic diversity, and Shannon diversity grouped per body site (red = gill, green = skin, and brown = digesta). Each body site assessed for diversity differences across age (Kruskal–Wallis, Benjamini-Hochberg FDR 0.05). Gill microbial diversity: **(A)** richness, **(B)** Faiths PD, and **(C)** Shannon; Skin microbial diversity: **(D)** richness, **(E)** Faiths PD, and **(F)** Shannon; and Digesta microbial diversity: **(G)** richness, **(H)** Faiths PD, and **(I)** Shannon. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$).

colonization in marine fish over time. Fish were sampled at three age points including 43, 137, and 430 dph. At 430 dph, fish were either collected from an offshore sea pen (n = 20) or from the indoor environment. The indoor fish at 430 dph had been in the sea pen but were transferred back to the indoor environment to be used as broodstock (n = 12). These fish were in the indoor tanks for 79 days before sampling. Fish from 43 to 137 dph were always reared in indoor systems. At each

body site: gill (**Figures 2A–C**), skin (**Figures 2D–F**), and digesta (**Figures 2G–I**), microbial diversity was compared across fish ages. Additionally, fish from 430 dph were separated by either indoor or ocean net pen. When comparing richness measures, all three body sites were influenced by age with the gill ($P < 0.0001$, KW = 31.85, **Figure 2A**) being most influenced followed by digesta ($P < 0.0001$, KW = 24.88, **Figure 2G**) and then skin ($P = 0.0435$, KW = 8.127, **Figure 2D**). A similar pattern was observed for Faith's PD, which takes into account microbial phylogenetic diversity with all three body sites being influenced by age. The gill was most influenced ($P < 0.0001$, KW = 28.8, **Figure 2B**) followed by digesta ($P = 0.0002$, KW = 20.22, **Figure 2H**) and lastly skin ($P = 0.0038$, KW = 13.4, **Figure 2E**). Shannon diversity had the same pattern with gill ($P < 0.0001$, KW = 31.63, **Figure 2C**), digesta ($P < 0.0001$, KW = 27.91, **Figure 2I**), and skin ($P = 0.0015$, KW = 15.47, **Figure 2F**) all being influenced by fish age in the same order of impact. When comparing only samples at 430 dph, gill diversity (richness, Faith's PD, and Shannon evenness) was larger for fish which were transferred from the ocean net pen back into the indoor environment as compared to ocean net pen reared fish. This effect was also seen in the skin, but to a much smaller degree.

To model age and microbial diversity across the body sites, we performed a regression and Spearman correlation for each diversity measure. For this analysis, we excluded ocean net pen reared fish from 430 dph to compare only indoor fish (**Figure 3**). For richness, both gill and skin samples were positively associated with fish age while digesta samples were negatively associated with fish age (**Figure 3A**). For Faith's PD, both gill and skin again were positively associated with fish age (**Figure 3B**). Lastly for Shannon diversity, skin was positively associated with fish age while digesta was negatively associated with fish age (**Figure 3C**). These cumulative results suggest a general mechanism for alpha diversity changes in the marine fish YTK, *S. lalandi*, whereby alpha diversity may continue to increase over time in the gill and skin surfaces while digesta samples start highly diverse but then adapt or reduce in complexity over time.

Microbial Compositional Drivers Across Age and Rearing Condition

Next we wanted to understand how the composition of microbial diversity changed over time (age) and to also determine if there was evidence for succession. To determine if age was associated with microbial niche differentiation across body sites, we compared the fish body site microbiome independently at each of the four ages or conditions including 43 dph (**Supplementary Figures 3a,b**), 137 dph (**Supplementary Figures 3c,d**), 430 dph "indoor tank" (**Supplementary Figures 3e,f**), and 430 dph "seapen" (**Supplementary Figures 3g,h**). Body sites at each age group, even as early as 43 dph, had unique microbial communities measured using Unweighted and Weighted normalized Unifrac distance metrics (**Supplementary Table 2a**). For Weighted normalized Unifrac, based on the *F*-statistic, body site microbial communities were most differentiated at 430 dph, especially in the open sea pens. This result suggests that body site

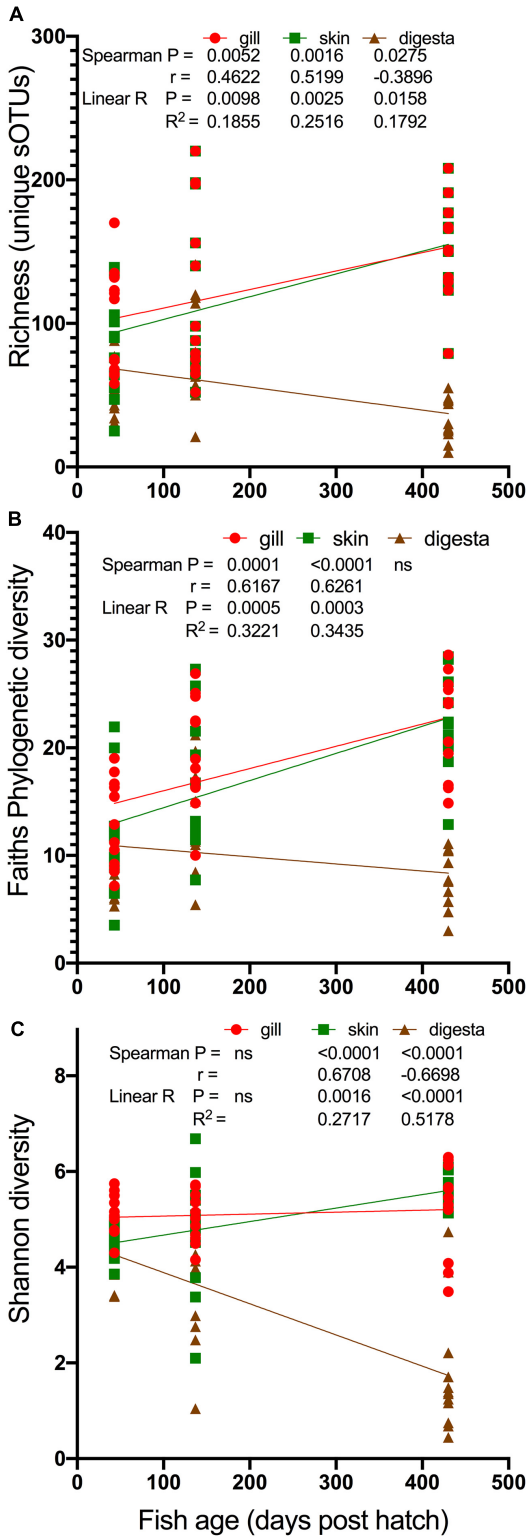

FIGURE 3 | Modeling of changes in alpha diversity: **(A)** richness, **(B)** Faiths PD, and **(C)** Shannon diversity over the age of the fish. Only fish reared in indoor systems included (430 dph seapen fish excluded). Statistical comparisons of both Spearman correlation and linear model (linear regression) calculated with results depicted on the legends.

microbial communities continue to differentiate throughout the lifetime of the fish.

We then sought to answer the question if certain body sites are more influenced by age. To do this, we compared microbiome differences of age and tank type within each body site independently (**Supplementary Figures 3i–n** and **Supplementary Table 2b**). For both Unweighted and Weighted normalized Unifrac distance comparisons, the gill microbiome samples were more differentiated across ages as compared to the skin and digesta (*F*-statistic). Furthermore, when observing the gill samples, the 430 dph fish reared in the indoor tank and ocean net pen were divergent on the PCoA (**Supplementary Figures 3i,j**). In addition, fish at 43 dph were also differentiated.

Next, we evaluated if overall fish mucosal microbiome similarity to the BE changed with age and if it did, which BE or water sample types were most influential (e.g., potential source reservoirs for fish microbiome colonization). For indoor reared fish at 43, 137, and 430 dph, we compared the microbiome of the gill, skin, and gut to various hatchery components including tank side, water from the tank, the inlet pipe into the tank, air stones, air diffusers, and feed. For feed, we evaluated 12 different feed types that were used throughout the production schedule ranging from days 1–12 (first feed) until harvest. The first feed type (live rotifers) consistently had a more similar microbial community to the gill, skin, and digesta samples across the different ages (**Supplementary Figure 4**) thus we used these samples (unenriched and enriched rotifers) for the feed comparison in the broader BE comparison. When including all possible BE sample types, a noticeable trend emerged where at the earliest age (43 dph), the microbial communities across all body sites were generally more similar to the BE (**Figures 4A–C**). Whereas at later ages, the microbiome of the gill and skin communities generally become more dissimilar from the inlet pipe and feeds, but became more similar to the air diffuser. The digesta samples (**Figure 4C**), however, consistently became more differentiated from the BE samples over time suggesting a stronger niche differentiation in the gut. To quantify this, we included only BE sample comparisons which were consistent in all ages – water, inlet pipe, and first feeds – and compared how the mucosal microbiomes of the fish disperse or converge toward the BE. For both gill and skin samples, the total differentiation of fish mucosal site to the three BE samples was least at 43 dph but increased with age (**Figures 4D,E**). The gill and skin samples were both more similar to the inlet pipe at 43 dph and became more divergent from the inlet pipe over time (137 and 430 dph). Digesta samples became more differentiated from all BE surfaces equally over time (**Figure 4F**). To estimate the total impact of these differences, we calculated the effect size (**Figure 4G**). For the gill, the dissimilarity differences across the BE samples explained 34.5% of the variation at 43 dph but then increased to 68.8% of the variation explained at 137 dph. For the skin, the largest jump in effect size occurred between 137 dph (25.6%) and 430 dph (61.5%; **Figure 4G**). These results indicate that niche differentiation occurs at varying rates depending on body site and that some BE microbial sources continue to have an influence on the fish mucosal microbiome throughout the lifespan of the fish, whereas other environmental sources may only be influential during early ontogeny.

Determining Which Built Environment Surfaces Contribute to Fish Microbiome

To identify the extent by which the BE contributes to the mucosal microbiome of the fish, we applied the popular microbial source tracking program SourceTracker2 which uses Bayesian statistics to estimate contributions of features from various sources to sink communities. SourceTrackr2 determined that contributions of the BE varied widely depending on both the body site and the age of the fish. At 43 dph, the tank side biofilm and air stones were the biggest sources of microbes to the gill and skin of the fish larvae, while the majority of microbes in digesta samples were from unknown or unsampled sources (**Figure 5A**). Rotifer feeds also contributed to the gill, skin, and gut microbiomes, but to a lesser extent compared to airstone and tank side (**Figure 5A**). At 137 dph, gill was again influenced by the airstone and air diffusers in the BE, while higher frequencies of skin and digesta samples were colonized by microbes from feeds (**Figure 5B**). However, microbes from unknown sources had the largest overall contribution at 137 dph across all body sites (**Figure 5B**). For 430 dph fish transferred from the ocean net pen back to the land based facility, both air diffusers and the water column were the largest microbial sources to the gill and skin microbiomes (**Figure 5C**). For the 430 dph net pen reared fish, gill, and skin samples were primarily colonized by microbes from unknown sources followed by small proportions from air diffusers, airstones, and water from pre-transfer. Common planktonic marine microbes from sea water and netpen biofouling were not collected in this study and thus is likely a meaningful "source" which would fall into "unknown sources" in this study. Interestingly, digesta samples for both 430 dph seapen and 430 dph indoor fish were primarily colonized from water samples from the 137 dph (**Figures 5C,D**). This would suggest that the water community which fish are exposed to prior to transfer to ocean net pen (at around 137 dph) is very important to the gut microbiome colonization and that these microbes remain in the gut even after long term growout in seapens. The finding that the microbiome of the fish digesta originates primarily from water sources rather than feed sources is intriguing. It is important to note, however, that the feeds used in this study were normal extruded pellet feeds with no added probiotics. Results from the Sourcetracker2 analysis reinforce and support the observations from the beta diversity comparisons, that fish mucosal sites are influenced uniquely by the BE which also show succession patterns as a function of age.

430 dph Seapen vs. Indoor

One of the primary questions in this dataset is understanding how the surrounding environment influences mucosal microbiomes. Specifically, we were interested in understanding the specificity and stability of these microbial communities as a function of ontongeny. To compare fish of the same age (430 dph) and genetic cohort, we sampled fish which were being reared in ocean net pens along with fish which had been in seapens but

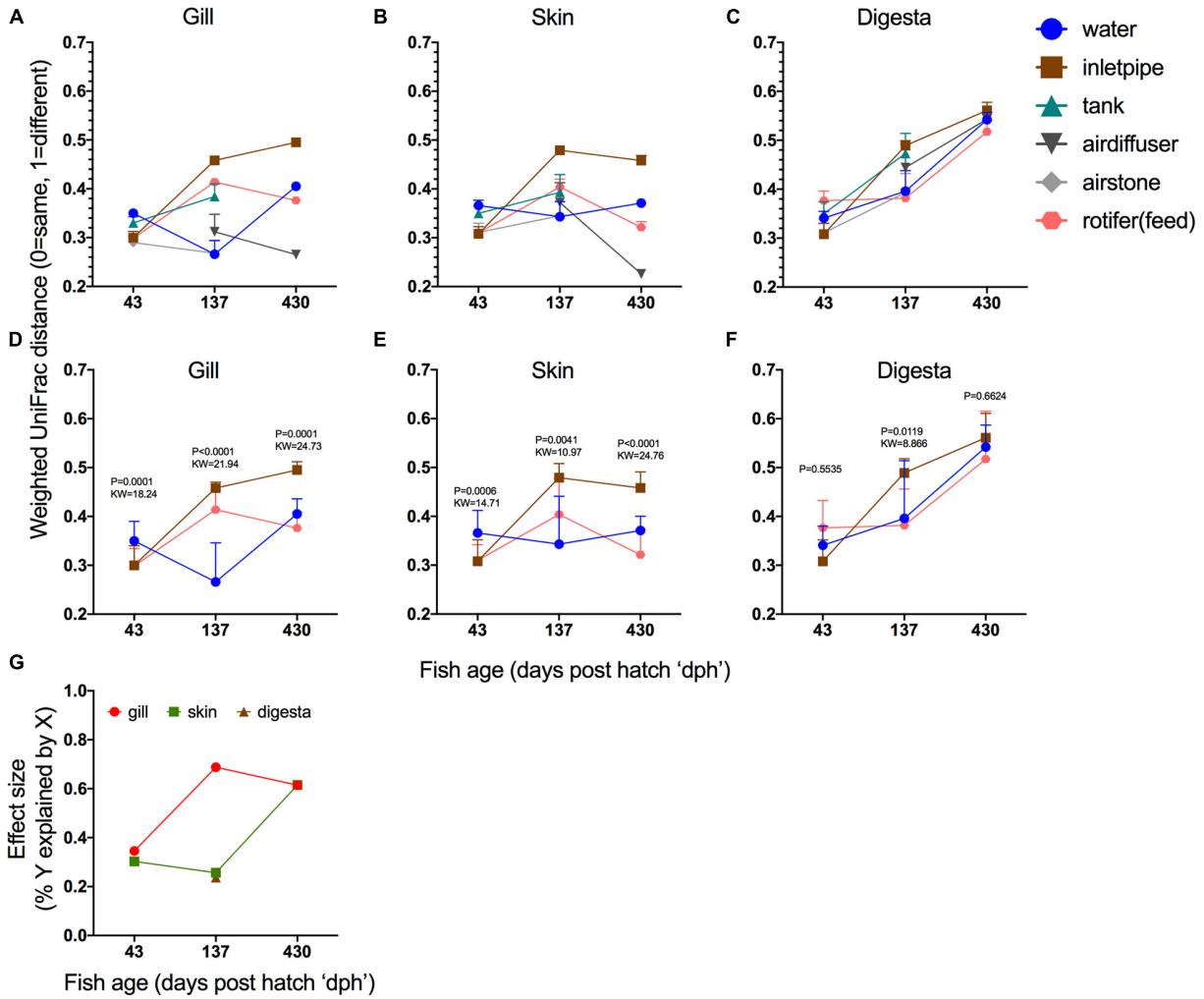

FIGURE 4 | Niche differentiation within body sites over time. Beta diversity distances (Weighted normalized UniFrac) of **(A)** gill, **(B)** skin, and **(C)** digesta samples compared to six different hatchery built environment putative microbial sources [water, inlet pipe, tank side, air diffuser, airstone, and first feed (rotifers)]. Statistical comparison of microbiome differentiation across three BE comparisons (water, inlet pipe, and first feed) over time and calculated independently across three body sites: **(D)** gill, **(E)** skin, and **(F)** digesta (Statistical test: Kruskal–Wallis, *P* value and KW test statistic reported in figure panel. **(G)** Results from the Kruskal–Wallis test for (d,e,f) depicted as effect size to demonstrate the rate of microbial community niche differentiation.

were brought back to the indoor facility. Digesta samples were previously shown to have large decreases in alpha diversity at 430 dph particularly when comparing the fish in the seapen vs the indoor fish. Interestingly, much of this microbial diversity loss can be attributed to a single uncultured representative ASV, from the family Mycoplasmataceae (phylum Tenericutes, class Mollicutes), which becomes more dominant in the fish gut with age especially in the outdoor seapen. This ASV was observed in 100% of the 430 dph fish yet was found in only 75% of the 43 dph and 137 dph fish, while less frequently observed in the BE (**Supplementary Figure 5a**). At 430 dph this ASV made up a large proportion of total reads in the seapen (mean = 0.71) and FT indoor tank (0.60) fish but significantly less abundant in younger fish at 137 dph (0.14) and 43 dph (0.02; **Supplementary Figure 5b**). Thus, although the Mycoplasmataceae is present in younger fish, the proportion of reads is much smaller. Since these are proportions, it's important to realize that this does not implicate a biomass change, but only representation in comparison to total microbial diversity.

DISCUSSION

Seafood is an important source of protein globally which has led to the steady positive growth in aquaculture over the past 30 years. Marine finfish production has tremendous opportunity for growth (Gentry et al., 2017) yet challenges and concerns have arisen over the sustainability of such practices (Bush and Oosterveer, 2019). One of the primary concerns is animal welfare and preventing disease transmission from farmed fish to wild stocks (Bush and Oosterveer, 2019; Weitzman et al., 2019). A potential solution to antibiotic overuse in agriculture

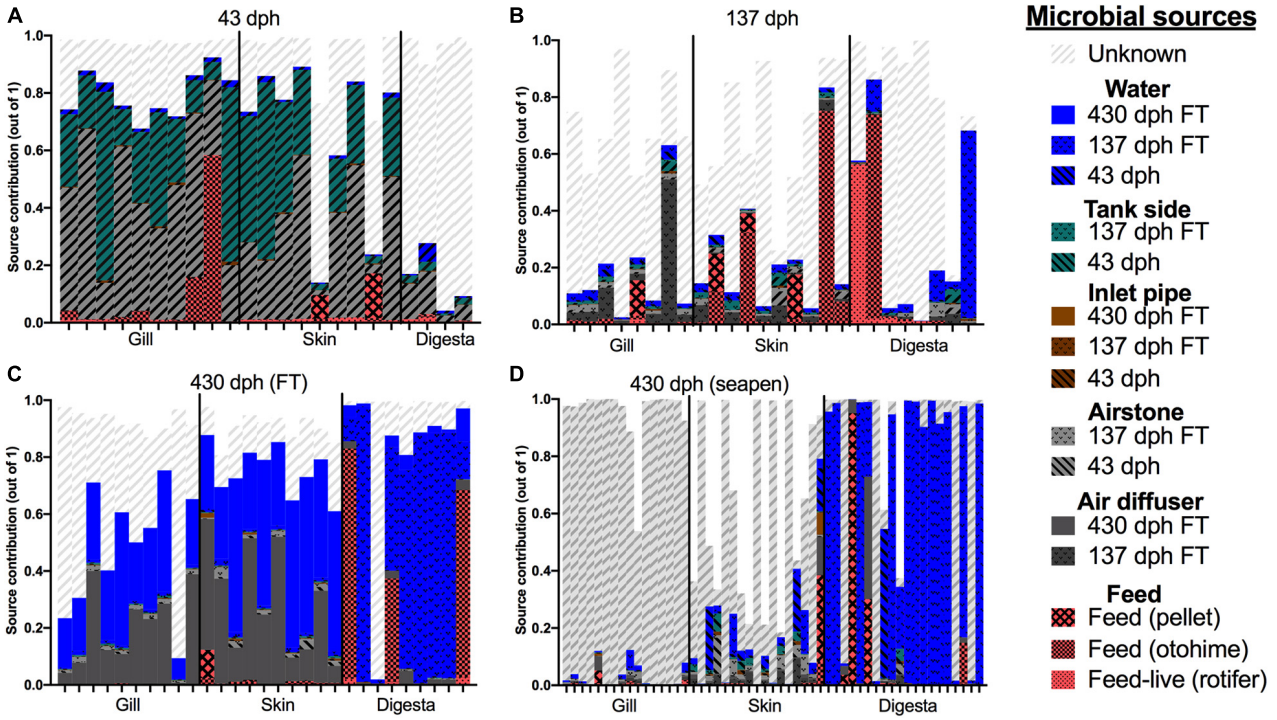

FIGURE 5 | SourceTracker2 analysis of individual microbiome contributions from the built environment onto various mucosal body sites across time: **(A)** 43 dph, **(B)** 137 dph, **(C)** 430 dph indoor, and **(D)** 430 dph seapen. Features with less than 100 counts across all samples excluded. "Unknown" indicates source population was not sampled or included thus would be the percentage of a given sample which has source microbes from an unknown location or undetermined source.

is the promotion of probiotics. The mucosal microbiome is an important component of fish health as microbes colonizing the gill, skin, and gastrointestinal tract can either be a source of infection or inversely, protect the animal from infection by inhibiting the colonization of pathogens, producing antimicrobial compounds, or eliciting an immune response (Gomez et al., 2013). Our research sought to evaluate how the mucosal microbiome develops and to estimate its stability in different body sites over time in the economically important cultured marine fish *S. lalandi*. We describe the potential sources of microbes from the "BE" (hatchery surfaces) that drive these changes across three unique body sites including the gill, skin, and digesta communities. Previous fish microbiome studies have focused primarily on one body site at a time, particularly the gut, while our approach aimed to more fully describe diversity dynamics across multiple mucosal body sites.

Gill microbiomes were the most sensitive to changes in the indoor and outdoor culture environment followed by skin with digesta demonstrating a more deterministic or enriched microbiome with ontogenic development. Specifically, while both gill and skin microbial communities increased in diversity with age, the digesta decreased. The progression of decreasing microbial diversity in the fish gut samples suggest that the gut environment is more deterministic rather than stochastic in microbial community composition. Conversely, the gill and skin generally increase in diversity with age which could be due to additive exposure and increased surface area over time. In addition to variable exposure to the external environment, individual body sites maintain unique physical and chemical properties that confer selection for specific microbial groups. Neutral (stochastic) theory ascribes that biodiversity formation and change over time occurs from random dispersal and exposure events and while it is largely conceptualized in macrofauna and flora (Hubbell, 2011), it can also be applied to microbial communities (Sloan et al., 2006). In contrast, a niche-based (deterministic) model describes how select species evolve and adapt to certain conditions as the result of interspecies interactions and niche differentiation. In this study, we demonstrate that while the gill and skin do have unique microbial communities, the processes for microbial colonization are largely stochastic whereas the gut environment demonstrates a more deterministic process for microbial colonization. In adult Atlantic salmon sampled from marine net pens, gut microbial diversity decreased as the age of fish was increased while the presence of most individual gut microbes were random and only a few deterministic, which was primarily driven by Mycoplasma (Heys et al., 2020). In zebrafish (Burns et al., 2016) and sturgeon (Abdul Razak and Scribner, 2020), both freshwater fish, higher proportions of gut microbes were non-neutral or deterministic as fish matured (older age). In catfish skin microbiomes, geographic location drove community composition with most microbes being neutral (Chiarello et al., 2019).

The implications of different body sites demonstrating a more neutral or deterministic microbiome is important for

understanding both the impact of environmental change on wild fish stocks as well as improving aquaculture production. Negative anthropogenic impacts to the marine environment include contaminant and nutrient pollution which can cause disturbances of primary productivity. In a wild marine fish, the Pacific chub mackerel, the composition of external mucosal microbiomes of gill and skin were most influenced by temporal changes, coinciding with temperature, along with gill alpha diversity positively correlated with age (Minich et al., 2020a). The gill is an important organ for excretion of nitrogenous waste (Sayer and Davenport, 1987; Wilkie, 2002) and gas exchange which is critical for highly active swimming fish like *Seriola* spp. (Yamamoto et al., 1981; Roberts and Rowell, 1988). In aquaculture settings, microbes which produce compounds causing off-flavor in flesh (Auffret et al., 2013) have been found to be enriched and primarily taken up through the gills of fish (From and Hørlyck, 1984; Klausen et al., 2005). Since the gill is a critical component of maintaining homeostasis, and in this study appears most susceptible to changing environmental conditions, further research is needed to understand how changes in the microbiome may negatively or positively impact fish physiology. Additionally, skin is an important physical barrier for disease prevention. The skin microbiomes of two coastal pelagic marine fish, *Scomber japonicus* and *S. lalandi*, were strongly influenced by increased temperature that coincided with increased proportions of a potential marine pathogen, *Photobacterium* spp. (Horlick et al., 2020; Minich et al., 2020a).

Body site microbiomes of *S. lalandi* were most similar to the BE surfaces at the earliest age (43 dph). As fish aged, digesta samples diverged from all BE surfaces, while gill and skin were differentially influenced by specific BE surfaces. In Atlantic salmon reared in freshwater indoor hatcheries, microbial diversity from both the tank side and water column were highly correlated with the fish skin and gut, but not other BE surfaces (Minich et al., 2020b). Understanding which surfaces likely contributed to the various body sites over time was calculated using SourceTracker2 analysis. At 43 dph, the biofilm from the tank side along with the aeration equipment (airstones) were the largest contributors to the gill and skin communities whereas much of the digesta microbes were from unknown sources. Aeration equipment in tilapia culture has been implicated as a source of *Acinetobacter* in culture systems (Grande Burgos et al., 2018). While feed had a marginal impact on the microbial community of the various fish body sites, it was not consistent and was generally lower than the surrounding BE surfaces. Although diet has been shown to have a strong influence on gut microbiome development (Nayak, 2010; Tarnecki et al., 2017), the importance of live feeds as contributors to the gut microbiome is debated (Ringø, 1999; Bakke et al., 2013). One explanation is that the microbes colonizing the live feeds have low specificity for successful colonization of the fish gut. Likewise, since the overall exposure to and density of BE surfaces and associated microbes, including the water, is much greater than that of the live feeds (Walburn et al., 2019), feed-associated taxa may be outcompeted in the gut environment.

A unique opportunity of this study was to compare mature fish (430 dph) from an ocean net pen to fish that had been in the ocean but were transported back to an indoor system to be used as broodstock. We are not aware of any other study which has looked at the microbiome transition from ocean to indoor in a marine fish. Selective breeding programs rely on the ability to develop broodstock which are used to maintain genetic lines from previous grow out populations (Symonds et al., 2014). Ocean net pen fish generally had lower microbial diversity than indoor reared fish for all fish body sites, but was most pronounced in the gill. This further suggests that the mucosal, even in adult fish that are least susceptible to BE impacts, has a high capacity to change which is critical when considering time scales for probiotic effects (Vadstein et al., 2018; Dawood et al., 2019; Ramírez et al., 2019). Probiotic treatments in fish are common but little is known about dosage for a given treatment along with frequency of administration for having a lasting effect. If the normal microbial community of a fish gill or skin can change rapidly, this would suggest that a sustained administration rather than a "one-time treatment" would be required for maintaining mucosal health in fish. For gill and skin communities, the water column and aeration surfaces contributed the most for indoor reared fish while fish reared in the net pens had many bacteria of unknown sources, presumably from the ocean, e.g., seawater. Digesta samples, however, were primarily colonized by hatchery water associated microbiota and to a lesser extent feeds. The opposite explanation is also true that in land-based systems, fish feces could be contributing more to the water column microbiome as compared to the oceanic conditions where feces is more quickly exported out of the system. These vast differences and the speed at which microbiomes develop and change is a plausible explanation for differences between wild and farmed *Seriola* (Ramírez and Romero, 2017). The most abundant microbe in the *Seriola* digesta was an unresolved Mycoplasmataceae which was strongly associated with transfer of fish from indoor rearing systems to the ocean net pen. Mycoplasma are important gut microbes which can colonize the gut very early in development. Several plausible explanations exist for this observation. First, it is possible that in land-based systems, fish are simply not as heavily exposed to Mycoplasma. Second, it is possible that Mycoplasma microbial density or diversity is higher in ocean net pen systems compared to the indoor system thus allowing the Mycoplasma to dominate the gut microbiome. Lastly, an alternative explanation is that Mycoplasma outcompetes other microbes in the fish gut especially as the fish increase in age. However, since the data are compositional, it is not possible to determine absolute microbial densities thus requiring additional experimentation to resolve. All mucosal environments were influenced by the BE over time with the strongest effects at early fish development. Digesta samples in particular became less influenced by the BE over time and demonstrated a strong selective or deterministic pressure on microbiome development with increasing age. This progression of decreasing microbial diversity in the fish gut suggests that the gut environment is more deterministic rather than stochastic in microbial community composition whereas the gill and skin generally increase in diversity with age which could be due to additive exposure.

One of the limitations of this study is that we did not perform quantitative measures of the microbial communities. Part of

the reason for this is that these methods can often involve invasive or destructive sampling of tissues. Since we largely utilize non-invasive sampling techniques, at least for the fish samples, performing quantitative measures is a challenge. Nonetheless, future studies should focus on developing non-invasive methods for accessing the quantitative measures of microbial quantities in both the BE and the fish mucous.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Data is publicly available on European Nucleotide Archive (EBI: ERP120036) and through Qiita (Study ID: 12227 and Analysis ID 25157).

ETHICS STATEMENT

The animal study was reviewed and approved by Port Stephens DPI: From the hatcheries, the microbiome was passively sampled from fish while undergoing routine biometric measurements (length and weight). For sea cage reared fish, the microbiome was sampled from euthanized fish grown in the netpen during routine health checks (as part of another study).

AUTHOR CONTRIBUTIONS

JM, BN, AE, SF, and EA contributed to the conception and design of the study. JM collected microbiome samples in the field. JM performed DNA extractions at USC in the lab of AE followed by microbiome processing and data analysis at UCSD. RK helped with mentorship on data analysis and provided feedback for microbiome analyses. JM wrote the first draft of the manuscript. All authors contributed to the manuscript revision, and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.676731/full#supplementary-material

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Dietary Lipid Effects on Gut Microbiota of First Feeding Atlantic Salmon (*Salmo salar* L.)

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Decline in fish oil and fish meal availability has forced the aquaculture sector to investigate alternative and sustainable aquafeed ingredients. Despite that several studies have evaluated the effect of fish oil replacement in aquaculture fish species, there is a knowledge gap on the effects of alternative dietary lipid sources on the gut microbiota in early life stages of *Salmo salar*. The present study evaluated the influence of dietary administration of two different lipid sources (fish oil and vegetable oil) on the intestinal microbiota of first feeding Atlantic salmon (*S. salar*) up to 93 days post first feeding (dpff). The two diets used in this study, FD (fish oil diet) and VD (blend of rapeseed, linseed and palm oils diet), were formulated to cover the fish nutritional requirements. Apart from the lipid source, the rest of the feed components were identical in the two diets. Hindgut samples were collected at 0, 35, 65, and 93 dpff. Moreover, fertilized eggs, yolk sac larvae, rearing water and feed were also collected in order to assess a possible contribution of their microbiota to the colonization and bacterial succession of the fish intestines. To analyze the bacterial communities, amplicon sequencing was used targeting the V3–V4 region of the 16S rRNA gene. The findings indicate that feeding on either fish oil or vegetable oil-based diet, fish growth variables (mean wet weight and total length) did not differ significantly during the experiment ($p > 0.05$). No significant differences were also found between the two dietary groups, regarding their gut bacteria composition, after the analysis of the 16S rRNA sequencing data. Instead, gut microbiota changed with age, and each stage was characterized by different dominant bacteria. These operational taxonomic units (OTUs) were related to species that provide different functions and have been isolated from a variety of environments. The results also show little OTUs overlap between the host and rearing environment microbiota. Overall, this study revealed the occurrence of a core microbiota in early life of Atlantic salmon independent of the feed-contained oil origin.

Keywords: *Salmo salar*, larvae, gut microbiota, dietary intervention, fish oil replacement

INTRODUCTION

Fishmeal and fish oil have been the main ingredients in diets for farmed carnivorous fish species, providing the fed fish with the necessary proteins and lipids for high growth performance and resulting in a nutritionally rich final product (International Fishmeal and Oil Manufacturers Association [IFOMA], 2001; Turchini et al., 2010). Due to the declining availability of fishmeal and fish oil, their contents in feed are reduced (Hardy, 2010) and substituted by a variety of alternative feed ingredients. As changes in fish diet ingredients can alter the gut microbiota of fish species, it is important to evaluate the impact of these new diets with lower fish-meal and -oil contents on the composition of the gut microbial communities for reared fish species (for a review see Ringø et al., 2016).

In Atlantic salmon (*S. salar*), a carnivorous fish with significant economic value in European aquaculture (FAO, 2004), the effect of the alternative aquafeed ingredients on the gut microbiota have been evaluated previously and, in some cases, it was revealed that changes associated with intestinal disorders and slower growth performance, were related to the fishmeal diets (e.g., Green et al., 2013; Navarrete et al., 2013; Schmidt et al., 2016; Gajardo et al., 2017; Booman et al., 2018; Egerton et al., 2020). These studies, however, have focused mainly on alternative protein sources and on juveniles and adult stages.

Although feed is considered as the main factor that affect the gut bacterial communities in fish species, data from previous studies have also shown variations in gut microbial communities across development stages which seem to be affected not only by the provided feed but also from the microbial communities of the rearing environment (Bakke et al., 2013, 2015; Stephens et al., 2016; Dehler et al., 2017; Egerton et al., 2018). For example, recent work by Minich et al. (2020) recognizes the strong association between the build environment, i.e., tank biofilm and water from the hatchery installation, and Atlantic salmon mucosal microbiota. In a different salmonid species (rainbow trout), gut microbiota was detectable before first feeding commenced, potentially due to contact with the surrounding water and yok sac digestion, indicating that gut microbiota establishment initiates at first feeding and that diet-type affect the bacterial composition (Ingerslev et al., 2014a,b).

Moreover, it has been reported, that fish egg fragments are consumed from the newly hatched larvae, and their microbiome can affect gut microbiota colonization in fish species (Olafsen, 1984; Beveridge et al., 1991; Nikouli et al., 2019). The significant stage of the mouth opening, in aspect of larval microbiota manipulation, have been also recognized in shrimp larvae by Wang et al. (2020) in aquaculture conditions. In addition, evidence also suggest that as early life stages are more prone in environmental/climate changes, then probably is more crucial to study the microbiome shaping on these stages (Lowe et al., 2021). On the other hand, studies have shown that host development considered to had greater effect than hatching environment on the gut microbiota colonization and succession (Califano et al., 2017; Nikouli et al., 2019; Xiao et al., 2021).

Apart from a few studies, which have investigated the gut microbial communities in early life stages of Atlantic salmon (Llewellyn et al., 2016; Dehler et al., 2017; Lokesh et al., 2019), there is a knowledge gap on the effects of a different dietary lipid source on the gut microbiota in early life stages of this fish species, as only Clarkson et al. (2017) have partially investigated the impact of fish oil replacement by vegetable oils during a dietary experiment in diploid and triploid populations of Atlantic salmon. The objective of the present study was to evaluate the influence of total replacement of fish oil with a blend of terrestrial alternative oils (rapeseed, linseed and palm oils) on the intestinal microbiota of first feeding Atlantic salmon. We also characterized the bacterial communities of the rearing environment to determine their contribution in the early colonization and the succession of the fish intestines.

MATERIALS AND METHODS

Experimental Design and Sampling

The study was carried out within the Norwegian animal welfare act guidelines, in accordance with EU regulation (EC Directive 2010/63/EU), approved by the Animal Ethics and Welfare Committee of the Norwegian University of Science and Technology (case number 16/10070). The experiment was conducted at the Ervik hatchery (Frøya, Norway) as described previously in Jin et al. (2019). Briefly, a fast-growing Atlantic salmon aquaculture strain was cultivated from fertilized eggs until 93 days post first feeding (dpff). The two diets used in this study, FD (fish oil diet) and VD (blend of rapeseed, linseed and palm oils diet), were formulated to cover the fish nutritional requirements. Apart from the lipid source, the rest of the feed components were identical in the two diets (see **Supplementary Table 1**).

Each dietary treatment was tested in duplicated groups of 200 Atlantic salmon individuals (0.23 g $\pm$ 0.03/fish). On sampling days (0, 35, 65 and 93 dpff) 10 fish from each tank were randomly collected and sacrificed by immersion in 40 mg/L Benzocaine (BENZOAK VET, ACD Pharmaceuticals AS, Oslo, Norway). Furthermore, duplicate samples of rearing water (100 ml/tank) were collected and filtered through 0.2 μm membrane filters (GTTP, Millipore, United States) using a low (<1,500 mmHg) vacuum apparatus. For gut microbiota analysis, hindguts were removed by aseptic dissection and rinsed with ultra-pure water. Moreover, 10 fertilized eggs (EG), 10 yolk sac larvae (YS), and 0.25 g of the provided feeds were sampled in order to assess the contribution of their microbiota on the colonization of Atlantic salmon gut.

DNA Extraction and Sequencing

DNA was isolated from Atlantic salmon (eggs/yolk sac larvae/hindguts) and environmental (water/diets) samples by using the QIAGEN QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol "DNA Purification from Tissues." Bacterial communities were characterized by 16S rRNA amplicon sequencing. All samples analyzed individually and pooled prior the 16S rRNA analysis as follow: (a) DNA extracts from 5 individual fish samples (eggs, yolk sac larvae and hindguts) were pooled, resulting in two

pooled samples from each time point/fish tank (**Supplementary Table 2**) and (b) the DNA from the rearing water samples were pooled, resulting in 1 water sample per replicate tank ("STW"—initial stock tank, "FW"—rearing water from fish oil (FD) group and "VW"—rearing water from vegetable oil (VD) treatment.

PCR amplification and sequencing was performed at MRDNA Ltd.[1] (Shallowater, TX, United States) facilities on a MiSeq using paired end reads (2 × 300 bp) following the manufacturer's guidelines. A total of 37 samples (representing 30 pooled fish samples, 5 pooled water samples and 2 feed samples) was used in the final amplicon library. The 16S rRNA gene V3-V4 variable region PCR primers S-D-Bact-0341-b-S-17 and S-D-Bact-115 0785-a-A-21 (Klindworth et al., 2013) with barcodes on the forward primer (**Supplementary Table 3**) were used in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, United States) under the following conditions: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 53°C for 40 s, and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. After that, the samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. Then the pooled and purified PCR product was used to prepare illumina DNA library.

Data Analysis

Sequencing raw data were processed with the MOTHUR software (version 1.40.5) (Schloss et al., 2009, 2011) and the operational taxonomic units (OTUs) were classified with the SILVA database release 132 (Quast et al., 2013; Yilmaz et al., 2014) following the methodology described in Nikouli et al. (2018). Identification of closest relative of the Most abundant OTUs was performed with Nucleotide Blast[2]. Raw sequence data from this study have been submitted to the Sequence Read Archive[3] with BioProject accession number PRJNA520982. Statistical analysis and graphical illustrations were performed in the Palaeontological STudies (PAST) software (Hammer et al., 2001) and in the R Studio platform Version 1.1.419 (RStudio Team, 2020), with 3.4.3 R version and enveomics.R package, Version 1.2.0 (Rodriguez-R and Konstantinidis, 2016).

RESULTS

Fish Growth Performance

The growth performance of the fish was evaluated throughout the experiment (see **Supplementary Table 4** and **Supplementary Figure 1**) and at none of the sampling points the mean wet weight or total length differed significantly across replicate tanks or between dietary treatments (FD and VD) ($p > 0.05$; **Supplementary Figure 1**). The initial (D0) mean weight was 0.23 ± 0.03 g (±SD). After 93 days (D93) the final mean weight was 4.58 ± 1.74 g for the group FD and 4.54 ± 1.78 g for the group VD. Regarding total mean length, the initial (D0) was 29.9 ± 1.6 cm which increased to 76.0 ± 8.9 cm and 73.8 ± 9.2 cm at D93 for the groups FD and VD, respectively.

Bacterial Diversity

The analysis of the 16S rRNA sequencing data revealed a total of 4,548 unique OTUs, with the rarefaction curves (**Supplementary Figure 2**) and the OTUs richness coverage based on the Chao1 index (**Supplementary Table 5**) indicating satisfactory sequencing depth. Diversity was considerable higher for rearing water (STW, FW, VW) than gut and diet samples, both in terms of OTU richness (**Table 1**) and evenness (**Supplementary Table 5**).

Taxonomic classification showed the presence of 21 bacterial phyla (**Figure 1** and **Supplementary Figure 3**). OTUs which were not classified to known bacterial phyla were only 3.0% of the relative abundance and are assigned as "Bacteria_unclassified." Proteobacteria (37.2%), Firmicutes (23.9%), Actinobacteria (18.8%), and Bacteroidetes (12.8%) were the dominant bacterial phyla in the dataset. The remaining 18 phyla (Planctomycetes, Verrucomicrobia, Patescibacteria, Dependentiae, Acidobacteria, Gemmatimonadetes, Fusobacteria, Cyanobacteria, Deinococcus-Thermus, Fibrobacteres, Armatimonadetes, Nitrospirae, Spirochaetes, Elusimicrobia, Omnitrophicaeota, Tenericutes, Chloroflexi, and Kiritimatiellaeota) were present with relative abundance ≤ 2%.

S. salar Microbiota

Comparing Atlantic salmon microbiota between the different life stages, fertilized eggs (EG) had the highest observed and estimated (Chao1) OTU richness (172 ± 100 and 222 ± 114, respectively). At the yolk sac stage (YS), the OTU richness decreased to 87 ± 0.7 and increased again at first feeding (D0). After that, OTU richness was on the same level until D93 when it decreased (**Table 1**). Proteobacteria was the dominant bacterial phylum in the samples, mainly due to γ- and β-Proteobacteria (**Supplementary Figure 4**). β-Proteobacteria was the dominant subphylum in pre-feeding stages (EG, YS, D0), with representatives mainly from the Burkholderiaceae and Chitinibacteraceae families (**Supplementary Figure 5**). However, in fertilized eggs (EG), OTUs representing β-Proteobacteriales were classified only at class level (44.1% of the total reads). γ-Proteobacteria dominated the period with active feeding (D35—D93) in both dietary treatments, with Pseudomonadaceae, Xanthomonadaceae, Vibrionaceae, Enterobacteriaceae, Moraxellaceae, and Aeromonadaceae as the most abundant families. However, their relative abundances differed between the two dietary treatments (**Supplementary Figure 6**). Actinobacteria, the dominant bacterial phylum at the late stages (D35 and D65) in vegetable oil dietary group (VD), was due to the high relative abundance of mainly Propionibacteriales, Corynebacteriales, and Micrococcales representatives. The presence of Firmicutes and Bacteroidetes was due to the classes Bacilli and Bacteroidia.

[1] www.mrdnalab.com

[2] https://blast.ncbi.nlm.nih.gov/Blast.cgi

[3] https://www.ncbi.nlm.nih.gov/sra/

TABLE 1 | Amplicon sequencing results of 16S rRNA gene diversity reported in all sample categories.

Samples	Reads	Observed OTUs richness	No. of the most dominant OTUs (cumulative relative dominance ≥ 80%)	Most abundant OTU (% of total reads) and closest relative (≥97%)
EG	22,151 ± 7168.6 N = 2	172 ± 99.7	16	SOTU0011 (23.9%)—*Methylotenera versatilis*
YS	14,382 ± 3186.2 N = 2	87 ± 0.7	10	SOTU0013 (19.4%)—*Delftia acidovorans*
D0	21,081 ± 1712.6 N = 2	132 ± 26.2	14	SOUT0009 (32.3%)—*Iodobacter fluviatilis*
D35F	7,658 ± 5011.0 N = 4	121 ± 64.8	46	SOTU0017 (9.3%)—*Pseudomonas viridiflava*
D65F	2,735 ± 1660.5 N = 4	110 ± 29.9	56	SOTU0070 (7.9%)—*Janthinobacterium agaricidamnosum*
D93F	2,003 ± 637.1 N = 4	93 ± 6.4	51	SOTU0005 (11.5%)—*Cloacibacterium normanense*
D35V	25,175 ± 27875.9 N = 4	135 ± 46.2	33	SOTU0005 (10.4%)—*Cloacibacterium normanense*
D65V	4,812 ± 1975.0 N = 3	132 ± 11.7	37	SOTU0005 (11.1%)—*Cloacibacterium normanense*
D93V	1,170 ± 608.3 N = 4	79 ± 25.3	46	SOTU0004 (7.0%)—*Weissella cibaria*
FD	21,022 N = 1	259	7	SOTU0004 (38.6%)—*Weissella cibaria*
VD	20,699 N = 1	216	8	SOTU0004 (37.8%)—*Weissella cibaria*
STW	53,280 N = 1	2,422	259	SOTU0001 (9.4%)—*Polynucleobacter necessaries*
FW	76,806 ± 11852.5 N = 2	1,683 ± 183.8	52	SOTU0001 (14.5%)—*Polynucleobacter necessaries*
VW	53,618 ± 8553.9 N = 2	1,100 ± 137.2	35	SOTU0001 (20.8%)—*Polynucleobacter necessaries*

Pre-feeding S. salar life stages: Eggs (EG), yolk sac larvae (YS) and "D0." Feeding stages of (a) fish oil group (FD): D35F, D65F, and D93F and (b) vegetable oil group (VD): D35V, D65V, and D93V. Rearing water: Pre-feeding tank (STW), fish oil group (FW) and vegetable oil group (VW). Feed: vegetable oil (VD) and fish oil feed (FD). N, Number of biological replicates analyzed; D, Day.

Microbial Communities in Diets and Rearing Water

The bacterial communities in feed samples, consisted almost entirely of Firmicutes (relative abundance of 84.2 and 82.1% in FD and VD, respectively; **Figure 1**). The Firmicutes were affiliated to the Lactobacillaceae (38.5 and 36.6% in FD and VD, respectively) and Leuconostocaceae families (37.9 and 38.8% in FD and VD, respectively). The rearing water samples (VW, FW, WST) contained mainly Proteobacteria, Actinobacteria and Bacteroidetes species, with Burkholderiaceae (β-Proteobacteria), Sporichthyaceae (Actinobacteria) and Chitinophagaceae (Bacteroidetes) as the most abundant families (**Figure 1**). In contrast to the experimental diets, Firmicutes in water samples were detected in relative abundance ≤ 1%.

Similarities Between Bacterial Communities

Statistical analysis revealed no significant differences (Tukey's test, $p > 0.05$, **Supplementary Table 6**) in the bacterial community composition of the Atlantic salmon samples between the pre-feeding stages (EG, YS, D0). However, EG and D0 samples differed significantly from those taken during the feeding period (D35—D93) in both dietary treatments, with stage D35 in VD group as the only exception. YS bacterial communities differed significantly ($p < 0.05$) with the bacterial communities only at D93 in both dietary groups (FD and VD). The gut microbiota of the host did not reveal significant differences between the two dietary groups for the different stages ($p > 0.05$), again with stage D35 in VD group as the only exception (**Supplementary Table 6**).

Further comparison of the bacterial community composition of Atlantic salmon hindguts, based on a Bray–Curtis distance matrix (**Figure 2**), showed a clear separation between bacterial communities in gut and bacterial communities of the rearing environment (water and diets). Moreover, the bacterial communities of the host were more similar with respect to life stages than to the diet treatments (**Figure 2** and **Supplementary Figure 7**), and this is also indicated through the similarity percentages analysis (SIMPER) based on Bray–Curtis distance. According to the results of the analysis the average dissimilarity among the groups of the same life stages was 76.0%, whereas the average dissimilarity within groups of the same dietary treatment was 78.5% (FD) and 83.6% (VD).

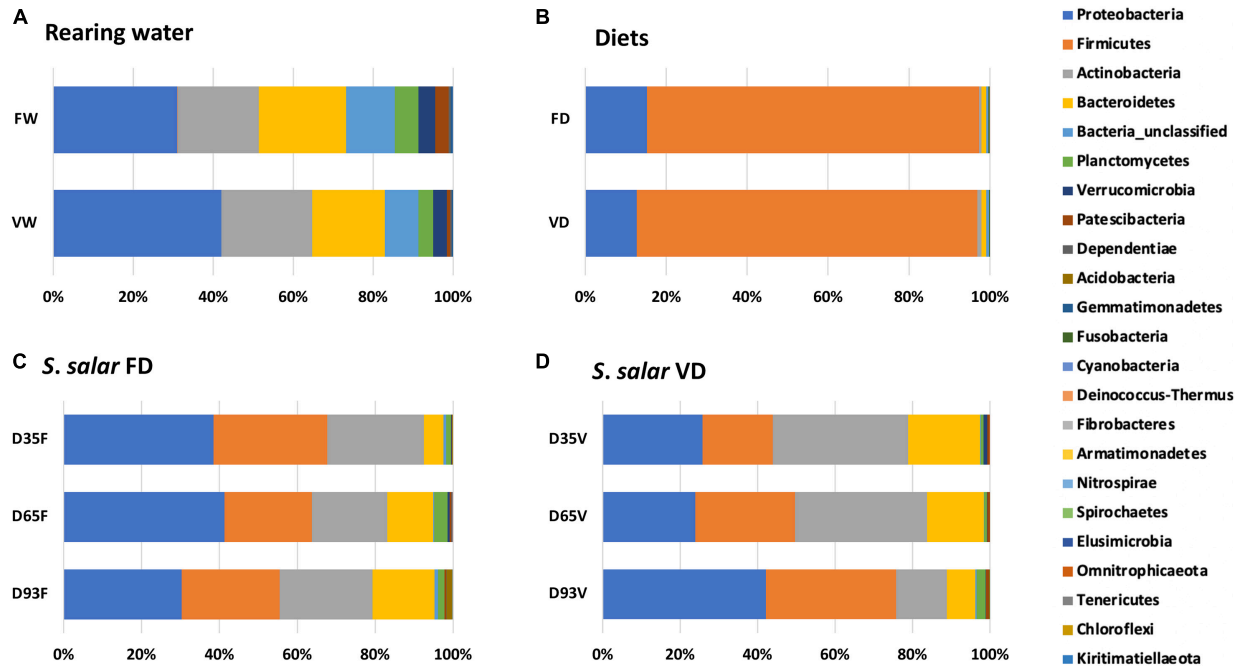

FIGURE 1 | Phylum composition of microbiota from rearing water **(A)**, diets **(B)** and hindgut of *S. salar* samples in Fish oil "FD" **(C)** and vegetable oil "VD" **(D)** dietary treatments, at D35, D65, and D93 post first feeding.

Common and Unique OTUs

Overall, only 2.3% of the OTUs were found in all sample types (rearing water, diets, pre- and after first feeding hindguts). 75.4% of OTUs occurred only in water samples (**Figure 3**). From the 1,004 OTUs detected in total in Atlantic salmon samples, 423 OTUs (9.3% of the OTUs) were unique in that type of samples. The majority of them (343 OTUs) were unique in the host at the active feeding stages, whereas 13 OTUs were shared among all samples independent of life stage or diet treatment.

DISCUSSION

In the present study, we evaluated the influence of dietary administration of two different lipid sources (fish oil and vegetable oil) on the gut bacterial communities of first feeding Atlantic salmon. Moreover, we characterized the bacterial communities from the rearing environment (rearing water and feeds) and the epibiotas of fertilized eggs and yolk sac larvae to determine their contribution in the bacterial colonization and succession of the gut. Previous studies suggest that the bacterial communities of the rearing environment, mainly from the rearing water and the feed, are important sources for community assembly of the intestinal microbiota of fish (Hansen and Olafsen, 1999; Nayak, 2010; McDonald et al., 2012; Scott et al., 2013; Bolnick et al., 2014; Eichmiller et al., 2016; Kashinskaya et al., 2018). For example, Schmidt et al. (2016), reported a significant effect on intestinal microbial communities in postsmolt Atlantic salmon following replacement of dietary fishmeal with plant ingredients. However, the results in the present study suggest that substitution of fish oil by vegetable oils did not significantly affect the composition of intestinal microbial communities in the same host species.

Furthermore, the results of the present study indicate little overlap between the bacterial communities of the host with that of the rearing environment (water and feed), whereas the life stage appeared to be the main factor affecting the structure of gut microbiota. These results are in agreement with previous findings from Llewellyn et al. (2016), who studied 96 wild-caught individuals of Atlantic salmon with different age and habitats and observed grouping of their intestinal bacterial communities based on the lifecycle stage. In addition, Lokesh et al. (2019), reported stage specific microbial enrichment in intestinal mucosa of the same host species (samples from embryonic stages up to 80 weeks post hatch). Similar stage specific signatures have also been reported across development in *Sparus aurata* (Nikouli et al., 2019), *Danio rerio* (Stephens et al., 2016), and *Gadus morhua* (Bakke et al., 2015) suporting further that the life stage seems to be the primary force shaping gut microbiota in juveniles' stages of fish. The change in microbiota with life stage can be due to both host-microbe (e.g., development in morphology and immune system) and microbe-microbe interactions (mutualism, commensalism and competition). The significance of these factors is, however, still not known.

Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes were the dominant bacterial phyla detected in the host samples for both dietary treatments in our study. These bacterial phyla seem to characterize the bacterial communities in individuals of Atlantic salmon at the freshwater life cycle stages (Llewellyn et al., 2016). These bacterial phyla are also commonly found in

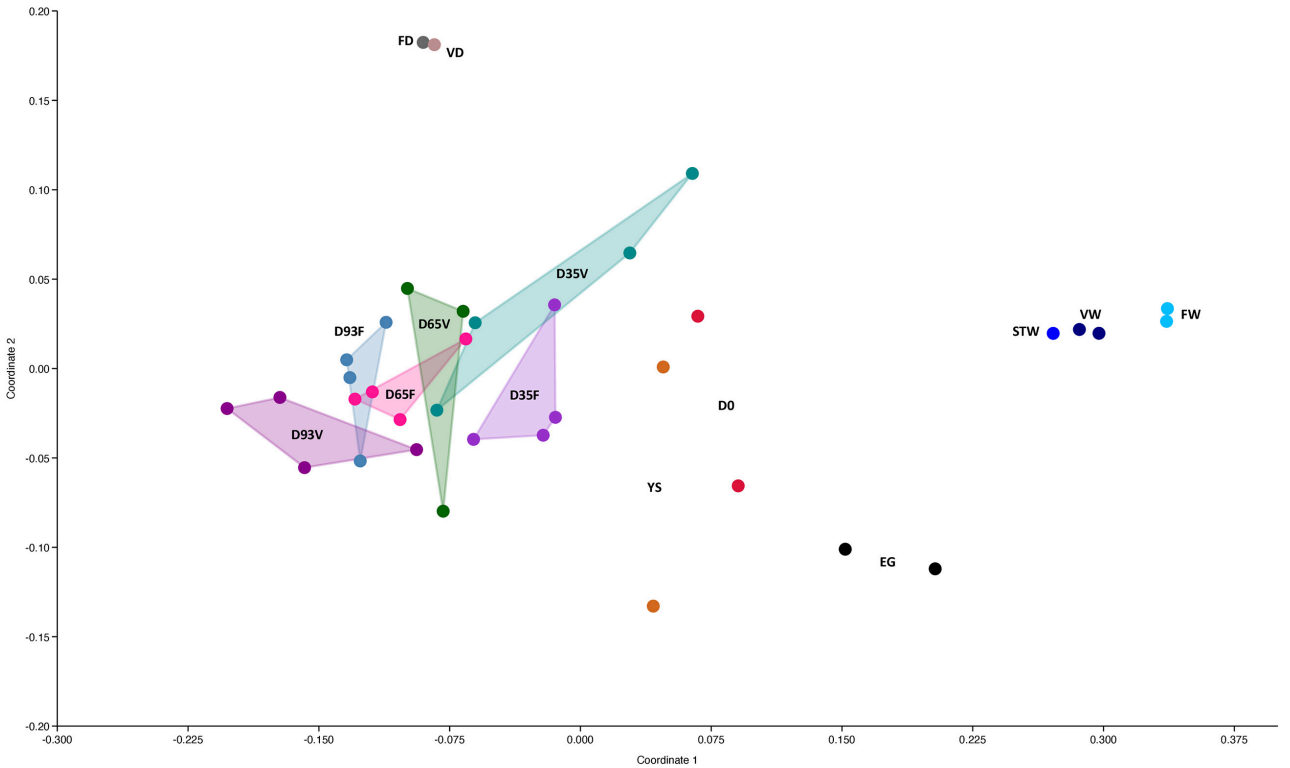

FIGURE 2 | Non-metric multidimensional scaling (nMDS) plot for all the bacterial communities of all sample categories based on Bray-Curtis distances. Pre-feding stages: Eggs (EG), yolk sac larvae (YS) and "D0". Feeding stages of (a) fish oil group (FD): D35F, D65F, and D93F and (b) vegetable oil group (VD): D35V, D65V, and D93V. Rearing water: Pre-feeding tank water (STW), Fish oil group rearing water (FW), vegetable oil group (VW). Feed: vegetable oil feed (VD) and fish oil feed (FD). D, Day.

the gut bacterial communities of both saltwater and freshwater fish species (Hansen and Olafsen, 1999; Nayak, 2010; McDonald et al., 2012; Navarrete et al., 2013; Bolnick et al., 2014; Kormas et al., 2014; Llewellyn et al., 2016; Stephens et al., 2016; Dehler et al., 2017; Tarnecki et al., 2017; Booman et al., 2018; Lokesh et al., 2019; Nikouli et al., 2018, 2019).

Despite the fact that the two experimental feeds contained almost entirely Firmicutes, the increase in relative abundance of Firmicutes in samples after the onset of feeding was not solely due to feed specific OTUs. It should also be noted that 26.4% of the bacterial representatives detected on fertilized eggs (EG) were not detected in the water of the incubation tank (WST). This support the view that the microbial communities of fish eggs may be vertically transmitted from their parents or horizontally from their breading tank (Hansen and Olafsen, 1989; Nikouli et al., 2019).

In agreement with previous studies (Schmidt et al., 2016; Lokesh et al., 2019; Nikouli et al., 2019) the observed species richness in water samples was always an order of magnitude higher than the richness of the host samples. Bacterial communities in rearing water did not show major shifts during the experiment. OTU0001 dominated at all time points, with closest relative the bacterial species *Polynucleobacter necessaries*. This species is commonly found in freshwater samples and it can contribute to the catabolism of urea and reduction of nitrate (Boscaro et al., 2013). The dominant bacterial species in Atlantic salmon samples are related with bacterial species from various habitats. The dominant OTU on fertilized eggs (OTU0011) was classified within the *Methylotenera* genus (β-Proteobacteria) and has previously been detected in fertilized eggs of the same host species by Lokesh et al. (2019). This genus consists of methylotrophic species that use methylamine as sole carbon, energy and nitrogen source (Kalyuzhnaya et al., 2006) and seem to be associated with RAS systems (Minich et al., 2020). The dominant OTU at the YS stage (OTU0013), seems to be related with *Delftia acidovorans* (β-Proteobacteria). Species of the genus *Delftia* are obligate anaerobes, organotrophic and non-fermentative organisms (Wen et al., 1999). They have previously been detected in the gut of healthy individuals of *Epinephelus coioides* (Sun et al., 2009), *Oncorhynchus mykiss* (Navarrete et al., 2012) and *Sparus aurata* (Kormas et al., 2014; Nikouli et al., 2018) and *S. salar* (Gajardo et al., 2016).

Just before onset on feeding (D0), the dominant OTU (OTU0009) showed similarities with the species *Iodobacter fluviatilis* of the Chitinibacteraceae (β-Proteobacteria) family. Species of this genus have been recorded mainly in sediment and water samples (Ryall and Moss, 1975; Wynn-Williams, 1983; Logan, 1989). Their presence on fish skin (*Oncorhynchus mykiss* and *Salmo trutta*) has been associated with skin lesions (Carbajal-González et al., 2011). However, they have previously been

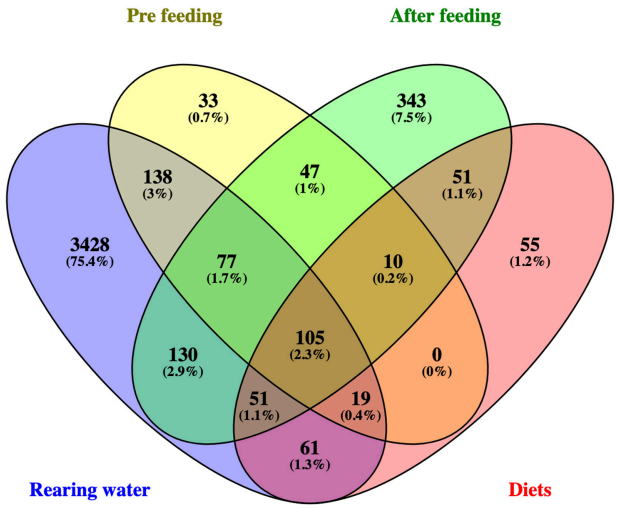

FIGURE 3 | Venn diagram demonstrating the number of the shared and unique operational taxonomic units (OTU) and their percentage of the total library, between S. salar samples [pre-feeding (EG,YS, D0) and post first feeding stages (D35, D65, D93 in both diets)], diets (FD and VD) and rearing water sample (STW, FW, VW).

detected in high relative abundance in healthy *Coreius guichenoti* individuals (Li et al., 2016) whereas the present study reports the presence of this bacterial species in Atlantic salmon gut microbiota for the first time.

After first feeding, although not statistically significant differences were found between the bacterial communities in the hindgut samples of the different life stages, each stage was characterized by different dominant OTUs. Moreover, gut bacterial communities differed also between dietary treatments regarding their dominant bacterial species (OTU). Chitinibacteraceae, the dominant bacterial family on D0 (with relative abundance 32.3%), was detected in ∼50× lower relative abundance (≤0.6%) in the rest of the samples. At D35 and D65 in FD treatment, the dominant OTUs (OTU0017 and OTU0070, classified as *Pseudomonas viridiflava* and *Janthinobacterium agaricidamnosum,* respectively), are described as plant (Alivizatos, 1986; Alimi et al., 2011; Taylor et al., 2011; Sarris et al., 2012) and mushroom pathogens (Lincoln et al., 1999; Graupner et al., 2015). According to recent findings, *Janthinobacterium lividum* (β-Proteobacteria) exhibits antimicrobial activity against multidrug resistant bacteria of clinical and environmental origin, such as Enterococci and Enterobacteriaceae (Baricz et al., 2018). Its presence in the gastrointestinal bacterial communities of Atlantic salmon, may have probiotic activity.

At D35 and D65, samples from the VD dietary treatment, were dominated by OTU0005, with closest relative *Cloacibacterium normanense* (Bacteroidetes). This OTU was also dominant at D93 in FD treatment. According to the literature, this species is frequently present in sewage treatment plants (Benedict and Carlson, 1971; Güde, 1980) where it contributes in the decomposition of complex organic compounds (Bernardet et al., 2002). Similar processes may take place in the intestinal system of Atlantic salmon at D35V, D65V, and D93F. The dominant OTU at D93 (OTU0004), also dominant in both provided feeds (FD, VD), was affiliated with *Weissella cibaria* (Firmicutes). This bacterial species belongs to the lactic acid bacteria, and has antimicrobial activity in the intestinal system of other fish species (Mouriño et al., 2016). Other *Weissella* spp. have been found in gut of *Oncorynchus mykiss* (Lyons et al., 2017; Mortezaei et al., 2020) and Atlantic salmon (Reveco et al., 2014; Godoy et al., 2015; Lokesh et al., 2019). It is worth noting that beside OTU0004, also OTU0013 and OTU0017 are associated with probiotic bacterial species (detected in all time points studied here, from EG to D93, independently of the dietary treatment). This observation suggests a co-evolutionary relationship of these bacterial species with the host studied here, and a possible specialized function in the hosts intestinal system.

CONCLUSION

The present study evaluated the effect of total fish oil replacement by a blend of terrestrial vegetable oils (rapeseed, linseed and palm oils) in the feed on the colonization and the bacterial succession in first feeding of Atlantic salmon, up to 93 days dpff. We demonstrated that feeding on either fish oil or terrestrial vegetable oil diets, did not result in significant differences in the intestinal gut microbiota and growth performance parameters (wet weight and total length). On the contrary, the composition of gut microbiota changed with age, and each stage was characterized by different dominant bacteria. These OTUs are related to species that may have probiotic activity to the host. Finally, this study revealed the occurrence of a core microbiota independent of the studied life stages and diet. These findings indicate that total fish oil replacement by terrestrial vegetable oils is feasible and can lead in low cost formulated feeds. Future work should aim on understanding the functional role of the detected core community which could lead in further feed, growth performance and host health optimization.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA520982.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics and Welfare Committee of the Norwegian University of Science and Technology (case no. 16/10070). The study was carried out within the Norwegian Animal Welfare Act guidelines, in accordance with EU regulation (EC Directive 2010/63/EU).

AUTHOR CONTRIBUTIONS

EN, KK, YO, IB, and OV: methodology. EN: formal analysis. EN and KK: data curation and writing—original draft preparation. EN, KK, YJ, YO, IB, and OV: writing—review and editing. OV: supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.665576/full#supplementary-material

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Interaction Between Dietary Lipid Level and Seasonal Temperature Changes in Gilthead Sea Bream *Sparus aurata*: Effects on Growth, Fat Deposition, Plasma Biochemistry, Digestive Enzyme Activity, and Gut Bacterial Community

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A 121-day feeding trial was undertaken to test the effects of two dietary lipid levels (16 and 21% L16, L21) in triplicated gilthead sea bream groups (initial weight: 67.5 g) reared at two different water temperatures (high, H 23°C and low, L 17°C) in the same recirculation system but exposed to a switch in temperature after 58 days. Fish kept at H were transferred to L (HL transition, autumn shift), and the fish kept at L were exposed to H (LH transition, summer shift), while continuing to receive the same diet to apparent satiation in each group. At the end of the trial, no significant diet effect on specific growth rate (SGR), feed intake (FI), and feed conversion rate (FCR) were detected in fish exposed to HL transition compared with those exposed to LH transition, while gross lipid efficiency (GLE) and lipid efficiency ratio (LER) were higher in L16. After temperature changes, L16 displayed higher SGR, FI, GLE, and LER, while mesenteric fat index was reduced. After temperature changes, the combined effects of low lipid diet and low temperature conditions resulted in higher pepsin activity, while trypsin, chymotrypsin, and lipase activities were generally higher at high lipid content. The combined effect of diet and temperature did not alter the metabolic plasma profile, except for the observed final higher aspartate aminotransferase (AST) and alkaline phosphatase (ALP) values when combining high dietary lipid (L21) and temperature changes. Different diets showed a significantly different gut microbiome layout, only at high temperature with L16 diet resulting in a higher load of *Lactobacillus*. On the contrary, no dietary impact on ecosystem diversity was observed, independently from the temperature. In addition, L16 diet in the HL transition favored an increase in *Weissella* and *Bradyrhizobium* genera in the gut microbiome, while in the final condition

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of LH transition, L21 diet favored a significant increase in *Streptococcus* and *Bacillus*. According to the results, the utilization of 16% dietary lipid levels in gilthead sea bream should be preferred during seasonal temperature changes in order to optimize feed utilization and gut health.

Keywords: gilthead sea bream (*Sparus aurata* L.), feeding strategies, growth, digestive enzyme, plasma biochemistry, gut microbiota, gut health

INTRODUCTION

Today, feeding strategy optimization related to environmental conditions is extremely necessary to pursue more intensive and more efficient aquaculture production in the Mediterranean basin. Feed production is the greatest cost in the aquaculture sector and can account for up to 60–80% of the overall expenses (Hasan et al., 2007; Luna et al., 2019). Dietary lipid supplementation has been largely developed to reduce feed cost and reduce the need for limited and expensive protein ingredients in many farmed fish species (Leaver et al., 2008; Bell and Koppe, 2010; Bonaldo et al., 2010) including gilthead sea bream (*Sparus aurata*), which is one of the most important marine finfish species farmed in the Mediterranean area (Guillen, 2019). Currently, commercial diet composition for the grow-out phase of this species consists, on average, of 43% protein and 20% fat (Koven, 2002; Vasconi et al., 2017; Arantzamendi et al., 2019). Among abiotic factors, water temperature is the key environmental factor, playing a crucial role on metabolism, nutrient utilization, fat deposition and welfare, in particular for this species which is highly susceptible to thermal seasonal changes and fluctuation (Ibarz et al., 2010; Sánchez-Nuño et al., 2018a). Although it is known that the optimal temperature range is between 18 and 26°C (Davis, 1988; Jobling and Peruzzi, 2010), this species is yearly subjected to large temperature fluctuations (from 11 to 26°C) in most farming conditions. Previous works found that increasing dietary lipids from 16 to 24% produced no significant differences in final body weight and specific growth rate at summer temperatures between 24 and 27°C (Velázquez et al., 2006; Bonaldo et al., 2010; Mongile et al., 2014). On the other hand, several studies have also been devoted to developing winter feeds for overcoming metabolic alterations, immune suppression, and nutritional disorders (Silva et al., 2014; Richard et al., 2016; Schrama et al., 2017). While most of these diets were formulated in order to test the effectiveness of functional ingredients such as immunostimulants and antioxidants, their lipid content ranged from 17 to 19.7%. In addition, especially at temperatures below 13°C, if there is an excess in dietary lipid, it can be accumulated as a fat depot in perivisceral tissue due to low metabolic activity (Ibarz et al., 2007, 2010).

To the best of our knowledge, few studies have been carried out to assess optimal lipid composition during water temperature changes. Sánchez-Nuño et al. (2018a) found that dietary lipid content 18% vs. 14% did not affect growth in fish subjected to temperature fluctuations from 22 to 14°C; however, the authors suggested adopting lower lipid levels to avoid excessive fat deposition and putative oxidative stress during recovery. Environmental temperature fluctuation during seasonal changes may also affect fish metabolism, digestive enzyme activity, and gut bacterial community, which may influence performance, tissue composition, and fish health (Couto et al., 2012; García-Meilán et al., 2013; Guerreiro et al., 2016; Zarkasi et al., 2016; Sepulveda and Moeller, 2020). To date, very limited studies have investigated how changes in water temperature interact with diet in shaping the gut microbiome structure in teleost species, and none of them deals with sea bream. The aim of the present study was to explore the effects of dietary lipid level and temperature switch on growth, digestive enzyme activity, plasma biochemistry, and gut microbiome structure during the on-growing of gilthead sea bream.

MATERIALS AND METHODS

Experimental Diets

Ingredients and proximate composition of the experimental diets are represented in **Table 1**. Two isonitrogenous (43.7%) extruded diets (sinking pellet size diameter 4.00 mm) were produced to contain a low 16% (L16) and high 21% (L21) dietary lipid level. Diets were formulated with fish meal and with a mixture of vegetable ingredients currently used for sea bream in aquafeed (Parma et al., 2016). Diets were produced by Sparos Lda (Olhão, Portugal).

Water Temperature Switch

Before the beginning of the trial (October), fish were adapted to the laboratory facilities at the constant water rearing temperature of 20°C for 10 days. At the beginning of the trial, triplicate tanks were randomly divided into two groups: one at high (H) temperature 23.17 ± 1.11°C and one at low (L) temperature 17.34 ± 0.92°C, respectively, and maintained at these constant temperatures for 58 days. In the RAS, water temperature was maintained warmer in six tanks by a heater (H03609-00.B-2012/01, Zodiac Pool Care, Saint-Barthélemy-d'Anjou, France), while the water in the remaining tanks was kept cooler (AWP 16 SP R407C, GENCOLD S.r.l., Cesena, Italy) for the whole experiment.

On day 58, fish were exposed to a switch in temperature [fish kept at H were transferred to L, HL, and the fish kept at L were transferred to H (LH)], while continuing to receive the same diet in each group. The thermal variation of 6°C occurred at a rate of 3°C day^{-1}. Thus, fish that were brought from 23°C to 17°C (HL) were subjected to summer–autumn temperature variation, while fish brought from 17°C to 23°C (LH) underwent spring–summer temperature changes. In order to exclude the effect of light regime on performance, photoperiod was maintained constant at 12-h

TABLE 1 | Ingredients and proximate composition of the experimental diets.

	L16	L21
Ingredients, % of the diet		
Fishmeal Super Prime	15.00	15.00
Soy protein concentrate	16.00	16.00
Wheat gluten	7.45	8.00
Corn gluten	9.00	9.00
Soybean meal 44	20.00	20.00
Wheat meal	16.65	12.00
Fish oil	6.50	8.55
Rapeseed oil	6.50	8.55
Vitamin and Mineral Premix *INVIVO* 1%	1.00	1.00
Antioxidant	0.20	0.20
Sodium propionate	0.10	0.10
MAP (monoammonium phosphate)	1.00	1.00
L-lysine	0.25	0.25
DL-methionine	0.35	0.35
Proximate composition,% on a wet weight basis		
Moisture	5.69	5.84
Protein	43.59	43.75
Lipid	16.30	20.81
Ash	6.29	6.24
Gross energy cal g^{-1}	4,819.12	5,051.63

Vitamins and mineral premix (IU or mg kg^{-1} diet; in vivo NSA: Portugal); DL-*alpha tocopherol acetate, 200 mg; sodium menadione bisulfate, 10 mg; retinyl acetate, 16,650 IU;* DL-*cholecalciferol, 2,000 IU; thiamine, 25 mg; riboflavin, 25 mg; pyridoxine, 25 mg; cyanocobalamin, 0.1 mg; niacin, 150 mg; folic acid, 15 mg; l-ascorbic acid monophosphate, 750 mg; inositol, 500 mg; biotin, 0.75 mg; calcium panthotenate, 100 mg; choline chloride, 1,000 mg, betaine, 500 mg; copper sulfate heptahydrate, 25 mg; ferric sulfate monohydrate, 100 mg; potassium iodide, 2 mg; manganese sulfate monohydrate, 100 mg; sodium selenite, 0.05 mg; zinc sulfate monohydrate, 200 mg; yttrium oxide, 100 mg.*

light and 12-h dark through artificial light (light intensity on the water surface 400 lx).

Fish and Rearing Conditions

The experiment was carried out at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. Gilthead sea bream juveniles were obtained from Panittica Pugliese (Torre Canne di Fasano, Brindisi, Italy). At the beginning of the trial, 30 fish (initial average weight: 67.50 ± 1.66 g) per tank were randomly distributed into 12 450-L square tanks. Experimental diets (L16 and L21) were administered to triplicate groups to visual satiation twice a day (h 8.30 and h 16.00) for 6 days a week. While temperatures were switched after intermediate day sampling, each tank continued to receive the same dietary treatment until the end of the trial. Tanks were provided with natural seawater and connected to a closed recirculating aquaculture system (RAS) with an overall water volume capacity of 6,000 L. The rearing system consisted of a mechanical sand filter (0.4 m^3 of silica sand, 0.4–0.8 mm. PTK 1200, Astral Pool, Servaqua S.A. Barsareny, Spain), ultraviolet lights (SH-63, BLUGEO S.r.l., Parma, Italy), and a biofilter (PTK 1200, Astral Pool, Servaqua S.A. Barsareny, Spain). The oxygen level was kept constant (8.0 ± 1.0 mg L^{-1}) by a liquid oxygen system regulated by a software program (B&G Sinergia snc, Chioggia, Italy). Ammonia (total ammonia nitrogen, TAN ≤ 0.1 mg L^{-1}), nitrite (NO_2 ≤ 0.2 mg L^{-1}), nitrate (NO_3 ≤ 50 mg L^{-1}), and salinity (25–30 g L^{-1}) were daily monitored spectrophotometrically (Spectroquant Nova 60, Merck, Lab business, Darmstadt, Germany). Sodium bicarbonate was added daily to keep pH constant at 7.8–8.0. The feeding trial lasted for a total of 121 days.

Sampling

The samples size for each analysis are reported in **Supplementary Table 1**. At the beginning, just before thermal change (58 days), and at the end of the experiment, all the fish in each tank were anesthetized by tricaine methanesulfonate at 100 mg L^{-1} and individually weighed. The proximate composition of the carcasses was determined on pooled samples at the beginning (10 fish per tank), before the temperature switch (three fish per tank), and at the end of the trial (five fish per tank).

Furthermore, wet weight of the viscera, liver, and perivisceral fat were individually recorded for intermediate (six fish per tank) and final (five fish per tank) pools to determine viscerosomatic index (VSI), hepatosomatic index (HSI), and mesenteric fat index (MFI). Moreover, liver pooled samples (from six individuals per tank) were taken out at the end of the trial and stored at −20°C until analyzed to access the fat liver content in animals subjected to temperature switch. At 5 h post meal (hpm), three fish per tank (n = 9/treatment) on day 58 (before temperature changes) and five fish per tank (n = 15/treatment) on day 121 were sampled and dissected to obtain their whole gastrointestinal tract; then they were first stored at −80°C and subsequently freeze dried until digestive enzyme activity analysis according to Busti et al. (2020a).

Digesta content (n = 3 fish per tank on intermediate sampling day 58, n = 9 fish per diet treatment; n = 3 fish per tank on final sampling day 121st, n = 9 fish per diet treatment) from posterior intestine was also individually sampled and immediately stored at −80°C for gut microbiota investigation according to Parma et al. (2016).

Blood was collected from the caudal vein in the three fish per tank on intermediate sampling (n = 9 fish per treatment) and in the five fish per tank (n = 15 fish per treatment) on the final sampling. Samples were then centrifuged (3,000 × g for 10 min at 4°C), and plasma aliquots were stored at −80°C until analysis according to Bonvini et al. (2018a).

All experimental procedures were evaluated and approved by the Ethical–Scientific Committee for Animal Experimentation of the University of Bologna, in accordance with European directive 2010/63/UE on the protection of animals used for scientific purposes (protocol ID 942/2019).

Calculations

The formulae employed to calculate growth performance, somatic indices, nutritional indices, and relative variations were used according to Bonvini et al. (2018b) and Parma et al. (2020) as follows:

Specific growth rate (SGR) (% day^{-1}) = 100 * (ln FBW - ln IBW)/days (where FBW and IBW represent the final and the initial body weights).

FI = Feed intake (% ABW^{-1} day^{-1}) = [(100*total ingestion)/(ABW)/days].

Feed conversion ratio (FCR) = feed intake/weight gain.

Viscerosomatic index (VSI) (%) = 100 * (viscera weight/body weight).

Hepatosomatic index (HSI) (%) = 100 * (liver weight/body weight).

Mesenteric fat index (MFI) (%) = 100 * (mesenteric fat weight/body weight).

Protein efficiency rate (PER) = (FBW – IBW)/protein intake.

Gross protein efficiency (GPE) (%) = 100 * [(% final body protein * FBW) - (% initial body protein * IBW)]/total protein intake fish.

Lipid efficiency rate (LER) = (FBW - IBW)/lipid intake.

Gross lipid efficiency (GLE) (%) = 100 * [(% final body lipid * FBW) - (% initial body lipid * IBW)]/total lipid intake fish.

Relative variation = (final considered value - initial considered value)/initial considered value.

Proximate Composition Analysis

Diets and whole bodies were analyzed for proximate composition. Moisture content was obtained by weight loss after drying samples in a stove at 105°C overnight. Crude protein was determined as total nitrogen (N*6.25) after performing Kjeldahl's method. Ash content was estimated by incineration in a muffle oven at 450°C overnight (AOAC, 2010). Total lipids were determined according to the extraction method of Bligh and Dyer (1959). The same method was performed also on final liver pool samples in order to estimate their fat content. Gross energy was determined by a calorimetric bomb (Adiabatic Calorimetric Bomb Parr 1261; PARR Instrument, IL, United States).

Digestive Enzyme Activity Analysis

Stomach and proximal intestine, including the pyloric ceca, of each individual were separately homogenized in distilled water (1:3 w/v) and were centrifuged at 4°C, 13,000 × *g*, for 10 min. Supernatants were stored at –20°C until being processed. Using the stomach homogenate, pepsin activity was measured according to the methodology described in Anson (1938). In brief, 10 μl of the enzyme extract was diluted in 1 mL of 0.1 M HCl-glycine buffer (pH 2.0) containing 0.5% bovine hemoglobin. The mixture was incubated for 20 min at room temperature (approximately 25°C). The reaction was terminated by adding 0.5 ml of 20% trichloroacetic acid (TCA) and was cooled at 4°C for 15 min to facilitate precipitation. After centrifuging at 13,000 × *g* for 15 min at 4°C, 200 μl of the supernatant was used to measure absorbance at 280 nm. One unit of enzyme activity was defined as 1 μg tyrosine released per minute using a specific absorptivity of 0.008 μg^{-1} cm^{-1} at 280 nm.

In the proximal intestine homogenate, trypsin and chymotrypsin activity were measured using Nα-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA) and *N*-glutaryl-L-phenylalanine *p*-nitroanilide (GAPNA) as substrates, according to Erlanger et al. (1961, 1966), respectively. For each of these enzymes, substrate stock (0.5 mM of BAPNA or GAPNA in dimethyl sulfoxide) was brought to the working concentration by 1/10th dilutions using 50 mM Tris-HCl and 20 mM $CaCl_2$ buffer (pH 8.5). The change in absorbance at 405 nm was measured over 10 min at room temperature, for 10–15 μl of the enzyme extract and 200 μl of substrate per each microplate well. For these enzymes, one unit of activity was defined as 1 μmol *p*-nitroaniline released per minute using coefficients of molar extinction of 8,270 M^{-1} cm^{-1} at 405 nm.

Amylase activity was measured following the 3,5-dinitrosalicylic acid (DNSA) method (Bernfeld, 1955). In brief, 30 μl of enzyme extract and 300 μl of substrate [2% soluble starch in 100 mM phosphate and 20 mM $NaCl_2$ buffer (pH 7.5) were incubated at 37°C for 30 min]. The reaction was stopped by the addition of 150 μl of DNSA and was heated in boiling water for 5 min. After cooling on ice, 1.5 ml of distilled water was added to the mixture, and the absorbance was measured at 530 nm. One unit of amylase activity was defined as the amount of enzyme needed to catalyze the formation of 1 μg of maltose equivalent per minute.

Lipase activity was measured using 4-nitrophenyl myristate as substrate, according to Albro et al. (1985). Briefly, 10 μl of enzyme extract was added to 50 μl of sodium taurocholate (0.4 mg ml^{-1}) and 130 μl of 100 mM Tris-HCL buffer (pH 8.0) per each microplate well. The change in the absorbance at 405 nm was measured over 10 min at room temperature. One unit of amylase activity was defined as the amount of enzyme needed to catalyze the production of 1 μg of *p*-nitrophenol per minute.

All the activities were expressed in units per g of wet weight of fish, considering both the total amount of tissue used for enzyme determination and the live weight of each sampled fish.

Metabolic Parameters in Plasma

The chemistry profile evaluated in the study was determined using an automated analyzer (AU 480; Olympus/Beckman Coulter, Brea, CA, United States) using dedicated methods (Olympus system reagent, OSR). The type of reaction used in the assay and OSR identification number were reported in brackets after the reported variables. The profile included glucose (GLU; exochinase reaction, OSR6121), urea (urease reaction, OSR6134), creatinine (CREA, Jaffè method, OSR6178), uric acid (Uric Ac, uricase reaction, OSR6198), total bilirubin (Tot Bil, colorimetric reaction, OSR6112), bile acid (Bil Ac, colorimetric method, OSR17000801), cholesterol (CHOL, enzymatic method, OSR6116), triglycerides (TRIG, enzymatic method, OSR61118), high-density lipoprotein (HDL, enzymatic method, OSR6187), total protein (TP, biuret method, OSR6132), albumin (ALB, bromocresol green method, OSR6102), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatine kinase (CK), and lactate dehydrogenase (LDH) (enzymatic reaction, OSR6009, OSR6004, OSR6179, and OSR6128, respectively), calcium (Ca^{+2}, Arsenazo reaction, OSR6017), inorganic phosphorus (P, molybdate reaction, OSR6122), potassium (K^+), sodium (Na^+), and chloride (Cl) (ion selective electrode indirect method), iron (Fe; colorimetric reaction, OSR6186), and magnesium (Mg; xylidyl blue reaction, OSR6189). The albumin-to-globulin ratio (ALB/GLO), CaxP, and Na^+ to K^+ ratio (Na/K) were calculated. Plasma cortisol (CORT) concentration was determined using a

chemiluminescence immunoassay (Immulite cortisol, Diagnostic Product Corporation, Los Angeles, CA, United States) using an automated analyzer (Immulite XP2000, Siemens).

Gut Bacterial Community DNA Extraction and Sequencing

Total DNA was extracted and analyzed from individual distal intestine content obtained from three fish per tank (300 mg per fish) on day 58 and day 121, as previously reported in Parma et al. (2020). The analyses were performed to target the transient bacterial community. Amplification of V3–V4 hypervariable regions of the 16S rRNA bacterial gene was carried out using the 341F and 785R primers (Klindworth et al., 2013) with added Illumina adapter overhang sequences and 2 × KAPA HiFi HotStart ReadyMix (KAPA Biosystems). For this step, the thermal cycle consisted of an initial denaturation phase at 95°C for 3 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, and a final extension step at 72°C for 5 min. To purify PCR products and to prepare samples for Library for Illumina sequencing, the Illumina protocol "16S Metagenomic Sequencing Library Preparation" was followed, as used in several other publications (Biagi et al., 2019; Musella et al., 2020). Sequencing was performed on Illumina MiSeq platform using a 2 × 250-bp paired-end protocol according to the manufacturer's instructions (Illumina, San Diego, CA, United States). Raw sequences were processed using the QIIME2 pipeline[1] (Bolyen et al., 2019). High-quality reads, obtained by a filtering step for length (minimum/maximum = 250/550 bp) and quality with default parameters, were cleaned using DADA2 (Callahan et al., 2016) and clustered into amplicon sequence variants (ASVs) using VSEARCH (Rognes et al., 2016). Taxonomy was assigned using RDP classifier against SILVA database (Quast et al., 2013).

Alpha diversity was assessed using Faith's Phylogenetic Diversity (PD_whole_tree), Chao1 index for microbial richness, and observed_ASVs, while beta diversity was estimated by computing UniFrac distances, which were used as input for principal coordinate analysis (PCoA).

Statistical Analysis

All data are represented as mean ± standard deviation (SD). A tank was used as the experimental unit for analyzing growth performance, and a pool of three (on intermediate sampling, day 58) and five (on final sampling, day 121) fish was considered as the experimental unit for analyzing carcass composition, liver fat content, and nutritional indices, whereas nine (on intermediate sampling, day 58) and 15 (on final sampling, day 121) individual fish per treatment were used for analyzing somatic indices, digestive enzyme activity, blood biochemistry, and gut bacterial community profiles. Data of growth performance, nutritional indices, somatic indices, fat liver content, enzyme activity, and plasma parameters were analyzed by a two-way analysis of variance (ANOVA) with Tukey's *post hoc* test. In order to assess the amplitude of variations occurring before and after the temperature change, relative variations in growth parameters, morphometric indices, nutritional indices, digestive enzyme activity, and plasma biochemistry were calculated and analyzed by a two-way analysis of variance (ANOVA) with Tukey's *post hoc* test. The normality and homogeneity of variance assumptions were validated for all data preceding ANOVA using Levene's test for homogeneity of variance and Shapiro–Wilks normality test. All gut microbiota statistical analyses were performed using R project[2]. PCoA plots were generated using the "vegan"[3] and "Made4" packages (Culhane et al., 2005), and for all PCoAs, betadisper and permutest functions were used to assess homogeneity of dispersion of our data (in all tests, p-value was > 0.05). PERMANOVA (Permutational Multivariate Analysis Of Variance) using distance matrices was used to asses data separation among groups (function "Adonis" in "vegan," numbers of permutations = 999), in order to assess the influence of temperature changes in each diet group and the influence of diet in the two temperature conditions. When required, Wilcoxon and Kruskal–Wallis test was used to assess significant differences in alpha diversity and taxon relative abundance between groups. A p-value ≤ 0.05 was considered statistically significant, while a p-value between 0.05 and 0.1 was seen as a trend.

RESULTS

Growth

Results on growth performance parameters and nutritional indices are summarized in **Table 2**. Concerning the temperature change occurring in the overall experimental period (days 0–121 period), no significant effects of diet nor temperature ($p > 0.05$) on growth (FBW, WG, SGR), FI, and survival were detected, while FCR was significantly influenced by temperature ($p < 0.05$) with lower values in LH (animals first exposed to L temperature and then switched to H). At the same time, diet and temperature had a significant effect on LER and GLE ($p < 0.05$), both of which were found to be higher in animals fed L16, while among groups fed the same dietary treatment, they were slightly lower in HL. Over days 0–58 period, no dietary effect was detected on FBW, WG, SGR, FCR, FI, and survival ($p > 0.05$), while temperature had a significant effect ($p < 0.05$) on FBW, WG, SGR, and FI with higher values in fish reared at 23°C (H groups) compared with those at 17°C (L groups). FCR was moderately lower ($p = 0.0505$) at high temperature. A significant dietary effect was recorded on LER and GLE ($p < 0.05$) but not on PER and GPE ($p > 0.05$). At the same time, significant temperature effect ($p < 0.05$) was found on PER, GPE, and LER, with lower levels in animals held at 17°C (L).

In the period following the temperature switch (days 59–121), diet and temperature had a significant effect ($p < 0.05$) on SGR and FI, with higher values in animals fed diet L16 and maintained at H temperature. Significant temperature effects ($p < 0.05$) were also found on WG and FCR showing higher

[1]https://qiime2.org/

[2]https://www.r-project.org/

[3]http://www.cran.r-project.org/package-vegan/

TABLE 2 | Growth performance and nutritional indices of gilthead sea bream fed experimental diets and exposed to water temperature switch.

	Overall period days 0–121						
	L16		**L21**		***P-value***		
	HL	**LH**	**HL**	**LH**	***Inter***	***Temp***	***Diet***
Growth performances							
IBW	66.83 ± 1.07	68.13 ± 0.64	67.27 ± 2.05	67.80 ± 2.77	*0.727*	*0.412*	*0.964*
FBW	175.07 ± 9.91	181.01 ± 10.81	170.84 ± 19.28	169.65 ± 20.71	*0.708*	*0.803*	*0.421*
WG	108.24 ± 8.89	112.88 ± 10.79	103.57 ± 17.83	101.85 ± 19.33	*0.721*	*0.870*	*0.388*
SGR	0.80 ± 0.03	0.81 ± 0.05	0.77 ± 0.07	0.75 ± 0.08	*0.728*	*>0.999*	*0.311*
FCR	1.34 ± 0.01	1.30 ± 0.04	1.32 ± 0.01	1.29 ± 0.03	*0.750*	*0.036*	*0.352*
FI	1.01 ± 0.04	0.99 ± 0.05	0.96 ± 0.09	0.92 ± 0.05	*0.930*	*0.391*	*0.162*
Survival	78.89 ± 1.92	78.89 ± 1.92	76.67 ± 5.77	77.78 ± 3.85	*0.805*	*0.805*	*0.457*
Nutritional indices							
PER	1.67 ± 0.01	1.74 ± 0.02	1.70 ± 0.03	1.74 ± 0.08	*0.624*	*0.062*	*0.466*
GPE	29.5 ± 1.34	30.8 ± 0.41	29.9 ± 0.88	29.6 ± 1.53	*0.232*	*0.489*	*0.552*
GLE	66.9 ± 1.40^{b}	76.5 ± 1.91^{b}	54.4 ± 5.93^{a}	56.0 ± 5.02^{a}	*0.128*	*0.044*	*0.000*
LER	4.47 ± 0.03^{b}	4.64 ± 0.05^{b}	3.58 ± 0.05^{a}	3.67 ± 0.17^{a}	*0.425*	*0.036*	*0.000*
	Before temperature switch days 0–58						
	H	**L**	**H**	**L**	***Inter***	***Temp***	***Diet***
Growth performances							
IBW	66.83 ± 1.07	68.13 ± 0.64	67.27 ± 2.05	67.80 ± 2.77	*0.727*	*0.412*	*0.964*
FBW	134.74 ± 13.45^{b}	95.16 ± 4.13^{a}	136.12 ± 13.74^{b}	99.44 ± 8.42^{a}	*0.821*	*0.000*	*0.659*
WG	67.91 ± 12.53^{b}	27.03 ± 3.96^{a}	68.86 ± 12.22^{b}	31.64 ± 5.66^{a}	*0.745*	*0.000*	*0.622*
SGR	1.20 ± 0.15^{b}	0.57 ± 0.07^{a}	1.21 ± 0.13^{b}	0.66 ± 0.08^{a}	*0.558*	*0.000*	*0.527*
FCR	1.25 ± 0.04	1.35 ± 0.09	1.23 ± 0.05	1.28 ± 0.01	*0.410*	*0.051*	*0.205*
FI	1.45 ± 0.16^{b}	0.77 ± 0.05^{a}	1.44 ± 0.18^{b}	0.84 ± 0.10^{a}	*0.622*	*0.000*	*0.715*
Survival	100.00 ± 0.00	100.00 ± 0.00	98.89 ± 1.92	98.89 ± 1.92	*>0.999*	*>0.999*	*0.195*
Nutritional indices							
PER	1.83 ± 0.07	1.69 ± 0.11	1.85 ± 0.06	1.78 ± 0.01	*0.461*	*0.043*	*0.250*
GPE	33.0 ± 1.16^{b}	28.1 ± 0.47^{a}	30.8 ± 1.17^{ab}	29.6 ± 2.55^{ab}	*0.070*	*0.008*	*0.718*
GLE	71.3 ± 10.36	77.5 ± 11.72	65.4 ± 2.35	52.0 ± 15.36	*0.163*	*0.586*	*0.039*
LER	4.89 ± 0.19^{b}	4.52 ± 0.30^{b}	3.89 ± 0.13^{a}	3.73 ± 0.02^{a}	*0.360*	*0.044*	*0.000*
	After temperature switch days 59–121						
	L	**H**	**L**	**H**	***Inter***	***Temp***	***Diet***
Growth performances							
IBW	134.74 ± 13.45^{b}	95.16 ± 4.13^{a}	136.12 ± 13.74^{b}	99.44 ± 8.42^{a}	*0.821*	*0.000*	*0.659*
FBW	175.07 ± 9.91	181.01 ± 10.81	170.84 ± 19.28	169.65 ± 20.71	*0.708*	*0.803*	*0.421*
WG	40.33 ± 4.50^{a}	85.85 ± 6.94^{b}	34.71 ± 5.63^{a}	70.21 ± 17.12^{b}	*0.407*	*0.000*	*0.100*
SGR	0.42 ± 0.08^{a}	1.02 ± 0.04^{b}	0.36 ± 0.02^{a}	0.84 ± 0.15^{b}	*0.288*	*0.000*	*0.045*
FCR	1.55 ± 0.12^{b}	1.30 ± 0.02^{a}	1.53 ± 0.12^{ab}	1.29 ± 0.05^{a}	*0.900*	*0.002*	*0.802*
FI	0.66 ± 0.08^{a}	1.29 ± 0.07^{c}	0.56 ± 0.04^{a}	1.07 ± 0.11^{b}	*0.193*	*0.000*	*0.008*
Survival	98.6 ± 2.4	98.6 ± 2.4	97.1 ± 5.0	98.6 ± 2.5	*0.709*	*0.709*	*0.697*
Nutritional indices							
PER	1.45 ± 0.09^{a}	1.75 ± 0.04^{b}	1.47 ± 0.11^{a}	1.75 ± 0.15^{b}	*0.850*	*0.001*	*0.892*
GPE	24.5 ± 4.88	31.6 ± 0.41	28.6 ± 3.78	30.0 ± 2.63	*0.169*	*0.059*	*0.532*
GLE	59.1 ± 11.72^{ab}	76.2 ± 5.94^{b}	37.0 ± 19.49^{a}	57.8 ± 14.35^{ab}	*0.822*	*0.044*	*0.034*
LER	3.88 ± 0.24^{bc}	4.68 ± 0.11^{c}	3.10 ± 0.22^{a}	3.68 ± 0.32^{ab}	*0.445*	*0.001*	*0.000*

Data are given as the tank mean (n = 3) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$). L16, low-lipid 16% diet; L21, high-lipid 21% diet; HL, constant temperature exposure to high (H) 23°C until temperature switch (days 0–58), then to constant low (L) 17°C until end of trial (days 59–121); LH, constant temperature exposure to low (L) 17°C until temperature switch (days 0–58), then to constant high (H) 23°C until end of trial (days 59–121); H, constant temperature exposure to high (H) 23°C; L, constant temperature exposure to low (L) 17°C. WG, weight gain, g. Calculations of growth performance and nutritional indices are reported in the section "Calculations."

WG and lower FCR in H temperature. At the same time, significant ($p < 0.05$) dietary and temperature effects were found on GLE and LER, with the highest values in fish fed L16 and kept at H and the lowest levels in individuals fed L21 and reared at L (17°C). No significant dietary effect ($p > 0.05$) was found on PER and GPE. A significant temperature effect ($p < 0.05$) was noticed on PER, which showed higher in animals reared at H temperature. No significant temperature effect was detected on GPE ($p < 0.05$). Relative variations in growth performance and nutritional indices calculated between day 58 and day 121 (before–after temperature switch) are represented in **Supplementary Table 2**. WG, SGR, and FI showed a higher increment/lower reduction in L16 compared with L21 (diet effect $p \leq 0.05$). A significant temperature effect was found on the relative variation in FBW, WG, SGR, FCR, and FI with lower increment in HL compared with LH. In addition, the relative variation in FI displayed a significant interaction effect ($p < 0.05$). Diet had no significant effect on the relative variations in nutritional indices ($p < 0.05$), while temperature affected PER, GPE, and LER relative variations showing decreasing values in HL compared with LH (temperature effect < 0.05). No significant dietary nor temperature effect were found on GLE relative variation ($p < 0.05$).

Proximate body composition and somatic indices data are shown in **Table 3**. Before temperature switch (day 58), protein displayed significant effects of diet, temperature, and interaction ($p < 0.05$) with higher amounts in L16 and H temperature. For lipid content, there were significant effects of temperature and interaction ($p < 0.05$), where groups fed L21 retained both the highest and the lowest percentages for H and L, respectively. Ash body percentage was significantly affected ($p > 0.05$) only by diet, with higher values in groups fed L16. Significant dietary and temperature effects (p < 0.05) were also found for moisture. At the same time, no significant dietary effect ($p > 0.05$) was found on HSI, MFI, and VSI, while a significant temperature effect occurred on HSI ($p < 0.05$) with higher levels in fish kept at low temperature (L 17°C).

At the end of the trial (day 121), no significant dietary and temperature effects were found in body protein, lipid, and ash percentages ($p > 0.05$). Moisture showed a significant dietary effect ($p < 0.05$), displaying a higher level in L21. Fat liver content was not significantly affected by diet nor temperature

TABLE 3 | Body composition and somatic indices of gilthead sea bream fed experimental diets and exposed to water temperature switch.

	Before temperature switch—day 58						
	L16		**L21**		***P-value***		
	H	**L**	**H**	**L**	***Inter***	***Temp***	***Diet***
Whole body composition, %							
Protein	17.54 ± 0.05^{b}	16.90 ± 0.37^{ab}	16.83 ± 0.07^{a}	16.89 ± 0.46^{a}	*0.008*	*0.022*	*0.007*
Lipid	13.13 ± 1.11^{ab}	13.11 ± 0.75^{ab}	14.21 ± 0.39^{b}	12.39 ± 1.29^{a}	*0.011*	*0.035*	*0.436*
Ash	3.94 ± 0.27	3.92 ± 0.08	3.64 ± 0.51	3.55 ± 0.14	*0.796*	*0.687*	*0.022*
Moisture	64.37 ± 1.06^{a}	64.84 ± 0.28^{ab}	64.67 ± 0.48^{ab}	65.70 ± 0.57^{b}	*0.316*	*0.013*	*0.044*
Somatic indices							
HSI	1.98 ± 0.10^{a}	2.40 ± 0.16^{b}	1.90 ± 0.07^{a}	2.33 ± 0.11^{b}	*0.934*	*0.000*	*0.396*
MFI	1.42 ± 0.34	1.45 ± 0.19	1.53 ± 0.28	1.64 ± 0.81	*0.846*	*0.673*	*0.421*
VSI	9.05 ± 0.62	9.62 ± 0.94	10.04 ± 1.54	9.13 ± 0.21	*0.236*	*0.309*	*0.222*
	After temperature switch—day 121						
	L	**H**	**L**	**H**	***Inter***	***Temp***	***Diet***
Whole body composition, %							
Protein	17.41 ± 0.55	17.46 ± 0.12	17.36 ± 0.30	16.99 ± 0.40	*0.161*	*0.287*	*0.102*
Lipid	13.68 ± 0.28	14.63 ± 0.41	13.81 ± 1.26	13.78 ± 0.51	*0.119*	*0.145*	*0.249*
Ash	3.87 ± 0.13	3.84 ± 0.11	3.72 ± 0.21	3.70 ± 0.20	*0.977*	*0.782*	*0.099*
Moisture	64.05 ± 0.32	63.46 ± 0.56	64.52 ± 1.25	64.61 ± 0.70	*0.255*	*0.405*	*0.012*
Somatic indices							
HSI	2.42 ± 0.54^{b}	1.74 ± 0.26^{a}	2.56 ± 0.38^{b}	1.60 ± 0.22^{a}	*0.141*	*0.000*	*0.993*
MFI	1.11 ± 0.39	1.23 ± 0.55	1.38 ± 0.49	1.52 ± 0.41	*0.924*	*0.301*	*0.022*
VSI	8.38 ± 1.26^{a}	9.46 ± 1.19^{ab}	9.92 ± 1.23^{b}	8.84 ± 2.60^{ab}	*0.015*	*0.299*	*0.012*
Lipid liver	10.80 ± 0.66	13.25 ± 4.32	11.81 ± 1.68	12.76 ± 0.77	*0.598*	*0.249*	*0.853*

Data are given as the mean (n = 9 diet^{-1} on day 58; n = 15 diet^{-1} on day 121) ± SD. In each line, different superscript letters indicate significant differences among treatments (P ≤ 0.05). L16, low-lipid 16% diet; L21, high lipid 21% diet; H, constant temperature exposure to high (H) 23°C; L, constant temperature exposure to low (L) 17°C.

HSI = Hepatosomatic index (%) = 100(liver weight/FBW).*

MFI = Mesenteric fat Index (%) = 100(mesenteric fat weight/FBW).*

VSI = Viscerosomatic index (%) = 100(viscera weight/FBW).*

SD, standard deviation.

($p > 0.05$). Concerning somatic indices, diet showed a significant effect on MFI and VSI ($p < 0.05$) with MFI values higher in L21 than in L16, while an additional interaction effect in VSI was also observed, where animals brought to low temperature (L) displayed higher values in L21, and fish exposed to rise in water temperature (H) displayed higher levels in L16. HSI was significantly affected by temperature with higher values observed in the L groups for both dietary regimes.

Relative variations in proximate body composition and somatic indices calculated between day 58 and day 121 (before–after temperature switch) are represented in **Supplementary Table 3**. No significant dietary or temperature effects were found on relative variations in protein, lipid, ash, and moisture ($p > 0.05$). Relative variation in HSI was not significantly affected by diet ($p > 0.05$); however, HL groups displayed a relative increment, while LH groups showed decreasing values (temperature effect $p < 0.05$). MFI and VSI relative variations were not significantly affected by diet nor temperature ($p > 0.05$).

Digestive Enzyme Activity

Digestive enzymes activities measured before and after water temperature change are shown in **Figure 1**. Before temperature switch, pepsin activity was significantly ($p < 0.05$) higher in fish fed on diet L21, but no significant differences were observed between groups maintained at low or high temperature. After temperature switch, the only significant differences observed were associated with interactions between the effects of dietary lipid level and low temperature; the highest and lowest activities were measured in fish fed on low-lipid and high-lipid diets, respectively, maintained at low temperature. On the other hand, no significant effect of temperature change was evidenced, irrespective of diet composition.

In the case of trypsin, a significant effect of rearing temperature and none of dietary lipid level was evidenced during the first part of the assay, with higher values measured in fish maintained at 17°C when compared with those at 23°C. After temperature inversion, significantly higher values were measured in fish fed on high lipids and maintained at high temperature when compared with those fed on low lipids and maintained at low temperature. On the other hand, significantly higher values of chymotrypsin activity were measured in fish fed on high dietary lipids, both before and after temperature switch.

Amylase activity was not significantly affected either by diet or temperature during the initial period of the experiment, but a significant interaction of diet × temperature occurred ($p < 0.05$).

After temperature inversion, no significant effect of dietary lipid level was evidenced on amylase activity, while significantly higher values of this enzyme were measured in fish maintained at high temperature.

Relating to lipase activity, while no significant effect of diet or temperature were measured during the initial period, these were evidenced after temperature change. Significantly lower activity was linked to the consumption of low lipid diet, and within the same dietary treatment, a higher activity was detected in fish kept at 23°C compared with those at 17°C.

Relative variations in digestive enzyme activity calculated between day 58 and day 121 are represented in **Supplementary Table 4**. Relative variations in digestive enzymes were not significantly affected by diet nor temperature ($p > 0.05$), except for amylase, which displayed a significant effect of temperature. In particular, a lower reduction was observed for LH treatment, and this reduction was less evident under L21 than under L16.

Plasma Biochemistry

The results of plasma parameters are shown in **Tables 4**, **5**. Before temperature change, significant dietary effect ($p < 0.05$) was displayed in Tot Bil, Ca^{2+}, P, Na^{+}, CORT, and CaxP, having higher levels in animals fed L21, except for CORT that appeared to be more elevated in groups treated with L16. At the same time, a significant temperature effect was noticed on TRIG, TP, AST, LDH, HDL, ALP, and Fe where all subjects reared at L temperature tended to have higher values than those at H temperature, except for ALP and Fe. Significant dietary and temperature effects ($p < 0.05$) were observed on ALB/GLO before temperature switch (day 58). Moreover, significant interaction effect on CREA was found in the same period of time ($p < 0.05$). Before temperature switch, no significant dietary and temperature effects ($p > 0.05$) were found in GLU, urea, uric Ac, CHOL, ALB, CK, K^{+}, Cl, Mg, and Na/K.

At the end of the experiment (day 121) diet significantly affected TP, ALB, AST, LDH, Ca^{2+}, K^{+}, Na^{+}, Cl, and Na/K on day 121 ($p < 0.05$). Plasma AST, LDH, K^{+}, Na^{+}, and Cl were higher in L21-supplied individuals and contrariwise for TP, ALB, Ca^{2+}, and Na/K. At the same time, temperature significantly affected GLU, CREA, Bil Ac, and CK ($p < 0.05$). Among them, GLU, CREA, and Bil Ac presented higher levels in individuals maintained at H temperature, while plasma CK concentration showed the opposite trend. Significant dietary and temperature effects ($p < 0.05$) were observed on Tot Bil, ALP, and ALB/GLO. Uric Ac was significantly affected by diet, temperature, and interaction ($p < 0.05$). Moreover, a significant interaction effect on TRIG was found at the same time ($p < 0.05$); no significant dietary and temperature effect ($p > 0.05$) was observed on urea, CHOL, P, Fe, Mg, CORT, CaxP, and HDL.

Relative variations in plasma parameters calculated between day 58 and day 121 are represented in **Supplementary Table 5**. Uric Ac, AST, and CK showed a higher relative increment in L21 compared with L16, while Ca^{2+} was significantly reduced in fish fed L21 compared with those fed L16 (dietary effect $p < 0.05$). HL animals displayed higher TP, AST, CK, LDH, and HDL relative increments compared to LH ones, while GLU, Bil Ac, ALP, and ALB/GLO relative increments were higher in LH animals than HL ones (temperature effect $p < 0.05$). In addition, significant interaction was found on relative variation of CK ($p < 0.05$). No significant dietary or temperature effect was found on relative variations of Urea, CREA, Tot Bil, CHOL, TRIG, ALB, P, K, Na^{+}, Fe, Cl, Mg, CORT, and CaxP, Na/K ($p > 0.05$).

Fecal Bacterial Community Profiles Before and After Water Temperature Changes

The 16S rRNA gene sequencing was performed on a total of 71 distal intestine content samples, yielding 1,724,306 high-quality

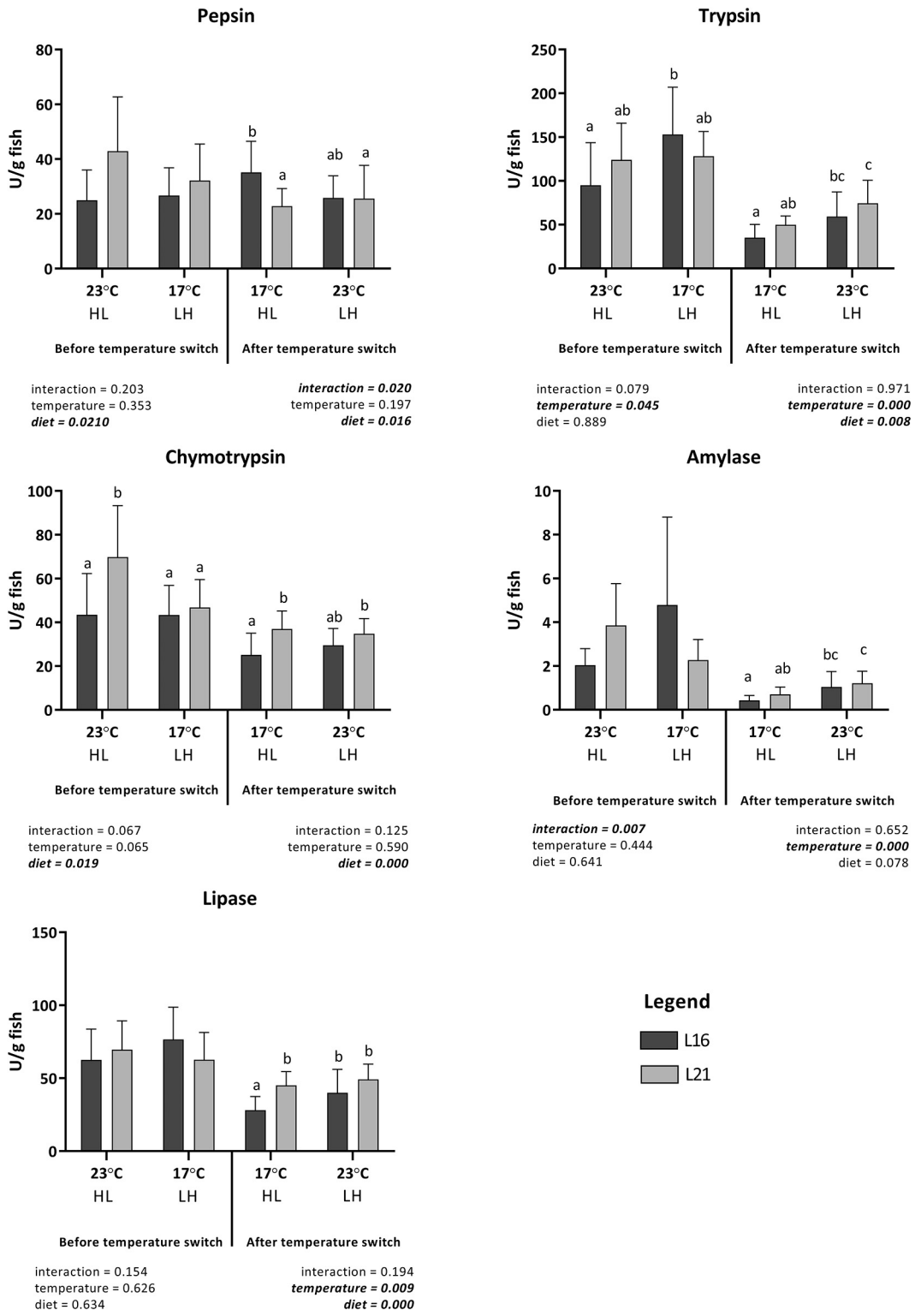

FIGURE 1 | Digestive gut enzymes activity (expressed as U g fish body weight^{-1}) of gilthead sea bream fed the experimental diets and exposed to temperature switch over 121 days. Data are given as the mean of triplicate tank individual samples (n = 3 per tank before temperature change, n = 5 per tank after temperature change) ± SD. Different letters indicate significant difference (two-way ANOVA $P \leq 0.05$) between treatments. L16, low-lipid 16% diet; L21, high-lipid 21% diet. HL, constant temperature exposure to high (H) 23°C until temperature switch (days 0–58), then to constant low (L) 17°C until end of trial (day 59–21); LH, constant temperature exposure to low (L) 17°C until temperature switch (days 0–58), then to constant high (H) 23°C until end of trial (day 59–121).

TABLE 4 | Plasma biochemistry values for gilthead sea bream fed the experimental diets and exposed to two different temperatures before temperature switch.

	Before temperature switch—day 58						
	L16		**L21**		***P - value***		
	H	**L**	**H**	**L**	***Inter***	***Temp***	***Diet***
GLU	90.56 ± 7.02	98.63 ± 26.72	82.78 ± 11.63	86.89 ± 8.64	*0.701*	*0.243*	*0.066*
Urea	6.11 ± 1.09	7.20 ± 2.93	6.56 ± 0.76	6.82 ± 1.50	*0.484*	*0.259*	*0.947*
CREA	0.20 ± 0.03	0.22 ± 0.03	0.23 ± 0.03	0.20 ± 0.03	*0.022*	*0.449*	*0.747*
Uric Ac	0.03 ± 0.04	0.03 ± 0.03	0.01 ± 0.00	0.01 ± 0.00	*0.987*	*0.987*	*0.053*
Tot Bil	0.05 ± 0.03^{ab}	0.04 ± 0.02^{a}	0.07 ± 0.01^{b}	0.06 ± 0.01^{ab}	*0.800*	*0.211*	*0.006*
Bil Ac	36.10 ± 20.78	31.03 ± 6.01	50.68 ± 15.98	38.38 ± 17.74	*0.686*	*0.340*	*0.234*
CHOL	231.00 ± 43.91	248.25 ± 28.00	240.78 ± 41.48	253.88 ± 58.17	*0.892*	*0.325*	*0.615*
TRIG	811.11 ± 524.56	1116.13 ± 584.34	823.11 ± 231.47	1205.25 ± 541.40	*0.819*	*0.048*	*0.764*
HDL	48.44 ± 13.36^{a}	66.25 ± 8.89^{b}	52.11 ± 8.25^{ab}	61.00 ± 12.60^{ab}	*0.248*	*0.001*	*0.836*
TP	3.56 ± 0.27	3.79 ± 0.50	3.51 ± 0.22	3.74 ± 0.23	*0.989*	*0.043*	*0.627*
ALB	0.95 ± 0.08	0.96 ± 0.14	0.89 ± 0.05	0.92 ± 0.05	*0.801*	*0.607*	*0.096*
AST	22.11 ± 15.98	51.13 ± 46.11	17.89 ± 9.57	43.50 ± 26.50	*0.858*	*0.007*	*0.534*
ALP	691.44 ± 316.46^{bc}	295.38 ± 178.67^{a}	936.33 ± 425.29^{c}	418.22 ± 129.45^{ab}	*0.539*	*0.000*	*0.070*
CK	92.89 ± 84.00	498.13 ± 833.58	28.00 ± 14.17	153.00 ± 131.14	*0.314*	*0.062*	*0.144*
LDH	455.56 ± 416.36^{ab}	1,195.75 ± 1,089.30^{b}	226.00 ± 131.44^{a}	1,013.88 ± 837.50^{ab}	*0.922*	*0.004*	*0.400*
Ca^{+2}	12.14 ± 0.65	12.00 ± 0.85	12.70 ± 0.43	12.38 ± 0.61	*0.687*	*0.298*	*0.044*
P	11.18 ± 0.88^{ab}	10.55 ± 1.62^{a}	12.50 ± 1.73^{b}	12.54 ± 1.03^{b}	*0.485*	*0.526*	*0.001*
K^{+}	5.03 ± 1.07	4.84 ± 0.87	4.29 ± 0.40	5.00 ± 0.54	*0.090*	*0.327*	*0.270*
Na^{+}	184.44 ± 6.71	182.75 ± 3.54	187.67 ± 4.95	188.78 ± 9.02	*0.526*	*0.895*	*0.043*
Fe	129.89 ± 37.03^{ab}	97.75 ± 27.44^{a}	147.78 ± 32.20^{b}	115.50 ± 31.03^{ab}	*0.995*	*0.007*	*0.119*
Cl	162.91 ± 4.18	162.08 ± 3.25	164.77 ± 5.01	167.41 ± 8.34	*0.366*	*0.637*	*0.067*
Mg	2.62 ± 0.16	2.77 ± 0.17	2.70 ± 0.16	2.67 ± 0.15	*0.114*	*0.284*	*0.849*
CORT	20.83 ± 9.08	28.24 ± 15.17	14.42 ± 9.87	10.68 ± 14.70	*0.211*	*0.677*	*0.010*
ALB/GLO	0.37 ± 0.02^{b}	0.34 ± 0.01^{a}	0.34 ± 0.01^{a}	0.33 ± 0.02^{a}	*0.498*	*0.001*	*0.004*
CaxP	136.11 ± 16.11^{ab}	127.50 ± 26.00^{a}	159.22 ± 27.46^{b}	155.50 ± 19.73^{ab}	*0.757*	*0.437*	*0.003*
Na/K	38.56 ± 9.53	39.00 ± 8.70	44.00 ± 4.12	38.22 ± 3.77	*0.197*	*0.267*	*0.330*

*Data are given as the mean (n = 9 diet^{-1} on day 58; n = 15 diet^{-1} on day 121) ± SD. Different letters indicate significant difference (two-way ANOVA, P ≤ 0.05) between treatments. L16, low-lipid 16% diet; L21, high-lipid 21% diet; H, constant temperature exposure to high (H) 23°C; L, constant temperature exposure to low (L) 17°C. GLU, glucose (mg dl^{-1}); urea (mg dl^{-1}); CREA, creatinine (mg dl^{-1}); uric Ac uric acid (mg dl^{-1}); Tot Bil, total bilirubin (mg dl^{-1}); Bil Ac, bile acid (μmol dl^{-1}); CHOL, cholesterol (mg dl^{-1}); TRIG, triglycerides (mg dl^{-1}); HDL, high-density lipoprotein; TP, total protein (mg dl^{-1}); Alb, albumin (g dl^{-1}); Ast, aspartate aminotransferase (U L^{-1}); Alp, alkaline phosphatase (U L^{-1}) CK, creatine kinase (U L^{-1}); LDH, lactate dehydrogenase (U L^{-1}); Ca^{+2}, calcium (mg dl^{-1}); P, inorganic phosphorus (mg dl^{-1}); K^{+} potassium (mEq L^{-1}); Na^{+}, sodium (mEq L^{-1}); Fe, iron (μg dl^{-1}); Cl, chloride (mEq L^{-1}); Mg, magnesium (mg dl^{-1}); CORT, cortisol (μg dl^{-1}); ALB/GLO, albumin/globulin; CaxP, calcium*phosphorus; Na/K, sodium/potassium.*

reads (mean ± SD, 24,286 ± 6,505) and clustered into a total of 2,726 ASVs, of which 2,434 were assigned at family level and 2,002 were assigned at genus level. In order to assess whether the different diets (L16 and L21) result in a specific gut microbiome response to water temperature changes, for each dietary regime, the gut microbiome was sampled before and after the HL (autumn shift) and LH (summer shift) temperature transitions. The correspondent variations in the gut microbiome profiles were assessed by the PCoA of the unweighted UniFrac distances between samples collected at the different temperatures, the taxa most explaining sample segregation are superimposed on the bidimensional space. Finally, for each diet and temperature transition, changes in the gut microbiome internal diversity are shown according to three different metrics: PD_whole_tree, Chao1, and observed_ASVs.

According to our findings (**Figures 2A,B**), under the L16 dietary regime, only the HL transition (autumn shift) resulted in a significant variation in the overall gut microbiome composition, both in terms of overall compositional structure ("Adonis," $p = 0.001$) and in terms of reduction in the internal ecosystem diversity (Kruskal–Wallis test $p = 0.002$; $p = 0.002$; $p = 0.006$). Particularly, the transition to low temperature brings about the reduction in *Bacillus* in the fish gut microbiome. Conversely, in fishes fed with the L21 diet, both the LH (summer shift) and HL (autumn shift) resulted in significant gut microbiome compositional changes (**Figures 2C,D**, "Adonis," $p < 0.01$). However, in these conditions, no significant variations in the gut microbiome compositional diversity were observed.

We next investigated whether the different diets were associated with specific gut microbiome compositional structure in fishes grown at high or low temperatures. To this end, the PCoA of the unweighted UniFrac distances of the gut microbiome composition of fishes consuming L16 or L21 diet is provided at both warm (**Figure 3A**) and cold (**Figure 3B**)

TABLE 5 | Plasma biochemistry values for gilthead sea bream fed the experimental diets and exposed to two different temperatures after temperature switch.

	After temperature switch—day 121						
	L	**H**	**L**	**H**	***Inter***	***Temp***	***Diet***
GLU	74.57 ± 15.51^{a}	99.73 ± 13.99^{b}	73.67 ± 15.40^{a}	106.14 ± 31.67^{b}	*0.737*	*0.000*	*0.878*
Urea	8.05 ± 1.80	8.89 ± 0.95	8.16 ± 1.80	8.60 ± 1.67	*0.677*	*0.187*	*0.839*
CREA	0.17 ± 0.02^{ab}	0.19 ± 0.03^{b}	0.16 ± 0.02^{a}	0.18 ± 0.03^{ab}	*0.558*	*0.002*	*0.299*
Uric Ac	0.03 ± 0.03^{a}	0.03 ± 0.03^{a}	0.03 ± 0.02^{a}	0.08 ± 0.06^{b}	*0.034*	*0.014*	*0.014*
Tot Bil	0.03 ± 0.02^{a}	0.04 ± 0.02^{b}	0.02 ± 0.02^{a}	0.03 ± 0.01^{a}	*0.081*	*0.036*	*0.011*
Bil Ac	11.02 ± 2.40^{a}	35.77 ± 25.98^{bc}	13.87 ± 6.61^{ab}	38.81 ± 34.62^{c}	*0.987*	*0.000*	*0.633*
CHOL	214.15 ± 26.58	219.53 ± 26.11	216.57 ± 42.01	213.83 ± 34.33	*0.653*	*0.884*	*0.856*
TRIG	316.07 ± 118.68	473.40 ± 249.43	477.50 ± 265.41	353.58 ± 172.86	*0.018*	*0.772*	*0.719*
HDL	75.43 ± 10.66	70.73 ± 9.51	67.93 ± 14.49	70.67 ± 10.66	*0.236*	*0.753*	*0.228*
TP	3.85 ± 0.35^{b}	3.64 ± 0.27^{ab}	3.51 ± 0.33^{a}	3.40 ± 0.36^{a}	*0.577*	*0.078*	*0.002*
ALB	0.98 ± 0.10^{c}	0.96 ± 0.08^{bc}	0.85 ± 0.07^{a}	0.87 ± 0.11^{ab}	*0.391*	*0.918*	*0.000*
AST	19.08 ± 11.47^{ab}	14.93 ± 9.86^{a}	35.00 ± 29.77^{b}	32.33 ± 17.51^{ab}	*0.887*	*0.515*	*0.002*
ALP	83.71 ± 33.37^{a}	216.47 ± 117.78^{b}	168.87 ± 70.95^{ab}	256.42 ± 126.14^{b}	*0.372*	*0.000*	*0.016*
CK	135.50 ± 163.01	54.40 ± 45.02	81.20 ± 74.99	49.50 ± 39.93	*0.338*	*0.032*	*0.251*
LDH	411.38 ± 280.24	266.13 ± 250.62	751.00 ± 788.72	657.50 ± 568.17	*0.857*	*0.406*	*0.013*
Ca^{+2}	12.48 ± 0.58^{b}	12.43 ± 0.69^{ab}	11.79 ± 0.63^{a}	12.35 ± 0.78^{ab}	*0.091*	*0.159*	*0.036*
P	10.90 ± 1.09	11.03 ± 0.80	11.02 ± 1.38	11.17 ± 1.55	*0.981*	*0.660*	*0.691*
K^{+}	5.11 ± 0.60^{ab}	4.83 ± 0.66^{a}	5.60 ± 0.54^{b}	5.54 ± 0.51^{b}	*0.480*	*0.262*	*0.000*
Na^{+}	187.57 ± 4.65	187.40 ± 3.91	189.27 ± 4.70	191.79 ± 7.04	*0.327*	*0.392*	*0.030*
Fe	102.00 ± 21.49	99.80 ± 23.04	95.93 ± 18.20	89.00 ± 17.85	*0.666*	*0.406*	*0.128*
Cl	164.20 ± 4.68	162.99 ± 2.91	166.83 ± 4.58	166.37 ± 5.21	*0.747*	*0.473*	*0.012*
Mg	2.70 ± 0.23	2.76 ± 0.18	2.69 ± 0.23	2.81 ± 0.20	*0.617*	*0.107*	*0.732*
CORT	21.18 ± 16.40	16.89 ± 9.17	13.97 ± 11.42	17.65 ± 7.99	*0.196*	*0.921*	*0.295*
ALB/GLO	0.34 ± 0.01^{b}	0.36 ± 0.01^{b}	0.32 ± 0.02^{a}	0.34 ± 0.02^{b}	*0.624*	*0.000*	*0.000*
CaxP	136.29 ± 17.88	137.53 ± 17.32	130.40 ± 21.54	138.67 ± 25.71	*0.529*	*0.394*	*0.670*
Na/K	37.14 ± 4.42^{ab}	39.67 ± 6.15^{b}	34.13 ± 3.76^{a}	34.86 ± 3.53^{a}	*0.460*	*0.185*	*0.002*

*Data are given as the mean ($n = 9$ diet^{-1} on day 58; $n = 15$ diet^{-1} on day 121) ± SD. Different letters indicate significant difference (two-way ANOVA, $P \leq 0.05$) between treatments. L16, low-lipid 16% diet; L21, high-lipid 21% diet; H, constant temperature exposure to high (H) 23°C; L, constant temperature exposure to low (L) 17°C. GLU, glucose (mg dl^{-1}); Urea (mg dl^{-1}); CREA, creatinine (mg dl^{-1}); Uric Ac, uric acid (mg dl^{-1}); Tot Bil, total bilirubin (mg dl^{-1}); Bil Ac, bile acid (μmol dl^{-1}); CHOL, cholesterol (mg dl^{-1}); TRIG, triglycerides (mg dl^{-1}); HDL, high-density lipoprotein; TP, total protein (mg dl^{-1}); Alb, albumin (g dl^{-1}); Ast, aspartate aminotransferase (U L^{-1}); Alp, alkaline phosphatase (U L^{-1}); CK, creatine kinase (U L^{-1}); LDH, lactate dehydrogenase (U L^{-1}); Ca^{+2}, calcium (mg dl^{-1}); P, inorganic phosphorus (mg dl^{-1}); K^{+}, potassium (mEq L^{-1}); Na^{+}, sodium (mEq L^{-1}); Fe, iron (μg dl^{-1}); Cl, chloride (mEq L^{-1}); Mg, magnesium (mg dl^{-1}); CORT, cortisol (μg dl^{-1}); ALB/GLO, albumin/globulin; CaxP, calcium*phosphorus; Na/K, sodium/potassium; SD, standard deviation.*

growth temperature. For each temperature, the internal gut microbiome diversity corresponding to both diets is also provided. According to our findings, only at high temperature did the different diets show a significantly different gut microbiome layout ("Adonis," $p = 0.01$), with L16 diet resulting in a higher load of *Lactobacillus*. On the contrary, no dietary impact on ecosystem diversity was observed, independent from the temperature.

The overall composition of the sea bream gut microbiome at different phylogenetic levels is represented in **Figure 4**: phylum in **Figure 4A** and family in **Figure 4B**. For all experimental groups, the most abundant taxa were Firmicutes, Proteobacteria, and Actinobacteria, which represented about 88% of the whole gilthead sea bream gut microbiota (**Figure 4A** and **Supplementary Table 6**). At family level, the gilthead sea bream gut bacterial community was dominated almost entirely by *Lactobacillaceae*, which represented around 60% of the whole ecosystem in all groups (**Figure 4B**). Interestingly, focusing on the genus level, specific compositional differences were detectable among the groups studied (Wilcoxon rank-sum test $p < 0.05$) (**Figure 5**). In particular, according to our data, for sea bream receiving L16 diet, the HL transition (autumn shift) resulted in a significant decrease in *Bacillus* and *Planctomycetaceae*, while for fish fed with L21 diet, the same shift resulted in the reduction of *Planctomyces* (Wilcoxon rank-sum test $p = 0.008$, $p = 0.016$, $p = 0.033$, respectively). On the other hand, for both diets, the LH transition (summer shift) gave a significant increase in *Methylobacterium* (Wilcoxon rank-sum test $p = 0.012$, $p = 0.033$). Finally, the L16 diet in the HL transition (autumn shift) favored an increase in *Weissella* and *Bradyrhizobium* genera in the gut microbiome, resulting in a significantly higher relative abundance of these genera in the final condition compared with fish fed with L21 diet in the same condition (Wilcoxon $p = 0.014$, $p = 0.026$), while L21 diet in the final condition of LH (summer shift) transition favored a significant increase in *Streptococcus* and *Bacillus* genera compared with L16 diet in the corresponding condition (Wilcoxon $p = 0.015$, $p = 0.011$).

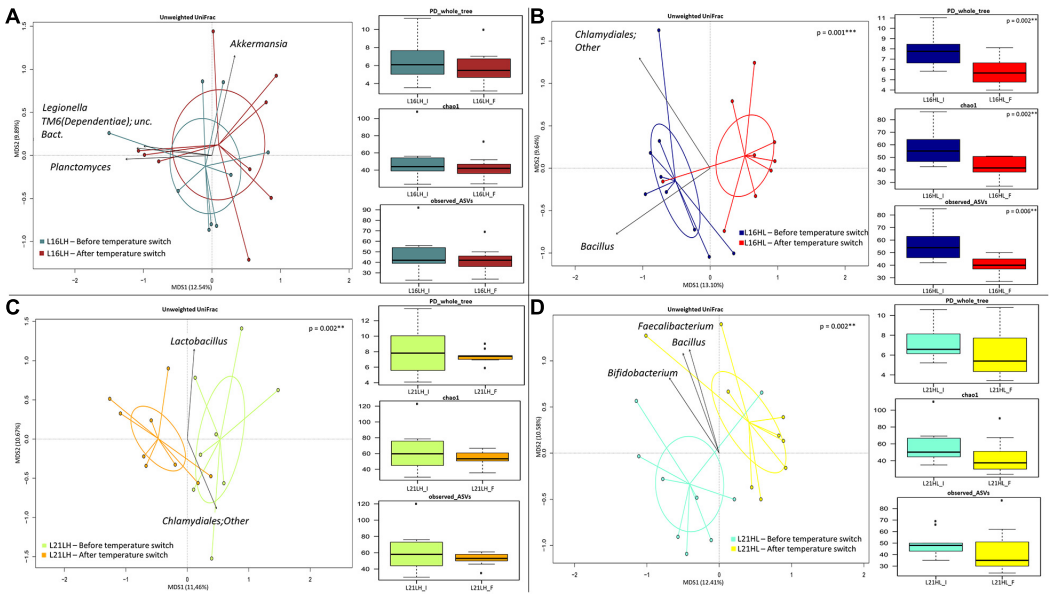

FIGURE 2 | Beta diversity and alpha diversity of gut microbiota of gilthead sea bream fed with the experimental diets and exposed to temperature switch over 121 days. **(A,B)** PCoA based on unweighted UniFrac distances between gut microbiota structure of animals fed with L16 diet and exposed, respectively, to summer shift (LH transition) and autumn shift (HL transition). Samples are significantly separated, only in the autumn shift condition (permutation test with pseudo-F ratios Adonis; $p = 0.001$). **(C,D)** PCoA based on unweighted UniFrac distances between gut microbiota structure of animals fed with L21 diet and exposed, respectively, to summer shift (LH transition) and autumn shift (HL transition). Samples are significantly separated in both conditions (permutation test with pseudo-F ratios Adonis; $p = 0.002$, $p = 0.002$). Black arrows are obtained by fitting the genus relative abundance values for each sample within the ordination space (function envfit of the vegan R package, with a p-value < 0.01). In each panel, boxplots show alpha diversity values, measured by Faith's Phylogenetic Diversity (PD_whole_tree), Chao1 index, and amplicon sequence variants (observed_ASVs). Only for the HL group **(B)**, all metrics showed a significant reduction (Kruskal–Wallis test $p < 0.01$) of alpha diversity in the final condition of group fed with L16 diet and subjected to a temperature switch toward autumn temperature (HL). L16, low-lipid 16% diet; L21, high-lipid 21% diet; HL, constant temperature exposure to high (H) temperature of 23°C before, and to low (L) temperature of 17°C after, temperature switch; LH, constant temperature exposure to low (L) temperature of 17°C before, and to high (H) temperature of 23°C after, temperature switch. Temperature switch occurred on day 58.

DISCUSSION

Though many studies have been conducted on the effect of water temperature on growth, physiological responses, and health in gilthead sea bream, so far, very few have investigated the possible interaction between temperature switch simulating seasonal variation and dietary lipid level, and no work exists on its capability to affect gut microbiota.

The growth parameters observed throughout the overall trial (fish encountering temperature switch between 23 and 17°C and vice versa), within 16 or 21% dietary lipid levels, showed similar performance in terms of growth (FBW, WG, and SGR). However, overall FCR was higher in animals entering low temperature (17°C, HL) in both diets. This significant difference was mainly due to the observed negative effect of temperature on FCR when fish moving from high to low temperature exhibited higher values and higher relative increments. Similarly, our study agrees with the "winter growth arrest" described by Sánchez-Nuño et al. (2018a), where gilthead sea bream brought from 22°C down to 14°C showed a doubling of FCR and a fourfold drop of SGR. Before the temperature change (day 0–58), temperature alone regulated fish growth rather than dietary lipid, and no differences in overall performance were detected within the temperature regimes tested. Our findings are in agreement with previous studies that found no differences in growth and feed utilization when feeding sea bream juveniles at increasing dietary lipid levels at constant high temperatures (Velázquez et al., 2006; Bonaldo et al., 2010; Mongile et al., 2014). Interestingly, in our study, after temperature change (day 59–121), low dietary lipid gained more influence, bringing a compensatory growth effect. In fact, 62 days after temperature change, L16 diet seemed to compensate better for the differences in SGR occurring between days 0 and 59. Furthermore, L16 was better accepted (higher FI values and higher relative increment) by animals passing from 17 to 23°C. It should also be mentioned that the differences in the initial body weight between high and low temperature recorded after the temperature switch could have also interfered in the final overall results.

Concerning lipid efficiency, overall results of LER and GLE indicated that at the same temperature regime, low dietary lipids guaranteed better lipid utilization rather than high ones both before and after temperature change; again, low lipid diet and high temperatures led to better lipid utilization by fish, confirming previous study statements (Velázquez et al., 2006; Bonaldo et al., 2010; Mongile et al., 2014). Feeding 16 or 21% lipid diets did not make any difference in HSI, as observed in other previous studies (Velázquez et al., 2006; Bonaldo et al., 2010; Mongile et al., 2014; Melis et al., 2017). However, HSI increased

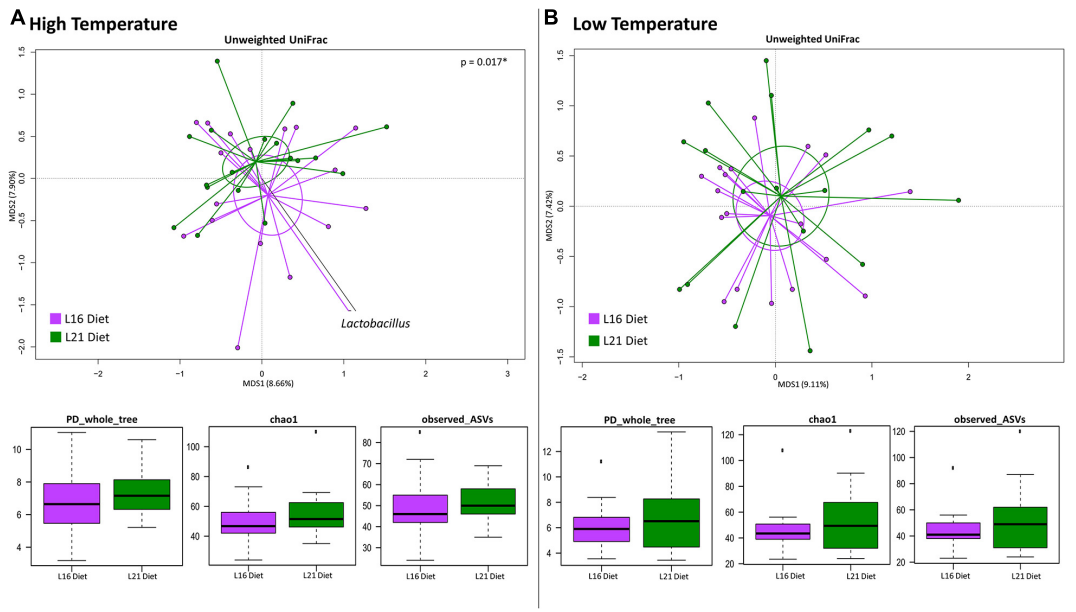

FIGURE 3 | Beta diversity and alpha diversity of gut microbiota of gilthead sea bream fed with the experimental diets at both warm and cold temperatures. **(A)** PCoA based on unweighted UniFrac distances between gut microbiota structure of animals fed with L16 and L21 diets and grown at warm temperature. Samples are significantly separated (permutation test with pseudo-F ratios Adonis; $p = 0.017$). **(B)** PCoA based on unweighted UniFrac distances between gut microbiota structure of animals fed with L16 and L21 diets and grown at cold temperature. Samples are not significantly separated (permutation test with pseudo-F ratios Adonis; $p > 0.05$). Black arrows are obtained by fitting the genus relative abundance values for each sample within the ordination space (function envfit of the vegan R package, with a p-value < 0.01). For both temperature conditions, all metrics used to assess alpha diversity did not show a significant variation between the two experimental diets (as highlighted by the boxplots in both panels). L16, low-lipid 16% diet; L21, high-lipid 21% diet.

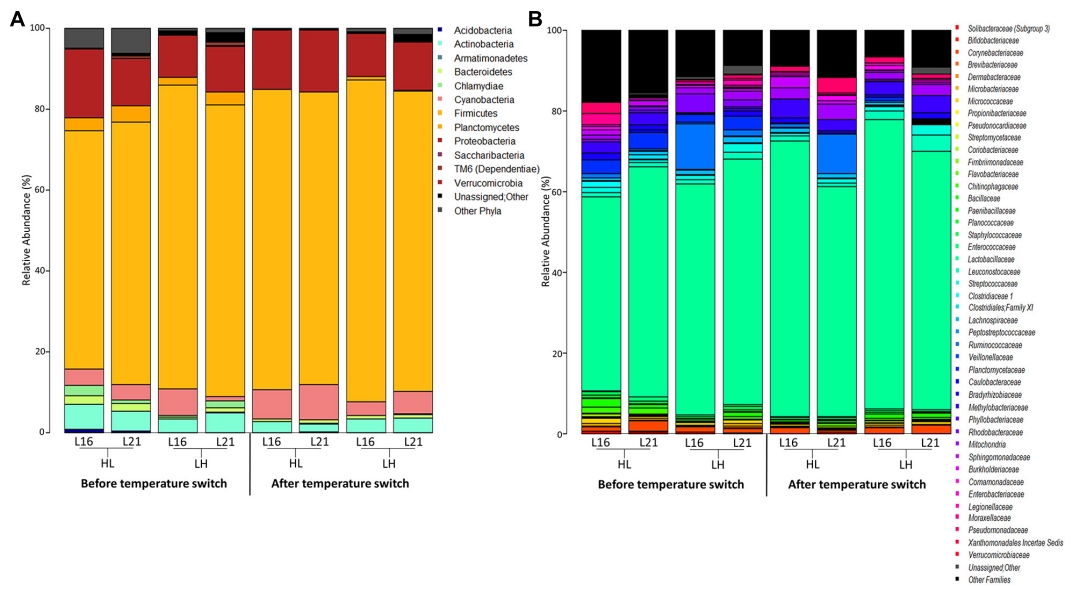

FIGURE 4 | Microbiota composition of distal gut content of gilthead sea bream fed with the experimental diets and exposed to temperature switch over 121 days. Bar plot summarizing the microbiota composition at phylum **(A)** and family level **(B)** of fish intestinal content. Only phyla with a relative abundance ≥ 0.1% in at least 10 samples, and families with relative abundance ≥ 0.1% in at least 10 samples are represented. L16, low-lipid 16% diet; L21, high-lipid 21% diet; HL, constant temperature exposure to high (H) temperature of 23°C before, and to low (L) temperature of 17°C after, temperature switch; LH, constant temperature exposure to low (L) temperature of 17°C before, and to high (H) temperature of 23°C after, temperature switch. Temperature switch occurred on day 59.

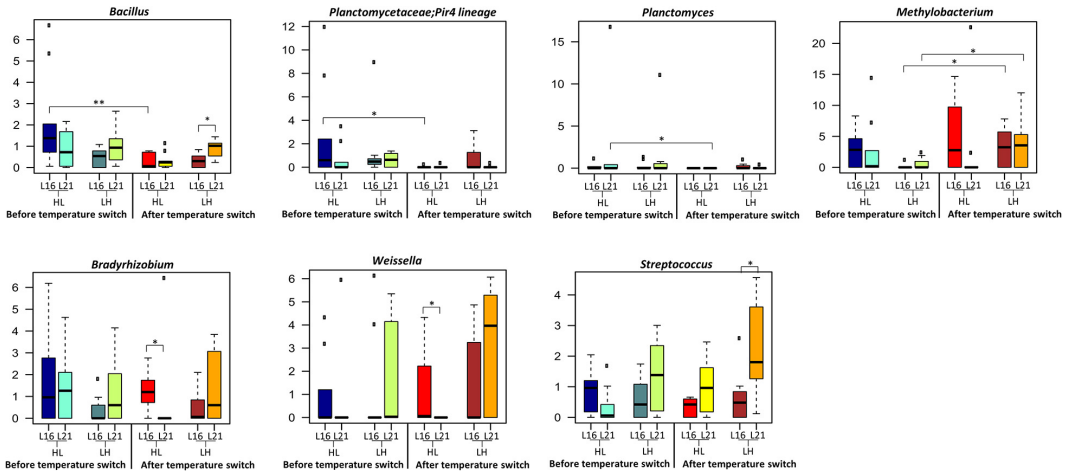

FIGURE 5 | Taxonomic composition of bacterial communities of distal gut content of gilthead sea bream fed with the experimental diets and exposed to temperature switch over 121 days. Distributions of relative abundance of genera that showed a significant variation between groups fed with different diets or after the temperature switch (Wilcoxon rank-sum test, $^{**}p \leq 0.01$; $^{*}p \leq 0.05$), only genera with a mean relative abundance $\geq$ 1.0% in at least one group were represented. The central box of each dataset represents the distance between the 25th and the 75th percentiles. The median between them is marked with a black line. L16, low-lipid 16% diet; L21, high-lipid 21% diet; HL, constant temperature exposure to high (H) temperature of 23°C before, and to low (L) temperature of 17°C after, temperature switch; LH, constant temperature exposure to low (L) temperature of 17°C before, and to high (H) temperature of 23°C after, temperature switch. Temperature switch occurred on day 59.

by 22–35% from H to L temperature, and though no statistical difference occurred in fat liver content, liver lipid content tended to be higher at 23°C. In contrast, most of the current literature, focused on metabolic and physiological responses of this species to low temperatures, observed an increase in the hepatosomatic index (HSI), which is explained by a higher mobilization of lipids due to fat mobilization and hepatic deposition caused by cold temperatures (Ibarz et al., 2005, 2007; Mininni et al., 2014). In our study, before temperature change, MFI, VSI, and their relative variations were not influenced by diet nor temperature. These findings are consistent with those of Mongile et al. (2014), where dietary lipid from 20% up to 24% did not have any effect in gilthead sea bream maintained at 27°C. However, in contrast with the above mentioned author's findings, 21% lipid diet caused slightly higher MFI levels after temperature change. In addition, a significant interaction indicated that the combined effect of temperature increase and L16 led to a higher VSI than L21.

To further explain the growth responses of gilthead sea bream after undertaking seasonal thermal changes, a spotlight on digestive enzymatic activity was performed. In our study, before the temperature change, pepsin activity was higher in fish fed high lipid (L21). However, after the temperature switch, a significant interaction indicated that the combined effect of low lipid (L16) and low temperature guaranteed a higher activity of this enzyme in animals subjected to a lower temperature of 17°C (HL). For both before and after temperature switch, our study reported no temperature-significant influence on pepsin activity. However, after the temperature switch, pepsin activity in L16 showed a general increasing pattern compared with that in the period before the temperature change, while its activity tended to decrease under L21. On the contrary, in on-growing cobia (*Rachycentron canadum*) reared at two different temperatures (30°C and 34°C), higher pepsin activity was attributed to animals reared at a high temperature of 34°C (Nguyen et al., 2019; Yúfera et al., 2019). Yet those subjects had higher FCR rather than others reared at 30°C and fed the same daily ration (Nguyen et al., 2019; Yúfera et al., 2019). The authors stated that in cobia reared at a higher temperature, increased pepsin activity could not improve growth owing to increased gastric transit rate (Yúfera et al., 2019). Unlike pepsin, trypsin appeared to be influenced by thermal changes. Before the temperature change, trypsin level was slightly higher at 17°C in each diet; then after the thermal switch, its activity displayed higher values at 23°C but relative decreasing changes ranging from 40 to 59% were observed for all the treatment. Our trypsin levels found before thermal change are consistent with results found in European sea bass (*Dicentrarchus labrax*) reared at three different water temperatures (17, 20, and 23°C) where the trypsin activity peaked at the lowest temperature of 17°C (Pereira et al., 2018). Temperature and dietary lipid level also affected FI, which could have resulted in variation of enzymatic activity. In addition, dietary lipid level may affect gastric transit rate as reported by Bonvini et al. (2018b) in European sea bass and by García-Meilán et al. (2013) in gilthead sea bream, with possible consequences on enzyme activity. In the present study, chymotrypsin activity was influenced only by diet, being more elevated in fish fed 21% dietary lipid level rather than 16% both before and after temperature change. Similarly, while temperature did not affect chymotrypsin, dietary regime was shown to improve consistently its activity in European sea bass reared at 17°C (Pereira et al., 2018). As regard amylase activity, while before the temperature change no dietary or thermal effect occurred, after the temperature switch, it was significantly impeded, with lower activity values and higher relative reduction in fish brought to 17°C (HL). This temperature

influence reinforces the hypothesis that when fish are subjected to colder temperatures, feeding absorption drops, while in the liver, a metabolic reassessment takes place for glycogen synthesis, accumulation, and storage (Silva et al., 2014; Melis et al., 2017; Sánchez-Nuño et al., 2018b). Similarly, lipase activity also was not influenced by diet or temperature before temperature change. Afterward, lipase showed a general reduction in activity with higher values in L21 and at high temperature. This is in contrast with Arantzamendi et al. (2019), where bile salt-activated lipase activity (BAL) of gilthead sea bream maintained at constant optimal water temperature (within 20 and 24.2°C) tended to increase with age throughout the life cycle.

Plasma cortisol and glucose levels are the first and main metabolites being released into the plasma as response markers to stress (Barton, 2002). Before the seasonal temperature change, fish fed L16 showed cortisol levels higher than L21 at each considered temperature, and a similar tendency, though not significant ($p = 0.066$), was noticed also in glucose. Afterward, cortisol was not influenced by any factor, while glucose increased in animals brought from 17 to 23°C (LH). Our findings on glucose levels are in contrast with cold-induced hyperglycemia observed in studies on gilthead sea bream undertaken in both outdoor and indoor conditions (Rotllant et al., 2001; Faggio et al., 2014; Matias et al., 2018).

Total protein is a liver impairment marker, and increase in concentration can be caused by structural liver alterations such as aminotransferase activity reduction, leading to a concurrent reduction in deamination capacity (Bernet et al., 2001). Among them, ALB was found to be the major plasma protein in 16–18°C acclimated gilthead sea bream, representing 25–30% of the TP. In our study, TP level was greater at lower temperatures, and after temperature change, both TP and ALB were found to be higher in animals fed lower lipid (L16). Our findings are in accordance with the significantly higher plasma TP levels of gilthead sea bream exposed to 13°C compared with those with the same thermal history maintained at 23°C described by Mateus et al. (2017). On the other hand, Sala-Rabanal et al. (2003) reported a decrease in plasma protein fractions in gilthead sea bream exposed to both acute or gradual water thermal decrease to 8, 12, and 14°C during 15 and 20 days long. AST, ALP, and LDH are non-specific plasma enzymes, indicators of tissue damage owing to pathological processes, toxic chemical exposure, or traumatic fish handling (Peres et al., 2013). In the present study, AST, ALP, and LDH were influenced only by temperature before the temperature change. While LDH and AST levels were higher at 17°C, ALP was very high in animals kept at 23°C. Then, after the temperature switch, ALP, AST, and LDH were found to be influenced by dietary lipids, with greater levels in response to high dietary lipid diet (L21). In the present study, the elevated blood LDH activity found at low temperatures before temperature changes could probably be caused by lactate accumulation in aerobic tissues such as red muscle and heart indicating an activation of the anaerobic component of metabolism during exposure to cold (Faggio et al., 2014; Feidantsis et al., 2020b). Before temperature switch, TRIG and HDL were more elevated at 17°C. These results are consistent with increased triglyceride levels found during the colder months, interpreted as a mobilization of the lipid deposits to use as fuels by Faggio et al. (2014). Though most previous studies revealed that cold water conditions for gilthead sea bream caused an imbalance in plasma ion levels (Rotllant et al., 2001; Gallardo et al., 2003; Sala-Rabanal et al., 2003; Vargas-Chacoff et al., 2009; Mateus et al., 2017), in the present study, they mostly changed according to dietary lipid content rather than temperature changes. Indeed, before the temperature change, calcium, phosphorus, and sodium were more elevated in animals fed high lipid diet (L21). Then, after temperature change, while potassium, sodium, and chloride remained higher in accordance with a high lipid diet, calcium was found to be more elevated in fish fed L16 diet. In our study, iron was the only ion influenced by temperature with higher values in animals kept at 23°C before the temperature change. In the present study, it should be mentioned that the high rate of temperature change (3° day^{-1}) could have induced physiological stress during the first days after the thermal switch. In fact, according to Feidantsis et al. (2020a,b). This species showed 3–5 days of adaptive cellular response to stress when a sudden thermal changes occurred in the range of 18–24°C.

The study of the gut microbiota has received great attention in the aquaculture sector as an indicator of productivity and fish health, and it is likely that its manipulation will be achieved in the near future in several fish species of commercial interest. Several studies have recently addressed the effect of diet (Huyben et al., 2020; Rimoldi et al., 2020), rearing density (Parma et al., 2020), age, sex (Piazzon et al., 2019), and genetic background (Piazzon et al., 2020) on the gut microbiota of gilthead sea bream; however more studies to detect dynamical changes of microbial composition during the farming cycle are necessary (Infante-Villamil et al., 2020). In the present study, at high phylogenetic levels, the overall gut microbiome structure was similar among groups, and the main represented taxa at phylum (Firmicutes, Proteobacteria, and Actinobacteria) and family (*Lactobacillaceae*) levels are consistent with previous trials on this species reared on similar aquafeed formulation and feeding protocols (Parma et al., 2016, 2020). According to our findings, the impact of the L16 and L21 diets on the overall gut microbiome was dependent on growth temperature. Indeed, only high temperature led the two diets associated with different gut microbiome compositional layouts with L16 diet resulting in a higher load of *Lactobacillus.* The dominance of *Lactobacillaceae* mainly *Lactobacillus* has been considered a valid indicator of optimal gut health condition in sea bream (Parma et al., 2016, 2020). Interestingly, the two diets performed differently in terms of microbiome response to the temperature transitions. In particular, while fish fed with L16 diet showed significant gut microbiome changes only at the autumn shift, parallel with a reduction in ecosystem diversity, for the L21 diet, both summer and autumn temperature shifts resulted in significant variations in the ecosystem. Temperature is known to modulate microbial diversity in animals especially in poikilothermic fish species (Sepulveda and Moeller, 2020); however, data explaining the interaction between diet and temperature changes in fish are scarce (Soriano et al., 2018; Busti et al., 2020b; Pelusio et al., 2020). Interestingly, among the few studies, which underlined the combined effect of temperature and dietary lipid level, Soriano et al. (2018), in yellowtail kingfish, detected a reduced bacterial abundance and richness associated

to a suboptimal low temperature and low dietary lipid level, suggesting that gut microbiome composition could maintain high relative abundance after the decrease in temperature only in the presence of appropriate nutritional conditions, pointing out the importance of optimal lipid level at low temperatures. On the other hand, in the present study, the temperature increase from 17 to 23°C showed a significant impact on the diversity (β-diversity) only in a high-lipid diet. As concerns the specific gut microbiome compositional changes, the decrease in temperature from 23°C to 17°C leads to a significant reduction in *Planctomycetaceae* and *Bacillus*. *Bacillus* is one of the most important beneficial taxa in fish species, which can make a positive contribution to nutrition, to the immune system, and to disease resistance toward pathogens by producing bacteriocins. This decreasing effect may be in line with the sensibility of sea bream to thermal reduction; however, it should be mentioned that, although there has been a significant decrease in this bacterial taxa only under L16, its value was higher in comparison with L21 at the same time point examined. After the temperature decrease from 23 to 17°C, fish fed L16 also showed a significantly higher abundance of *Weissella* in comparison to L21. This taxon, belonging to lactic acid bacteria (LAB), is of potential interest for its application as a probiotic in aquaculture (Mortezaei et al., 2020; Ringø et al., 2018, 2020) and has been shown to improve intestinal health and the hemato-parameters of hybrid surubim (*Pseudoplatystoma reticulatum* female × *P. corruscans*) male (Jesus et al., 2017).

In L16, the change toward high temperature was characterized by a significant increase in the relative abundance of *Methylobacterium*. Although with contradictory results, the abundance of *Methylobacteriaceae* in fish gut has been previously associated with environmental temperature change in tench, *Tinca tinca*, and the sparids pinfish, *Lagodon rhomboids* (Givens, 2012; Dulski et al., 2020). *Methylobacterium* has also been associated as beneficial microbial taxa with Nile Tilapia fed functional ingredients (Zheng et al., 2018). Focusing on the dietary effect after the increase in temperature, L21 showed a significantly higher abundance of *Bacillus* and *Streptococcus* compared with L16. Dietary lipid content and composition is known to potentially affect gut microbiota composition of animals, although very few studies in aquatic species are available. In mice, high-calorie diets can affect gut microbiota, reducing bacterial diversity and altering the ecosystem in favor of opportunistic taxa (Bruce-Keller et al., 2020). Also, in zebrafish, the increase in dietary fat from 5 to 15% led to reduced gut microbiome diversity (Falcinelli et al., 2015), and a high-fat diet (24% vs. 8%) fed to overfeeding affected the gut microbiome composition (Navarro-Barró et al., 2019). In this last-mentioned study, the authors revealed an increase in the abundance of Proteobateria, which has been proposed as a possible sign of gut microbiome imbalance in fish species. This is also in agreement with the observed increased taxa (*Enterobacteriaceae*) belonging to this phylum in sea bass gut microbiome, which experienced inflammatory gut mucosa after exposure to high temperature and low-oxygen condition (Busti et al., 2020b). In the present study, we did not observe a gut microbiome imbalance related to the lipid level tested, which remains within a general optimal requirement for this species. However, the significant increase in *Streptoccoccus* under L21 compared with L16 at the end of the trial may deserve specific attention. *Streptoccoccus* is considered one of the most common pathogens in aquaculture (Ringø et al., 2018). These taxa were indicative of dysbiosis in olive flounder, *Paralichthys olivaceus*, after antibiotic treatment (Kim et al., 2019), and in gilthead sea bream, its significant increase was associated with low fishmeal diet and high rearing density conditions (Parma et al., 2020). Finally, it should be mentioned that fish gut microbiome may also change within the same individual in different parts of the intestine due to their physiological differences (Piazzon et al., 2019), and according to Jones et al. (2018), bacterial community in the midgut of rabbitfish (*Siganus fuscescens*) hosted operational taxonomic units (OTUs) related to environmental sources, while hindgut hosted OTUs that appeared to be specialized in the role of fermentation. In this regard, further studies in gilthead sea bream should be carried out to define the interaction between microbial community and environmental changes in different traits of the intestine.

CONCLUSION

In conclusion, high dietary lipid levels, 21% did not improve growth and feed efficiency during seasonal temperature changes in comparison with low dietary lipid (16%). On the other hand, low dietary lipid improved feed intake, growth, and nutrient utilization after temperature changes, especially in fish entering optimal temperature (23°C), which simulated the spring to summer water temperature switch. In addition, after temperature switch, L16 reduced perivisceral fat. Low temperature (17°C) strongly affected overall growth performance and nutrient efficiency parameters in comparison with 23°C with major negative effects in fish experiencing summer to autumn temperature changes. After temperature changes, the combined effects of low-lipid diet and low-temperature conditions resulted in higher pepsin activity, while trypsin, chymotrypsin, and lipase were generally higher at high lipid content. The absence of a significant interaction in most of the plasma parameters examined supports the hypothesis that the combined effect of diet and temperature did not alter the metabolic plasma profile. However, the higher AST and ALP observed at the end of the trial in L21 may deserve further attention of possible negative effect on liver status when combining high dietary lipid and temperature changes. Gut microbiome composition were similar among all groups with the dominance of beneficial taxa (such as *Lactobacillus*) representative of a healthy ecosystem in this species especially in high temperature condition when L16 diet resulted in a higher load of *Lactobacillus*. In addition, after the temperature reduction, L16 was characterized by a higher abundance of the potential beneficial taxa *Weisella* spp., while the increase in temperature and L21 diet supports the growth of the potential pathogens *Streptococcus* spp. According to the results, the utilization of 16% dietary lipid levels in gilthead sea bream should be preferred when fish are exposed to temperature changes. Although the combined effects of temperature

and photoperiod was not addressed in this study, the results of the present study could give useful indication to optimize feeding strategy during summer to autumn and spring to summer temperature changes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical-Scientific Committee for Animal Experimentation of the University of Bologna (protocol ID 942/2019).

AUTHOR CONTRIBUTIONS

AB, NP, LP, and PG conceived and designed the experiment. NP, DS, and LP wrote the first draft of the manuscript. NP and LP carried out the fish maintenance and sample collection. DS, FD'A, and MC carried out the microbiota analysis and data processing. FD and EB carried out the plasma biochemistry analysis. MY, NG, and FM carried out the enzyme activity analysis. All the authors reviewed, improved the writing, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.664701/full#supplementary-material

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Dose-Dependent Effects of Histamine on Growth, Immunity and Intestinal Health in Juvenile Grouper (*Epinephelus coioides*)

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A 56 day feeding trial was conducted to examine the effects of different levels of dietary histamine on growth performance, immune response, and intestinal health of grouper (*Epinephelus coioides*). Seven isonitrogenous (46%), isolipidic (10%) diets were prepared with histamine supplement levels of 0 (T0), 0.05% (T1), 0.1% (T2), 0.15% (T3), 0.2% (T4), 0.25% (T5), and 0.3% (T6), respectively. The results showed that histamine supplementation had no significant effects on weight gain rate (WGR), specific growth rate (SGR), feed conversion rate (FCR), hepatosomatic index (HSI), and survival rate (SR) at the initial feeding period (day 0–28), but WGR and SGR had negative linear responses to the dietary histamine level at the whole feeding period (day 0–56), and a significant decrease was observed in groups T5 and T6 compared with T0 ($P < 0.05$). Supplementation of histamine decreased antioxidant capacity, immune response, the contents of serum interleukin-1 beta (IL-1β), intestinal-type fatty acid-binding protein (FABP2) and intestinal trefoil factor (ITF), and caused serious damage of intestine with significantly decreased VH and MFH of grouper, especially in fish fed with diets supplemented with high doses of histamine (0.25 and 0.3%). The intestinal microbial communities in treatments were different clearly with the control (T0), in terms of beta (β)-diversity boxplots and UPGMA phylogenetic tree based on unweighted unifrac distance. At the phylum level, the relative abundance of *Fusobacteria* was lower in group T0, while the abundance of *Firmicutes* was significantly lower in groups T5 and T6 ($P < 0.05$). At the genus level, the relative abundance of uncultured_bacterium_f_*Bacteroidales*_S24-7_group, uncultured_bacterium_f_*Lachnospiraceae,* and *Ruminiclostridium* were significantly higher in the control, while the abundance of *Cetobacterium* was significantly higher in groups T5 and T6 ($P < 0.05$). In conclusion, the present study suggested that up to 0.2% of dietary histamine did not result in a remarkable reduction in growth, immune response, and intestinal health; however, 0.25% or more dietary histamine could cause significant negative effects on growth performance, immune response, and intestinal health in *E. coioides*.

Keywords: histamine, growth performance, immune response, intestinal health, *Epinephelus coioides*

INTRODUCTION

With the rapid development of aquaculture industry worldwide in recently years, the quality of fish meal (FM), which served as the most primary protein source in aquatic feeds, has received more and more attention (Li et al., 2018; Wang et al., 2019; Ye et al., 2020; Zhai et al., 2020). High levels of histamine are usually presented in poor quality FM due to difference of original fish species, suboptimal conditions of preparation and transportation, improper storage, and so on (Higgs et al., 1995; Anderson et al., 1997; Visciano et al., 2012), resulting in a series of negative effects in aquatic animals, such as suppression of growth performance, reduction of feed utilization, inflammatory reaction, and intestinal diseases (Aksnes et al., 1997; Aksnes and Mundheim, 1997; Caballero et al., 1999; Tapia-Salazar et al., 2004). Therefore, histamine level is a useful and widely recognized parameter to assess FM quality and also as a vital safety indicator for food (Pike and Hardy, 1997; Tapia-Salazar et al., 2001; Tao et al., 2011; Biji et al., 2016).

The effects of dietary histamine exhibited variation in humans and different animal species. In humans, it was found that high dose of histamine (exceed 0.75 mg/kg body weight) may significantly enhance the risk of poisoning (Doeun et al., 2017). The European Union set regulatory ensures of histamine level below 0.2 g/kg in fresh fish and 0.4 g/kg in seafood products (Visciano et al., 2014). Moreover, suppression of growth, reduction of feed utilization and survival rate (Harry et al., 1975; Zhao et al., 2012), and gizzard lesions (Harry and Tucker, 1976) have been reported in chickens suffering from high doses of dietary histamine. The dietary histamine supplementation exerts detrimental effects in mysis (*Neomysis awatschensis* and *Neomysis japonica Nakazawa*) (Yang et al., 2010), rainbow trout (*Oncorhynchus mykiss*) (Moghaddam et al., 2015), and American eel (*Anguilla rostrata*) (Zhai et al., 2020). Li et al. (2018) found digestive system damage and liver inflammation in yellow catfish (*Pelteobagrus fulvidraco*) fed a diet supplemented with 0.1% histamine. Zhao et al. (2016) reported that high levels of histamine (4 g/kg) decreased the activity of digestive enzymes and exerted great damage to the morphology of the intestine and hepatopancreas in Chinese mitten crab (*Eriocheir sinensis*); however, dietary histamine supplementation had no significant effect on growth performance. In rainbow trout (*O. mykiss*), digestive tract damage was observed in fish fed diet containing 2 g/kg histamine (Watanabe et al., 1987; Fairgrieve et al., 1994), and dietary supplementation of histamine (2 g/kg) (Fairgrieve et al., 1994) or putrescine (13.3 g/kg) (Cowey and Cho, 1992) decreased feed consumption. Interestingly, Tapia-Salazar et al. (2001) reported that diet supplemented with 1.2–2.4 g/kg histamine/diet had a positive effect on weight gain in blue shrimp (*Litopenaeus stylirostris*).

Groupers as typical marine carnivorous fish have been widely cultured in several regions of China and Southeast Asia with the development of the intensive aquaculture industry. In China, annual production of groupers reached 183,127 tons in 2019 (China Fishery and Statistics Yearbook, 2020), for its fast growth, rich nutrition, high economic value, and consumer demand. To the best of our knowledge, study on the effects of dietary histamine on the performance of grouper is lacking, and its impact on intestinal health of fish is less reported (Zhao et al., 2016). Therefore, the present study was conducted to evaluate the effects of different doses of dietary histamine on growth performance, immune response, and intestinal health of grouper (*Epinephelus coioides*).

MATERIALS AND METHODS

Experimental Diets

A basal diet (T0; non-supplemented with histamine), served as control, was formulated (**Table 1**) based on nutritional requirements of grouper (*E. coioides*) as recommended by Yang et al. (2019). The experimental diets were prepared by supplementing the basal diet with 0.05% (T1), 0.1% (T2), 0.15% (T3), 0.2% (T4), 0.25% (T5), and 0.3% (T6) histamine (S20188; histamine [$C_5H_9N_3$] $\geq$ 98.0%; 111.15 g/mol; Shanghai yuanye Bio-Technology Co., Ltd., China). All the dietary ingredients were crushed to powders and through 60 mesh sieve and thoroughly mixed using the progressive enlargement method, then added the premixed fish oil, soybean oil and lecithin; subsequently, water with/without histamine was supplemented

TABLE 1 | Ingredients and proximate nutrient composition of the basal diet (% dry matter).

Ingredients	Content (%)
White fish meal[a]	43.0
Shrimp head meal	3.0
Soybean meal	16.88
Vital wheat gluten	7.00
Fish oil[b]	2.5
Soybean oil	2.5
Lecithin	2.0
Wheat meal	20.0
Choline chloride[c]	0.5
Vitamin premix[d]	0.5
Mineral premix[e]	0.5
$Ca(H_2PO_4)_2$	1.5
Antifungal agent	0.10
Antioxidant	0.02
Proximate nutrients composition	
Crude protein	45.90
Crude fat	10.32
Crude ash	9.17

[a]*White fish meal was obtained from Jiakang Feed Co., Ltd., Xiamen, China, imported from Peru (crude protein 68.34%, crude lipid 9.06%).*
[b]*Fish oil were obtained from Jiakang Feed Co., Ltd., Xiamen, China.*
[c]*Cholesterol was produced by Baiwei Biotechnology Holdings Co., Ltd., Hebei, China, which is extracted from pig, cattle, or sheep brain, and the minimum level is higher than 95%.*
[d]*Vitamin premix (mg kg^{-1} diet): vitamin A, 15; vitamin D3, 15; vitamin E, 75; vitamin K3, 50; vitamin B1, 50; vitamin B2, 75; vitamin B6, 75; vitamin B12, 0.3; nicotinic acid, 200; inositol, 350; D-calcium pantothenate, 200; folic acid, 9; D-biotin, 0.5.*
[e]*Mineral premix (mg kg^{-1} diet): $FeSO_4 \cdot 7H_2O$, 278; $CuSO_4 \cdot 5H_2O$, 41; $ZnSO_4 \cdot 7H_2O$, 463; $MnSO_4 \cdot 4H_2O$, 57; $MgSO_4 \cdot 7H_2O$, 2009; $CoSO_4 \cdot 7H_2O$, 3; Na_2SeO_3 0.6, $Ca(IO_3)_2$, 5.*

slowly and kept stirring until forming a dough. After pelletized, the diets were packed in plastic bags and stored at refrigerator (−20°C) until subsequent use. The histamine level in the basal diet was determined to be 158.7 mg/kg.

Animals and Experimental Conditions

The procedures for care and use of animals were approved by the Animal Care and Use Committee of Jimei University, China. The feeding trial was conducted in a recirculating water system and healthy grouper (*E. coioides*) were obtained from the Haikang Aquaculture Research Base of Dabeilong Aquaculture group. The whole study followed a strict series of rules and regulations promulgated by animal care and use committee of Jimei University, China. After acclimation (2 weeks), 30 fish (29 ± 0.52g; mean ± SE) were randomly allocated to each tank (containing 300 L seawater) and three tanks for each treatment were served as replicate, and thus 21 tanks (seven groups, and three repeats in each group) and 630 fish were used in the feed trial. Fish were hand fed one of seven diets for 56 days to apparent satiation two times daily at 08:30 and 18:30, respectively, and approximately 60% seawater was replaced daily. During the trial period, rearing water environment were monitored daily and maintained in a suitable range (temperature: 20–26°C; salinity: 30–32 g/L, pH: 7.5–8.2, dissolved oxygen [DO]: ≥7.5 mg/L; and total ammonia concentration: <0.2 mg/L).

Sample Collection

To determine the growth performance, 10 fish were randomly collected from each tank (three tanks for each treatment) after starvation for 24 h at days 28 and 56 respectively, thus 30 fish for each treatment were collected at each time point, batchwise anesthetized by 2-phenoxyethanol, and single-weighed for calculation of growth performance. Blood samples were taken from the caudal vein of 10 fish in each tank, immediately transferred into 1.5 mL Eppendorf tubes and held at 4°C overnight. Then, serum was collected and pooled following centrifugation at 10,000 r/min at 4°C for 10 min and stored in 1.5 mL Eppendorf tubes at −80°C for further analysis. Liver and intestine samples were dissected, weighed, and frozen immediately in liquid nitrogen and stored at −80°C for subsequent analysis. Foregut were randomly sampled from four fish in each tank, fixed with Bouin's fixative solution (75 ml saturated aqueous solution of picric acid, 25 ml formalin, 5 ml glacial acetic acid) for morphological determination. Intestine samples were collected from one fish per tank for intestinal microbiota analysis at day 56.

Measurement of Serum and Liver Biochemical Parameters

The total antioxidant capacity (T-AOC) and activities of alkaline phosphatase (AKP), acid phosphatase (ACP), and superoxide dismutase (SOD) in serum were evaluated spectrophotometrically using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions as previously described (Hu et al., 2019).

The liver sample was homogenized in ice-cold 0.86% physiological saline (pH: 7.2–7.4; *w: v* = 1:9), centrifuged at 3,000 rpm at 4°C for 10 min and the supernatant was collected. Protein concentration of the supernatant was determined with Coomassie brilliant blue method described by Hu et al. (2019). Malondialdehyde (MDA) level and activities of glutamic oxaloacetic transaminase (GOT) and glutamic propylic transaminase (GPT) of the supernatant were estimated using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer protocol.

Serum and Intestinal Inflammatory Factors

Interleukin-1 beta (IL-1β), serum amyloid A (SAA), and C-reactive protein (CRP) in serum were analyzed by enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). An ELISA kit was purchased from Shanghai Jianglai Biotechnology Co., Ltd. (Shanghai, China) for determination of fatty acid-binding protein 2 (FABP2) in serum, according to the manufacturer's instruction.

Intestinal samples were rinsed in 0.86% physiological saline of precooling (pH: 7.2–7.4; *w: v* = 1:9), homogenized and centrifuged (3,000 rpm, 4°C for 10 min). Protein concentration of the supernatant was measured by Coomassie brilliant blue method. Intestinal trefoil factor (ITF) was estimated by ELISA Kit provided by Shanghai Jianglai Biotechnology Co., Ltd. (Shanghai, China). The content of ITF was expressed as "pg per mg of protein."

Intestinal Morphology

Analysis of intestinal morphology by making Hematoxylin and Eosin (H&E)- stained sections as described in our previous study (Zhang J. J. et al., 2020). Briefly, the pre-fixed anterior intestinal samples with Bouin's fixative solution were subsequently dehydrated with a graded series of ethanol and cleared in xylene, then embedded in paraffin, finally sectioned at 6 um thickness. After that, sections were stained using Hematoxylin and Eosin (H&E) and mounted on glass slides. The sections were observed with positive fluorescence microscope (Leica TM 820, Nussloch, Germany), and muscular thickness (MT) and mucosal fold height (MFH) were measured using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, United States) as described in our previous study (Zhang J. J. et al., 2020).

Intestinal Microbiota Analysis

Total DNA of intestinal samples was extracted using a DNA extraction kit, and DNA integrity and quality were measured by electrophoresis on a 1% agarose gel. Then, the V3 + V4 regions of the 16S rRNA gene of intestinal bacteria was amplified with the forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by Polymerase Chain Reaction (PCR). The PCR product purity and concentration were determined with Nano-Drop®ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, United States). Subsequently, high-throughput sequencing was performed on an

Illumina HiSeq platform (Beijing Biomarker Biotechnology Co., Ltd., Beijing, China). The sequencing data have been uploaded to GenBank (Accession number, PRJNA718150). Analysis of intestinal microbiota abundance and diversity was performed using BMKCloud[1]. The detailed protocols were described in our earlier study (Yang et al., 2019).

Calculations and Statistical Analysis

The following formulas were used to calculate growth performances and feed utilization parameters: Weight gain rate (WGR,%) = 100 × (final body weight–initial body weight)/initial body weight; Specific growth rate (SGR,%/d) = 100 × (ln final body weight–ln initial body weight)/days of feeding trial; Feed conversion rate (FCR) = feed intake/(final body weight–initial body weight); Hepatosomatic index (HSI,%) = 100 × (liver weight/body weight); Survival rate (SR,%) = 100 × number of survived fish in sampling/initial number of fish allocated.

All data were subjected by one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test, which was conducted to examine significant differences among treatments using SPSS statistical package version 22.0 (SPSS Inc., Chicago, United States). The results are presented as mean values followed by the standard error of the mean (Mean ± SE) and significance was declared at the $P < 0.05$.

RESULTS

Growth Performance

The growth performance of grouper is presented in **Table 2**. With increasing histamine level, WGR and SGR tend to decrease, while FCR showed an increase trend, but no significant difference was observed at the initial feed period (day 0–28). Compared with group T0, significant increased FCR and decreased WGR and SGR were observed in the groups T5 and T6 at the whole feeding period (day 0–56) ($P < 0.05$). Neither HSI nor SR statistically differed across dietary treatments at the initial feed period (day 0–28) and the whole feeding period (day 0–56) ($P > 0.05$), and the SR were above 95.56% in all groups.

Serum Non-specific Immune Parameters

The serum non-specific immune parameters are presented in **Table 3**. At days 28 and 56, the serum ACP activities in fish fed the histamine supplemented diets were lower than those fed the control diet (T0), and significant difference was observed in fish fed the diet T6 ($P < 0.05$). Fish fed the diets T4, T5, and T6 at days 28 and 56 showed significant decreased AKP activities compared to those fed the diet T0 ($P < 0.05$), while fish fed the diet T6 exhibited the lowest AKP activity. Serum SOD activity decreased with the increment of dietary histamine in experimental diets and a significant decrease was observed in the treatment T6 compared with the T0 at days 28 and 56 ($P < 0.05$). The T-AOC activities in the histamine treatments at day 28 was significantly lower than that in the control ($P < 0.05$), with the exception of the group T2. T-AOC activity was affected negatively by all histamine treatments at day 56, while significant decrease was only observed in fish fed the diet T6 ($P < 0.05$).

Liver Biochemical Indices

The results of the liver biochemical parameters are displayed in **Table 4**. The MDA contents of fish fed the histamine supplemented diets (except for diet T1) at day 28 were significantly higher than those fed the diet T0 ($P < 0.05$). Meanwhile, compared with the group T0, significantly decreased activities of GOT and GPT were observed in group T6 ($P < 0.05$). At day 56, the MDA content in liver of fish fed diets T5 and T6 increased statistically compared with the control ($P < 0.05$). Moreover, GOT and GPT activities in groups T5 and T6 were significantly lower than those in group T0 ($P < 0.05$).

Inflammation Markers in Serum and Intestine

As can be seen from **Table 5**, serum amyloid A (SAA) and C-reactive protein (CRP) contents did not vary statistically in all dietary treatments at days 28 and 56. Compared with the control, the serum IL-1β content of fish fed diet T6 increased significantly at day 28 ($P < 0.05$). At day 56, serum IL-1β contents of fish fed the histamine supplemented diets increased statistically compare with those fed the diet T0 ($P < 0.05$), while the treatment T6 showed the highest value. In groups T4, T5, and T6, the contents of serum fatty acid-binding protein 2 (FABP2) and intestinal trefoil factor (ITF) increased significantly at day 28 as compared to the control and the highest values exhibited in fish fed diet T6 ($P < 0.05$). At day 56, the contents of serum FABP2 and intestinal ITF in group T6 were significantly higher than that in the control ($P < 0.05$).

Intestinal Morphology

Intestinal morphometrical parameters of grouper fed different diets at days 28 and 56 are shown in **Table 6**. There was a significant reduction of muscular thickness (MT) in fish fed diets T5 and T6 at days 28 and 56 compared with those fed the diet T0 ($P < 0.05$). In addition, the mucosal fold height (MFH) in groups T4, T5, and T6 at day 28 was lower significantly than the control, while the significant decrease was only observed in group T6 ($P < 0.05$). The intestine section of fish fed with diet supplemented with histamine displayed an inflammation and damage, characterized by a thin MT, reduced MFH, while the most serious impairment was observed in fish fed diets T5 and T6 with higher doses of histamine (0.25 and 0.3%) (**Figures 1**, **2**).

Intestinal Microbiota

The phylum level analysis demonstrated that *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, *Chloroflexi,* and *Acidobacteria* constituted common predominant bacterial phylum in all groups, and histamine supplemented diets significantly reduced the relative abundance of *Firmicutes* and *Bacteroidetes*, significantly increased the relative abundance of *Proteobacteria* and *Fusobacteria*, especially in groups T5

[1]www.biocloud.net

TABLE 2 | Effects of histamine on growth performance of grouper (*Epinephelus coioides*).

		Groups						
		T0	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
0–28 d	WGR (%)	138.59 ± 17.48	124.55 ± 3.90	122.56 ± 6.49	121.07 ± 8.27	120.24 ± 9.42	120.01 ± 8.60	119.05 ± 3.67
	SGR (%)	3.09 ± 0.25	2.89 ± 0.06	2.85 ± 0.10	2.83 ± 0.13	2.81 ± 0.16	2.81 ± 0.14	2.80 ± 0.06
	FCR	1.06 ± 0.08	1.13 ± 0.04	1.14 ± 0.06	1.20 ± 0.02	1.17 ± 0.10	1.18 ± 0.08	1.20 ± 0.04
	HSI (%)	2.24 ± 0.24	1.92 ± 0.23	1.78 ± 0.12	2.15 ± 0.28	2.09 ± 0.24	2.07 ± 0.16	2.03 ± 0.18
	SR (%)	98.89 ± 1.11	100.00 ± 0.00	96.67 ± 1.92	97.78 ± 2.22	97.78 ± 1.11	98.89 ± 1.11	97.78 ± 1.11
0–56 d	WGR (%)	235.43 ± 9.40^{a}	231.62 ± 8.41^{a}	228.60 ± 15.57^{ab}	217.59 ± 19.83^{ab}	216.57 ± 7.52^{ab}	214.08 ± 5.86^{b}	210.51 ± 9.90^{b}
	SGR (%)	2.16 ± 0.05^{a}	2.14 ± 0.04^{a}	2.12 ± 0.09^{ab}	2.06 ± 0.11^{ab}	2.06 ± 0.04^{ab}	2.04 ± 0.03^{b}	2.02 ± 0.06^{b}
	FCR	1.01 ± 0.06^{a}	1.10 ± 0.06^{ab}	1.13 ± 0.03^{abc}	1.13 ± 0.07^{abc}	1.13 ± 0.02^{abc}	1.19 ± 0.04^{bc}	1.26 ± 0.01^{c}
	HSI (%)	2.44 ± 0.14	2.28 ± 0.17	2.00 ± 0.15	2.37 ± 0.12	2.02 ± 0.12	2.34 ± 0.14	2.06 ± 0.11
	SR (%)	98.89 ± 1.11	95.56 ± 1.11	95.56 ± 2.22	96.67 ± 1.92	95.56 ± 1.11	95.56 ± 1.11	95.56 ± 1.11

Different characters in the same row data indicate significant differences ($P < 0.05$).

TABLE 3 | Effects of histamine on serum immune parameters of grouper (*Epinephelus coioides*).

		Groups						
		T0	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
28 d	ACP (U/100 mL)	7.34 ± 0.23^{a}	7.16 ± 0.15^{a}	7.03 ± 0.29^{a}	7.11 ± 0.14^{a}	6.80 ± 0.19^{ab}	6.72 ± 0.24^{ab}	6.19 ± 0.42^{b}
	AKP(U/100 mL)	30.50 ± 2.88^{a}	26.44 ± 2.87^{ab}	25.73 ± 1.84^{ab}	25.18 ± 0.44^{ab}	22.43 ± 1.26^{b}	22.33 ± 1.88^{b}	21.06 ± 1.20^{b}
	SOD (U/mL)	182.44 ± 3.38^{a}	180.57 ± 4.10^{a}	179.70 ± 3.69^{a}	155.58 ± 5.80^{b}	167.50 ± 9.46^{ab}	157.66 ± 3.99^{b}	131.83 ± 5.65^{c}
	T-AOC (mM)	1.56 ± 0.11^{a}	1.25 ± 0.13^{b}	1.29 ± 0.06^{ab}	1.23 ± 0.04^{b}	1.15 ± 0.03^{bc}	0.87 ± 0.17^{c}	1.12 ± 0.06^{bc}
56 d	ACP (U/100 mL)	7.15 ± 0.04^{a}	7.09 ± 0.06^{a}	7.15 ± 0.16^{a}	6.98 ± 0.22^{a}	6.88 ± 0.08^{ab}	6.84 ± 0.09^{ab}	6.60 ± 0.05^{b}
	AKP(U/100 mL)	27.21 ± 0.46^{a}	25.39 ± 0.70^{ab}	25.21 ± 1.44^{ab}	24.43 ± 0.42^{b}	22.08 ± 0.34^{c}	21.13 ± 0.36^{c}	20.56 ± 0.72^{c}
	SOD (U/mL)	155.14 ± 4.28^{a}	153.68 ± 6.56^{a}	149.19 ± 9.28^{ab}	143.08 ± 12.03^{ab}	142.79 ± 9.48^{ab}	143.25 ± 9.17^{ab}	123.26 ± 4.23^{b}
	T-AOC (mM)	1.66 ± 0.13^{a}	1.60 ± 0.07^{a}	1.59 ± 0.22^{a}	1.44 ± 0.04^{ab}	1.40 ± 0.20^{ab}	1.19 ± 0.08^{ab}	1.09 ± 0.18^{b}

Different characters in the same row data indicate significant differences ($P < 0.05$). ACP, acid phosphatase; AKP, activities of alkaline phosphatase; SOD, superoxide dismutase; T-AOC, total antioxidant capacity.

TABLE 4 | Effects of histamine on liver biochemical indices of grouper (*Epinephelus coioides*).

		Groups						
		T0	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
28 d	MDA (nmol/gprot)	2.37 ± 0.74^{a}	3.39 ± 1.86^{a}	8.83 ± 2.31^{b}	8.59 ± 2.43^{b}	9.66 ± 0.95^{b}	11.01 ± 0.62^{b}	11.13 ± 1.54^{b}
	GOT(U/gprot)	40.19 ± 0.87^{a}	39.90 ± 2.42^{a}	39.68 ± 1.20^{ab}	34.35 ± 2.54^{ab}	39.13 ± 1.62^{ab}	37.32 ± 2.95^{ab}	33.16 ± 1.15^{b}
	GPT(U/gprot)	97.04 ± 8.11^{a}	96.49 ± 13.12^{a}	85.78 ± 6.75^{ab}	73.61 ± 3.73^{ab}	75.09 ± 3.12^{ab}	85.18 ± 8.41^{ab}	62.55 ± 8.84^{b}
56 d	MDA (nmol/Gprot)	5.95 ± 1.11^{a}	8.21 ± 1.87^{ab}	8.22 ± 1.55^{ab}	8.51 ± 0.49^{ab}	8.78 ± 1.25^{ab}	11.19 ± 1.78^{b}	11.00 ± 1.73^{b}
	GOT(U/gprot)	39.57 ± 1.08^{a}	37.04 ± 1.44^{ab}	36.41 ± 1.64^{ab}	37.38 ± 1.17^{ab}	36.35 ± 0.91^{ab}	34.74 ± 1.41^{b}	35.19 ± 1.21^{b}
	GPT(U/gprot)	95.21 ± 7.11^{a}	78.40 ± 6.39^{ab}	87.18 ± 3.46^{ab}	80.32 ± 5.36^{ab}	80.11 ± 7.28^{ab}	71.73 ± 3.05^{b}	73.61 ± 4.56^{b}

Different characters in the same row data indicate significant differences ($P < 0.05$). MDA, Malondialdehyde; GOT, glutamic oxaloacetic transaminase; GPT, glutamic propylic transaminase.

and T6 ($P < 0.05$) (**Figure 3A** and **Table 7**). At the genus level, enhanced abundance of *Cetobacterium* was observed in all treatments (except T3) compared with the control (T0), as well as decreased abundances of *Ruminiclostridium* and uncultured *Bacteroidales*_S24-7_group and *Lachnospiraceae*, while significant changes were observed in high histamine groups (0.25 and 0.3%) ($P < 0.05$) (**Figure 3B** and **Table 7**).

The Beta (β)-diversity boxplots and UPGMA phylogenetic tree based on binary_jaccard distance were used to analyze the microbial similarities among different groups. From the data in **Figure 4A**, a clear separation was observed between histamine treated groups and the control (T0) at phylum level, suggesting that supplementation of histamine changed the intestinal microbial community in *E. coioides*, especially in groups T5 and T6. However, no significant differences were found for the intestinal microbial β-diversity in different treatments, although apparent difference was observed in groups T5 and T6 (**Figure 4B**).

TABLE 5 | Effects of histamine on inflammation markers of grouper (*Epinephelus coioides*).

		Groups						
		T0	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
28 d	IL-1β (ng/L)	57.69 ± 8.45^{a}	71.20 ± 0.62^{a}	73.67 ± 4.32^{ab}	79.99 ± 3.54^{ab}	73.85 ± 1.51^{ab}	80.20 ± 0.90^{ab}	101.79 ± 9.50^{b}
	SAA (μg/mL)	8.12 ± 0.43	8.80 ± 1.04	9.15 ± 1.25	8.80 ± 0.67	9.11 ± 1.02	8.95 ± 0.63	9.51 ± 0.83
	CRP (μg/mL)	7.61 ± 0.26	8.13 ± 0.40	8.12 ± 0.32	8.23 ± 0.21	8.27 ± 0.23	8.37 ± 0.15	8.40 ± 0.07
	ITF (pg/mgprot)	175.08 ± 5.71^{a}	204.53 ± 5.87^{abc}	201.15 ± 8.63^{abc}	194.40 ± 14.51^{ab}	213.74 ± 2.92^{bc}	225.38 ± 16.09^{c}	231.18 ± 4.13^{c}
	FABP2 (ng/mL)	15.79 ± 1.06^{a}	16.73 ± 1.98^{a}	18.96 ± 0.16^{ab}	19.25 ± 1.65^{ab}	21.45 ± 0.38^{b}	20.49 ± 1.13^{b}	21.91 ± 0.19^{b}
56 d	IL-1β (ng/L)	49.96 ± 9.51^{a}	77.62 ± 2.98^{b}	76.50 ± 3.00^{b}	78.92 ± 2.95^{b}	81.20 ± 4.96^{b}	74.92 ± 1.78^{b}	82.57 ± 4.33^{b}
	SAA (μg/mL)	7.39 ± 0.52	8.83 ± 0.71	9.28 ± 0.91	9.26 ± 1.06	8.13 ± 1.00	9.82 ± 1.08	10.14 ± 0.89
	CRP (μg/mL)	7.47 ± 0.40	7.72 ± 0.92	8.20 ± 0.51	7.95 ± 0.13	7.62 ± 1.10	8.25 ± 0.13	8.33 ± 0.88
	ITF (pg/mgprot)	153.51 ± 4.15^{a}	163.06 ± 9.67^{ab}	166.04 ± 1.76^{ab}	186.50 ± 7.44^{bc}	175.19 ± 9.39^{abc}	174.55 ± 10.87^{abc}	190.87 ± 3.65^{c}
	FABP2 (ng/mL)	10.97 ± 0.98^{a}	11.50 ± 0.79^{a}	12.27 ± 1.09^{a}	12.14 ± 0.83^{a}	13.11 ± 0.91^{ab}	13.81 ± 0.10^{ab}	15.51 ± 1.28^{b}

Different characters in the same row data indicate significant differences ($P < 0.05$). IL-1β, Interleukin-1 beta; SAA, serum amyloid A; CRP, C-reactive protein; ITF, intestinal trefoil factor; FABP2, intestinal-type fatty acid-binding protein.

DISCUSSION

An initial objective of the present study was to evaluate the effect of graded levels of dietary histamine on performance of grouper (*E. coioides*). In the present study, the SR was unaffected by dietary histamine, which is in line with the result of previous studies (Watanabe et al., 1987; Fairgrieve et al., 1994; Tapia-Salazar et al., 2001; Zhai et al., 2020). Furthermore, we have demonstrated that the growth performance was not statistically different among the dietary treatments at initial feeding period (0–28 days), whereas dietary histamine suppressed growth performance at the whole feeding period (0–56 days), especially when histamine supplementary doses were 0.25 and 0.3%. Similar results were reported in Nile tilapia (*Oreochromis niloticus*) (Reyes-Sosa and Castellanos-Molina, 1995), Atlantic halibut (*Hippoglossus hippoglossus*) (Aksnes and Mundheim, 1997), Atlantic salmon (*Salmo salar* L.) (Opstvedt et al., 2000), Japanese seabass (*Lateolabrax japonicus*) (Hu et al., 2013), and American eel (*A. rostrata*) (Zhai et al., 2020). In Chinese mitten crab (*E. sinensis*), however, histamine supplementation (1, 2, 4 g/kg) did not affect the growth performance (Zhao et al., 2016). Tapia-Salazar et al. (2001) studied the effect of dietary histamine supplementation in blue shrimp *Litopenaeus stylirostris* and observed that a quadratic effect between weight gain and histamine supplementation levels, while the optimum dietary histamine supplementation levels (1,200 and 2,400 mg/kg), had favorable effects on growth performance. He et al. (2018) also reported that optimum dietary histamine level (18 mg/kg) significantly improved growth performance of yellow catfish (*Pelteobagrus fulvidraco*). Diamine oxidase (DAO) is one of the crucial enzymes in charge of the exogenous histamine metabolizing and scavenging system in organisms (Smolinska et al., 2014). Several studies suggested that DAO activity exists diversity in animals due to different contents of histamine and histidine infeed (Waton, 1963; Yang et al., 2010), which is a major reason for the difference in histamine intolerance. It is possible, therefore, that the sensitivities of fish and shrimp to histamine are highly species-specific, which partly explained the discrepancy of growth performance, although the information is lacking in aquatic animals.

Serum non-specific immune parameters served as important indexes that reflect health status of animals (Yu et al., 2019; Zhai et al., 2020). AKP and ACP, two important phosphatase enzymes, play a key role in clearing extracellular invaders and immune defense (Ellis et al., 2011; Matozzo et al., 2011; Tripathi et al., 2012). In the current study, serum ACP and AKP activities decreased significantly in fish fed 0.25 and 0.3% histamine containing diets, suggesting suppressed immune response. In accord with our results, Zhai et al. (2020) noticed the reduction of serum ACP and AKP activities in American eel (*A. rostrata*) fed commercial diet (containing 217 mg/kg histamine) supplemented 300 mg/kg histamine. Another study in Chinese mitten crab (*E. sinensis*) had reported that after 6 h of histamine injection, ACP and AKP activities in the 1 and 50 mg/g (body weight) histamine treated groups were significantly lower than those in the control (Zhao et al., 2012). However, contrary results were reported that improvement of lysozyme activity in histamine treated *Tetrahymena* (Kovacs and Csaba, 1990) and increased AKP activities were observed in all histamine treated groups (50, 100, and 200 μg/kg) in rabbits (Tripathi et al., 2012). Phosphatase activity enhanced significantly during incubation of *Tetrahymena pyriformis* in histamine-supplemented cultures, and histamine was identified as a phagocytic stimulus affecting phosphatase synthesis and phagocytosis of hemocytes (Kovacs and Csaba, 1990; Ellis et al., 2011; Matozzo et al., 2011). These inconsistent effects of histamine may be due to the different administration regimes and species specificity. Generally, the serum T-AOC and SOD activities can provide an indication of the antioxidant status of organisms, while higher serum T-AOC and SOD activities might indicate higher antioxidant capacity (Reddy et al., 1991). In the present study, the significant reduction of serum T-AOC and SOD activity might indicate a diminished antioxidant capability by high dose of histamine (0.25 and 0.3%). However, Zhao et al. (2012) reported that injected histamine could increase SOD activity at 6–24 h in *E. sinensis*. In an *in vitro* study, enhancement of peroxide activity and superoxide anion production were observed in whole hemolymph of Sydney

TABLE 6 | Effects of histamine on foregut muscular thickness and mucosal fold height of grouper (*Epinephelus coioides*).

		Groups					
		T0	T1	T2	T3	T4	T5	T6
28 d	MT (μm)	101.31 ± 8.00^{a}	88.34 ± 2.43^{ab}	86.02 ± 7.08^{ab}	85.12 ± 4.55^{ab}	84.37 ± 3.87^{ab}	78.99 ± 3.83^{b}	72.31 ± 8.73^{b}
	MFH (μm)	251.63 ± 9.84^{a}	215.99 ± 20.34^{ab}	204.28 ± 20.07^{ab}	208.27 ± 15.74^{ab}	198.15 ± 16.86^{b}	173.30 ± 10.40^{b}	179.96 ± 13.60^{b}
56 d	MT (μm)	106.12 ± 9.63^{a}	98.00 ± 5.46^{ab}	84.72 ± 7.91^{ab}	85.74 ± 7.37^{ab}	97.28 ± 4.94^{ab}	83.34 ± 6.96^{b}	77.62 ± 6.29^{b}
	MFH (μm)	262.21 ± 17.60^{a}	221.55 ± 18.30^{ab}	213.96 ± 22.81^{ab}	217.76 ± 19.32^{ab}	216.79 ± 24.97^{ab}	214.41 ± 20.99^{ab}	194.50 ± 15.70^{b}

Different characters in the same row data indicate significant differences ($P < 0.05$). MT, muscular thickness; MFH, mucosal fold height.

rock oysters (*Saccostrea glomerata*) after treatment for 30 min with noradrenaline, another biogenic amine (Aladaileh et al., 2008). These paradoxes may be explained in that short-term histamine stress can rapidly improve the antioxidant capacity by compensatory mechanism; however, long-term dietary histamine damage the ability to respond to reactive oxygen intermediates, which subsequently result in oxidative damage and cause the noticed decline with T-AOC and SOD activities.

Liver biochemical indices provide generally effective information about the function and health status of liver. Malondialdehyde (MDA), an endogenous genotoxic product of enzymatic and oxygen radical-induced lipid peroxidation, is commonly used as a bioindicator of oxidative injury. Additionally, the reduction of liver GPT and GOT activities suggested probable liver damage or dysfunction (Zhang J. Z. et al., 2020). In this study, histamine was noticed to cause higher MDA level and lower activities of GPT and GOT in liver. In accordance with our results, histamine-rich diets resulted in liver injury with a significant improvement in plasma GOT and GPT activities (Li et al., 2018; Zhai et al., 2020). Study done in immunized rabbits observed that histamine may cause hepatic damage at different dosage by determining the characteristic ratios and changes in serum GPT and GOT activities (Tripathi et al., 2012). However, different from the above studies, dietary supplementation with 10 g/kg histamine did not significantly affect the plasma GOT and GPT activities in rainbow trout (Shiozaki et al., 2004). The reason for these controversial results is unclear but it may have something to do with different degree of histamine intolerance in rearing species. Interestingly, grouper fed diets supplemented low dose histamine (0.1, 0.15, and 0.2%) at day 28 showed significantly lower antioxidative capacity (estimating by determining the activity of SOD, the T-AOC, and the level of MDA) compared with those fed the control diet (T0), but no significant differences were observed at day 56. This suggested that grouper had the capacity to adapt their antioxidative physiology changes caused by a long-term exposure to low dose of dietary histamine to some degree.

It has been evidenced extensively that the enhancement of pro-inflammatory factors (e.g., IL-1β, ITF, and FABP2) sharpens the inflammatory extent and results in intestinal and local tissue injury in organisms (Andre et al., 2000; Sharma et al., 2004; Levy et al., 2009; Overland et al., 2009; Skov et al., 2012; Venold et al., 2012; Song et al., 2013; Couto et al., 2014; Lee et al., 2017; Impellizzeri et al., 2018). The present study showed that dietary histamine increased the intestinal ITF and serum IL-1β and FABP2 levels at days 28 and 56, especially in the high dose group (0.3% histamine), illustrating that supplementation of histamine induced inflammation in grouper. In line with our results, Zhai et al. (2020) reported that high level of dietary histamine resulted in liver inflammation and oxidative damage in juvenile American eel (*A. rostrata*). These results confirmed that histamine is biologically active in fish with regulating the inflammatory response (Mulero et al., 2007). Interestingly, the contents of intestinal ITF and serum IL-1β and FABP2 at day 56 were lower than those at day 28, indicating that grouper adapted to dietary histamine gradually during the long-term feeding period. CRP and SAA are two key acute phase proteins associated with an

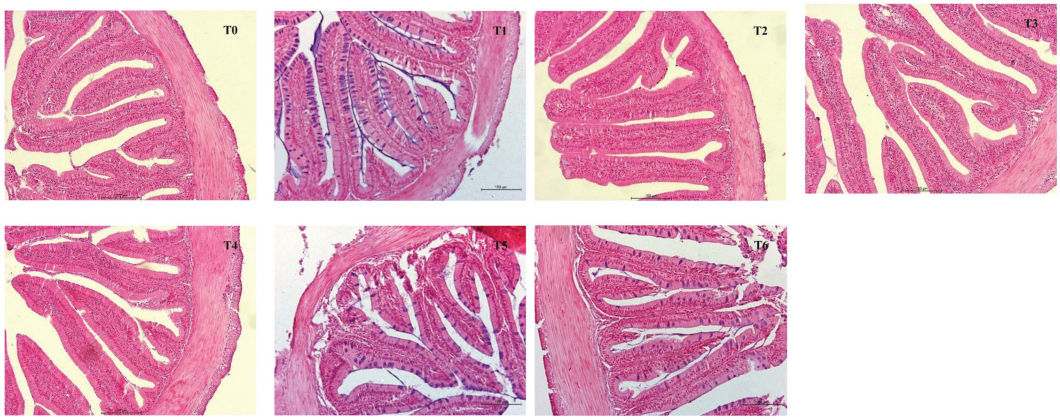

FIGURE 1 | The foregut morphological images from H&E-stained section of *E. coioides* fed the experimental diets for 28 days (100×).

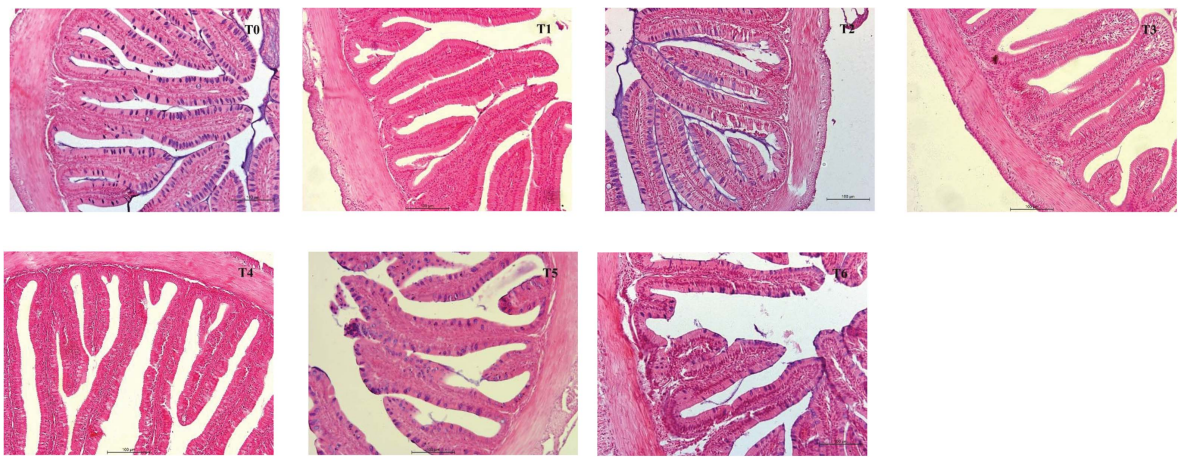

FIGURE 2 | H&E stained anterior intestine morphological sections of *E. coioides* fed the experimental diets for 56 days (100×).

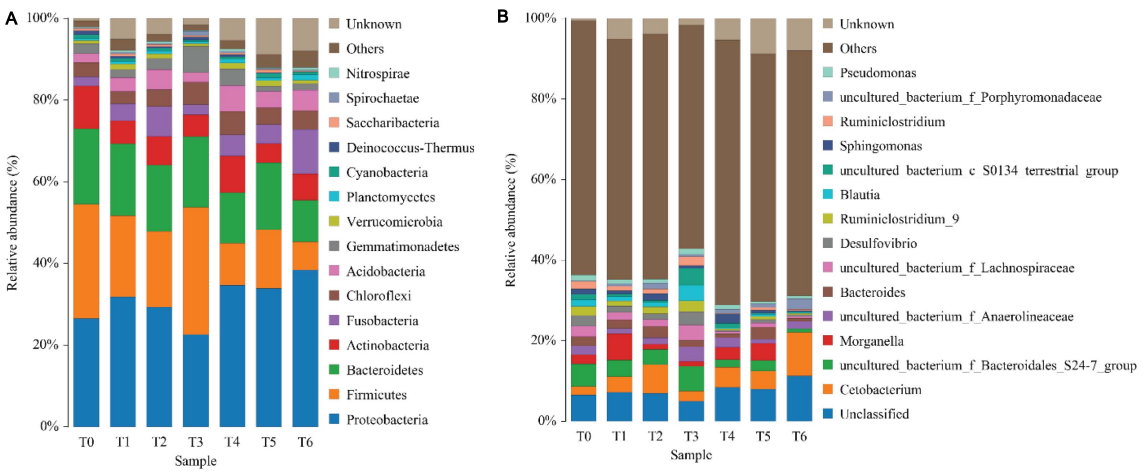

FIGURE 3 | Taxonomy classification of reads from 16 S rRNA V3–V4 regions at the phylum **(A)** and genus **(B)** taxonomic levels in intestinal microbiota of grouper *E. coioides* at day 56. Only top 15 most abundant (based on relative abundance) bacteria phylum and genus were exhibited in **(A,B)**, and other phylum and genus were all classified as "others."

TABLE 7 | MetaStat analysis of the abundance of intestinal bacterial phyla and genera ($\times 10^{-4}$) of grouper at day 56.

	Groups						
	T0	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
Phylum							
Proteobacteria	2040.69 ± 57.82^{c}	3130.35 ± 403.20^{bc}	2886.99 ± 91.96^{abc}	2251.97 ± 350.30^{ab}	3351.02 ± 150.71^{a}	3326.06 ± 133.75^{a}	3761.60 ± 644.49^{a}
Firmicutes	3700.38 ± 58.19^{a}	1961.52 ± 734.61^{bc}	1766.59 ± 757.48^{bc}	2805.52 ± 150.27^{ab}	994.64 ± 192.88^{c}	1409.50 ± 182.22^{c}	705.21 ± 182.13^{c}
Bacteroidetes	1847.51 ± 400.61^{a}	1738.90 ± 86.25^{a}	1576.15 ± 296.27^{ab}	1724.92 ± 154.73^{a}	1191.04 ± 135.70^{ab}	1600.42 ± 124.50^{ab}	986.52 ± 159.10^{b}
Actinobacteria	525.29 ± 5.24	552.38 ± 28.50	700.10 ± 176.10	526.15 ± 45.14	897.58 ± 229.03	464.96 ± 4.42	703.75 ± 430.76
Fusobacteria	313.50 ± 164.16^{b}	410.21 ± 196.24^{b}	1100.79 ± 479.88^{b}	256.0 ± 132.49^{b}	618.42 ± 31.93^{b}	542.80 ± 51.75^{b}	2067.00 ± 380.63^{a}
Genus							
Cetobacterium	21.73 ± 1.80^{b}	385.95 ± 184.59^{b}	739.99 ± 584.86^{b}	250.28 ± 130.14^{b}	460.92 ± 128.02^{b}	432.86 ± 109.06^{b}	2060.84 ± 379.77^{a}
uncultured_bacterium_f_Bacteroidales_S24-7_group	561.79 ± 181.85^{ab}	402.64 ± 104.45^{abc}	360.75 ± 191.52^{abc}	613.14 ± 31.71^{a}	177.56 ± 89.43^{bc}	255.64 ± 78.10^{abc}	95.32 ± 34.33^{c}
uncultured_bacterium_f_Anaerolineaceae	224.24 ± 87.73	128.02 ± 64.75	145.84 ± 28.56	361.63 ± 58.64	255.06 ± 172.24	92.03 ± 15.37	175.60 ± 67.39
Bacteroides	229.35 ± 42.64	217.53 ± 25.86	283.48 ± 158.23	162.93 ± 13.33	89.13 ± 38.18	304.73 ± 76.47	69.01 ± 23.18
uncultured_bacterium_f_Lachnospiraceae	368.89 ± 8.84^{a}	184.39 ± 97.95^{ab}	153.00 ± 97.84^{ab}	364.77 ± 105.68^{a}	52.77 ± 16.05^{b}	98.91 ± 27.92^{b}	49.87 ± 9.64^{b}
Desulfovibrio	259.41 ± 124.74^{ab}	152.03 ± 103.67^{ab}	136.93 ± 79.53^{ab}	331.51 ± 38.64^{a}	36.54 ± 14.25^{b}	86.27 ± 49.10^{b}	46.22 ± 15.91^{b}
Ruminiclostridium_9	233.07 ± 107.88^{ab}	121.92 ± 90.05^{ab}	160.40 ± 93.50^{ab}	264.29 ± 36.80^{a}	32.87 ± 13.49^{b}	42.70 ± 11.07^{b}	33.69 ± 1.35^{b}
Blautia	169.80 ± 80.76^{ab}	109.18 ± 68.33^{b}	105.18 ± 71.42^{b}	374.90 ± 175.21^{a}	22.85 ± 13.58^{b}	55.73 ± 20.30^{b}	29.77 ± 7.38^{b}
uncultured_bacterium_c_S0134_terrestrial_group	132.51 ± 60.92^{ab}	59.73 ± 44.25^{b}	49.68 ± 10.66^{b}	411.16 ± 218.66^{a}	116.70 ± 82.22^{ab}	26.90 ± 14.61^{b}	12.21 ± 4.20^{b}
Sphingomonas	131.44 ± 66.12	84.96 ± 17.95	168.32 ± 82.38	75.30 ± 18.66	149.41 ± 38.94	69.58 ± 1.89	30.13 ± 9.64
Ruminiclostridium	280.56 ± 2.83^{a}	110.24 ± 76.35^{bc}	111.06 ± 69.88^{bc}	218.30 ± 12.36^{ab}	17.46 ± 9.36^{c}	70.66 ± 26.14^{c}	26.85 ± 6.28^{c}
Bradyrhizobium	21.77 ± 8.28^{b}	55.43 ± 5.41^{b}	140.10 ± 84.68^{b}	30.20 ± 8.15^{b}	336.43 ± 131.07^{a}	108.38 ± 45.62^{b}	28.93 ± 9.39^{b}

Different characters in the same row data indicate significant differences ($P < 0.05$).

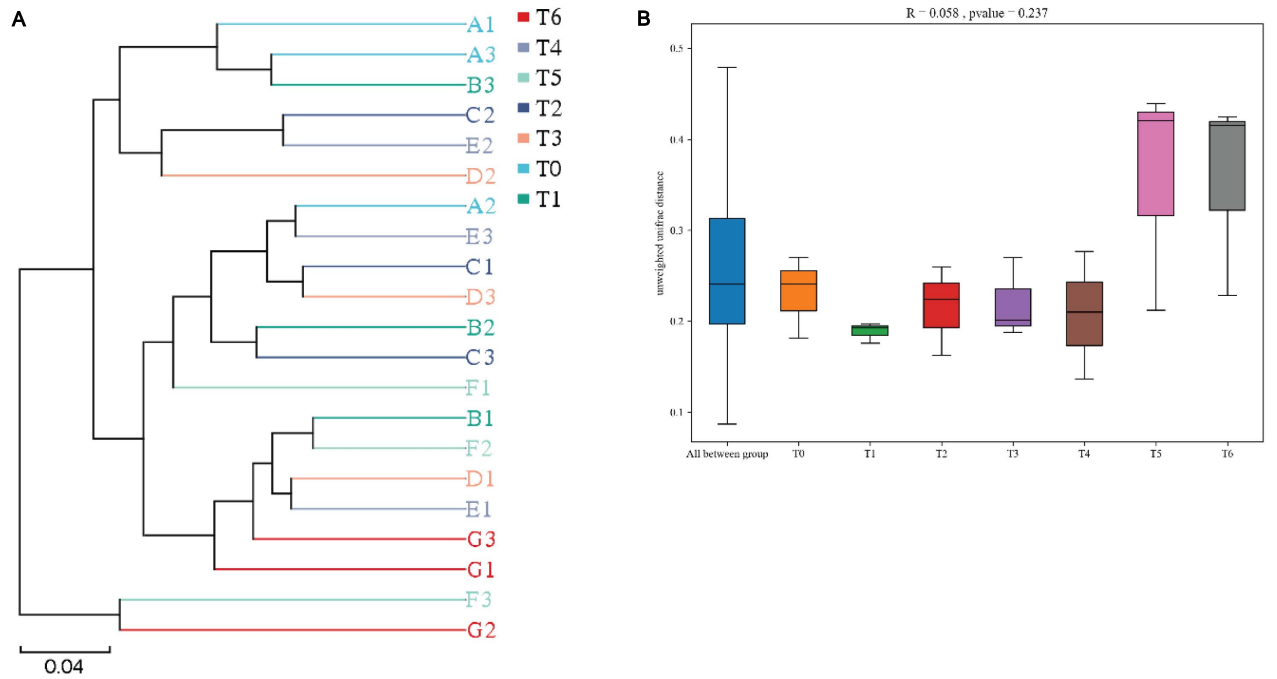

FIGURE 4 | Beta diversity of intestinal microbiota based on binary_jaccard distance of grouper *E. coioides* at day 56. **(A)** UPGMA-clustering trees at genus level. **(B)** Beta (β)-diversity boxplots based on phylum. A1, A2, and A3 refer to triplicates of group T0; B1, B2, and B3 refer to triplicates of group T1; C1, C2, and C3 refer to triplicates of group T2; D1, D2, and D3 refer to triplicates of group T3; E1, E2, and E3 refer to triplicates of group T4; F1, F2, and F3 refer to triplicates of group T5; G1, G2, and G3 refer to triplicates of group T6.

acute inflammatory response, with a significant enhancement of serum CRP and SAA levels following organism inflammation, injury, or infection (Pepys et al., 1978; Lindhorst et al., 1997; Pepys and Hirschfield, 2003; MacCarthy et al., 2008; Rosani et al., 2016; Franco-Martinez et al., 2019; Bello-Perez et al., 2020; Gursky, 2020; Williams et al., 2020). Derebe et al. (2014) reported that vitamin A deficiency may lead to an immune deficiency in mice with decreasing the abundances of SAAs in small intestine and liver. Another study in mice also demonstrated that animals may against lipopolysaccharides (LPS)-induced inflammation and tissue injury through increasing content of serum acute-phase SAA (Cheng et al., 2018). On the other hand, MacCarthy et al. (2008) reported that serum CRP-like protein level improved several folds in common carp (*Cyprinus carpio*) challenged with the pathogen *Aeromonas hydrophila*. A study done in rainbow trout showed that serum CRP concentration rose to a maximum at 6 or 9 days after exposure to formalin for 3.5 h at 300 ppm or 9.5 h at 30 ppm, respectively, and subsequently it began to decline until below normal at day 18 (Kodama et al., 2004). Kodama et al. (2004) also reported that the CRP content enhanced significantly to a maximum at day 3 after exposure to metriphonate (9.9 times higher than normal), then reduced to below normal. Interestingly, the present study showed that dietary histamine did not markedly affect serum CRP and SAA contents at days 28 and 56, although slight enhancement was observed in histamine-supplemented groups. A possible explanation for this might be that serum acute phase proteins (CRP and SAA) contents had recovered in some extent after starvation for 24 h. Unfortunately, serum CRP and SAA contents have not been immediately measured after feeding in this study. Taken together, exogenous high dose histamine not only causes liver oxidative damage but also induces inflammation on the digestive tract in both terrestrial and aquatic animals.

Intestinal morphometrical change is a helpful indicator of gut inflammation and health status (Venold et al., 2012). Intestinal integrity is mainly referred by MT, MFH, and abundance of goblet cells (Garcia-Ortega et al., 2016). Generally, higher MT and MFH indicated healthier intestinal structure, strongly linked to the suppression of enteritis and nutrient transport capacity. In this study, the reduction of MT and MFH demonstrated high doses of histamine (0.25 and 0.3%) exert harmfulness effect on intestinal morphology of grouper. Similar results were reported that high dose of histamine (103.5 mg/kg or more) may result in destruction of gastric and intestinal mucosal barrier in yellow catfish (*P. fulvidraco*) (He et al., 2018). As far as we know, to date, information about the effects of histamine on intestinal morphology in fish remains limited. Several studies indicated that histological and morphological pathology of digestive systems appeared in both rainbow trout and chicks when dietary histamine content exceeded 2 g/kg (Watanabe et al., 1987; Fairgrieve et al., 1994).

It has been extremely reported that intestinal microbiota might be of crucial importance to host health by improving intestinal morphology (Lee et al., 2014; Sayyaf Dezfuli et al., 2018; Torraca and Mostowy, 2018; Cani et al., 2019; Liu et al., 2019; Ortega et al., 2019), modulating metabolic, physiological,

and immunological processes (Al-Fataftah and Abdelqader, 2014; Reda and Selim, 2015; Wang et al., 2018; Heiss and Olofsson, 2019; Deng et al., 2020), and which is highly sensitive to dietary ingredients and components (Ringø et al., 2016). In the current study, the intestinal microbiota of grouper was dominated by *Proteobacteria*, *Firmicutes*, *Bacteroidetes,* and *Actinobacteria*, which is in keeping with our previous studies in grouper (Yang et al., 2019). Dietary 0.3% histamine significantly enhanced relative abundance of *Fusobacteria* compared with the control. Bacteria from this phylum includes several common pathogens in human (Tahara et al., 2014; Han, 2015; Harrandah et al., 2021) and aquatic animals (Meng et al., 2021; Wang et al., 2021), which can cause tissue necrosis and inflammatory response of the host. The relative abundances of *Firmicutes* and *Bacteroidetes* in histamine supplemented groups were lower than those in the control, and it has been reported that members of these phyla improve the functions of intestinal mucosal barrier and non-specific immunity of fish (Costantini et al., 2017; Duan et al., 2018; Wang et al., 2019; Meng et al., 2021). These results may partly explain why the growth performance and non-specific immunity of grouper in the control group were higher than those in histamine supplemented groups. Although significant difference was not observed in intestinal microbial diversity of grouper, higher histamine-treated diets changed bacterial composition in the intestine and increased its diversity, such as increased relative abundances of *Cetobacterium*, as well as decreased abundances of *Ruminiclostridium*, uncultured_bacterium_f_*Lachnospiraceae,* and uncultured_bacterium_f_ *Bacteroidetes*_S24-7_group. In accord with our study, Ye et al. (2020) reported that chilled trash fish generally contain high levels of biogenic amines (such as histamine) (Zhao et al., 2012, 2016), led to significantly decreased intestinal beneficial bacteria (e.g., *Bacteroidetes*_S24-7_group and *Lachnospiraceae*, etc.) in hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂), paralleled with suppression of growth performance. As for horse study, supplementation of *Saccharomyces cerevisiae* (*S. cerevisiae*) modulated positively the overall structure of intestinal microbiota with increased relative abundance of *Lachnospiraceae* (Garber et al., 2020), which is a family of butyrate-producing bacteria (Nicholson et al., 2012; Hamilton et al., 2020). On the other hand, high fat diet resulted in a reduction of the relative abundance of the *Bacteroidetes*_S24-7_group and *Lachnospiraceae* in mice, which might be one of the primary etiological mechanisms underlying obesity (Li et al., 2020). Similar to this study, the lower relative abundance of *Cetobacterium* is accompanied by greater growth performance in tilapia (*O. niloticus*) (Standen et al., 2015; Li et al., 2019). Moreover, combined with the poor growth performance and increased relative abundances of *Cetobacterium* in the soybean meal (SM) group, Wang et al. (2020) speculated that *Cetobacterium* may exert a harmful effect in bullfrog (*Lithobates catesbeianus*). However, these results are contrary to that of Meng et al. (2021) who suggested *Cetobacterium* has a beneficial effect on the production of vitamin B-12 in common carp (*Cyprinus carpio* L.). In the present study, combined with the results of intestinal morphology and inflammation markers, the increase of intestinal microbial diversity by supplemented histamine may be adverse to maintain homeostasis of intestine, indicating a tendency to induce inflammation, which is in line with previous study (Reveco et al., 2014). The above data suggested that the intestinal microbiota composition of grouper was greatly shaped by supplementation of high dose of histamine, which might negatively affect the growth performance, immune function, and intestinal morphology.

CONCLUSION

Diet supplemented with no more than 0.2% histamine did not negatively affect the growth performance, immune response, and intestinal health in grouper, while high doses (0.25 and 0.3%) of dietary histamine exert apparently negative effects on growth performance, immune response, and intestinal health in grouper. This study lays the foundation for future studies on exploring effective strategies to eliminate the adverse effects of histamine in marine fish.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI [accession: PRJNA718150].

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of Jimei University.

AUTHOR CONTRIBUTIONS

Z-YL: investigation and original draft. H-LY: data analysis and writing. L-HH: investigation and data analysis. WY: feed preparation and animal trial. C-XA: review and editing. Y-ZS: supervision, project administration, funding acquisition, review and editing. All authors contributed to the article and approved the submitted version.

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frontiers in Marine Science

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Reshaping of Gut Microbiota in Gilthead Sea Bream Fed Microbial and Processed Animal Proteins as the Main Dietary Protein Source

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The present study aimed to unravel the effects of partial (50%; 50LSAqua) and total (100%; 100LSAqua) replacement of fish meal (FM) by a commercial protein source (LSAqua SusPro) made of bacterial and processed animal proteins (PAP) in farmed juveniles of gilthead sea bream (*Sparus aurata*). The trial lasted 8 weeks, and the feasibility of replacement was assessed in terms of growth performance, histopathological scoring and composition of mucosal adherent microbiota from anterior intestine (AI). Specific growth rates (SGR) of 50LSAqua fish were undistinguishable from the CTRL group, whereas a slight but significant growth impairment was found with the total replacement. Histological signs of inflammation across the intestine were more evident at the highest level of FM replacement, and the total concentration of short chain fatty acids (SCFA) in stripped feces decreased in a dose dependent manner. Illumina sequencing of gut mucosal microbiota yielded a mean of 130,439 reads per sample assigned to 1,567 OTUs at 97% identity threshold. The bacterial richness was similar in all groups, but a significantly higher Simpson diversity index was found in 100LSAqua fish. At the phylum level, Proteobacteria were the most abundant in all groups, whereas Firmicutes decreased and Actinobacteria increased with the FM replacement. At a closer look, pro-inflammatory Gammaproteobacteria of the genus *Psychrobacter*, and *Acinetobacter* decreased with FM replacement, whereas the anti-inflammatory *Paracoccus*, *Arthrobacter*, and Actinomycetales increased, with a remarkable presence of the *Propioniciclava* genus in LSAqua groups. The inferred metagenome analysis suggested that these discriminant bacteria could be implicated in a counter-regulatory anti-inflammatory response. It also pointed to an over-representation of mucosal microbes that can potentially be involved in the natural production of antimicrobials in fish fed the experimental diets.

Keywords: fish meal, processed animal proteins, single cell proteins, gut microbiota, host defense, inflammation, *Sparus aurata*

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INTRODUCTION

Over the last two decades, the aquaculture industry is growing faster (∼7% annual growth rate) than any animal protein sector, with feed conversion ratios (FCR, dry feed intake/weight gain) (1.1–1.6) lower than in poultry (1.4–1.8), pork (2.6–4.4), and cattle (3.5–9) production (Clark and Tilman, 2017; Ritchie and Roser, 2019). However, feed is a major cost in farmed fish production and the reliance of marine aquaculture on marine feed ingredients still remains high (Tacon and Metian, 2015; Ytrestøyl et al., 2015). Historically, fish meal (FM) has been used as the main protein source of most farmed fish feeds because of its high protein content and excellent amino acids balance, but it is also a good source of vitamins, essential fatty acids, calcium, phosphorous and other minerals, which makes high replacement levels by alternative raw materials difficult (Naylor et al., 2009; Olsen and Hasan, 2012; Oliva-Teles et al., 2015). Traditionally, plant-based diets have been considered the most suitable alternative, and high levels of combined FM and fish oil (FO) replacement can be achieved in Atlantic salmon (*Salmo salar*) and in typically marine fish, such as European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) (Espe et al., 2006; Torrecillas et al., 2017; Simó-Mirabet et al., 2018; Aas et al., 2019; Perera et al., 2019). Nevertheless, these substitution diets need to be adequately supplemented with phosphorus, lysine, sulfur amino acids, protein hydrolysates, or nutraceuticals to meet the nutrient requirements for growth (Simó-Mirabet et al., 2018; Egerton et al., 2020), also preventing pro-inflammatory processes and impaired intestinal barrier permeability (Estensoro et al., 2016; Piazzon et al., 2017). However, even with diminished inclusion levels of FM, a shortage ranging from 0.4 to 1.32 million metric tons of FM could occur by 2050, significantly impairing the growth of the aquaculture industry (Jones et al., 2020). Thus, there is an urgent demand for suitable protein ingredients to maintain farmed fish performance, and stabilize the supply of feed ingredients during the expansion of aquaculture.

Among others, insect proteins, processed animal proteins (PAP) and single cell proteins (SCP) have the potential to play a major role in the development of future fish feeds (Nogales-Mérida et al., 2018; Révész and Biró, 2019; Turchini et al., 2019; Glencross et al., 2020). Certainly, in European sea bass, it is feasible to substitute up to 80% of FM meal by defatted *Tenebrio molitor* larvae meal without detrimental effects on nutrient digestibility, growth performance and markers of lipid/energy metabolism (Basto et al., 2021). PAPs from different animal by-products (feather meal, blood meal, meat, and bone meal from non-ruminants) also represent an excellent alternative to FM after the re-approval by the European Union of the use of PAPs in aquafeeds (EC Regulation No 56/2013). Indeed, its excellent palatability, competitive price, and high protein content with fewer carbohydrates than plant proteins make the locally produced agrifood by-products good candidates for more sustainable aquafeeds. However, a number of recent studies in gilthead sea bream highlighted that the maximum level of replacement is closely linked to diet composition and quality of processed PAPs (Moutinho et al., 2017; Davies et al., 2019; Karapanagiotidis et al., 2019; Psofakis et al., 2020; Fontinha et al., 2021). Likewise, SCP products prepared from different microbial sources, including microalgae, yeast and bacteria will contribute to the success of low FM diets in marine aquaculture (Jones et al., 2020). This was supported by studies conducted in Carangidae fish, Florida pompano (*Trachinotus carolinus*), where complete replacement of FM (12.82% of bacterial-SCP) was achieved with no signs of impaired growth performance (Rhodes et al., 2015). Recently, a high partial FM replacement with phototrophic bacteria was also proved with success in Asian sea bass (*Lates calcarifer*) (Delamare-Debouteville et al., 2019). In gilthead sea bream, FM substitution with fungi or microalgae SCP has also been tested (Vizcaíno et al., 2014; Rimoldi et al., 2020; Zamani et al., 2020), but the use of bacterial SCP as a main dietary protein source in this species is less evaluated. In any case, the long-term suitability of high inclusion levels of alternative protein sources is often questioned in aquafeeds, because the potential use of new feed ingredients requires the use of conventional methodologies, but also cutting-edge tools for unraveling the interactions between diets, host metabolism and gut microbiota, which has co-evolved with the host to develop a mutualistic relationship (Zhang and Davies, 2016; Agus et al., 2018). Thus, in fish and gilthead sea bream in particular, this living ecosystem is affected not only by intrinsic factors such as sex, age, genetics, or developmental stage, but also by external agents like diet, trophic level, season or captive state (Estruch et al., 2015; Piazzon et al., 2017, 2019, 2020; Egerton et al., 2018; Rimoldi et al., 2020).

Taking in mind this complex trade-off, the aim of the present study was to evaluate, in a high valuable farmed fish such as gilthead sea bream, the effects of the partial and total replacement of FM by a commercial product (LSAqua SusPro; LSAqua, Lambers-Seghers, Dendermonde, Belgium) made by a combination of PAP and bacterial SCP. The suitability of this protein concentrate was assessed successfully in shrimp (*Litopenaeus vannamei*) (Van Nguyen et al., 2020), and herein we aimed to test an improved formulation to go further in fishless aquafeeds for marine farmed fish with a high economic value for the European aquaculture. In that respect, gilthead sea bream is the main cultured fish in the Mediterranean basin and the third most important produced species in Europe[1]. In the current study, attention was focused on the growth performance and indicators of gut health, including histopathological scoring of anterior and posterior intestine (PI) sections, measurements of concentration of lactic acid and short chain fatty acids (SCFA) in stripped feces, and in-depth analysis of autochthonous microbiota from the anterior intestine (AI).

MATERIALS AND METHODS

Ethics Statement

Fish manipulation and tissue collection were carried out according to the Spanish (Royal Decree RD53/2013) and the current EU (2010/63/EU) legislations on the handling of

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experimental fish. All procedures were approved by the Ethics and Animal Welfare Committees of Institute of Aquaculture Torre de la Sal (IATS-CSIC, Castellón, Spain), CSIC (Permit number 112/2020) and "Generalitat Valenciana" (permit number 2020/VSC/PEA/0064).

Animals

Juveniles of gilthead sea bream (March 2020) were purchased from a Mediterranean hatchery (Piscimar, Burriana, Spain), and adapted for 2 months to the indoor experimental facilities of IATS-CSIC under natural photoperiod and temperature conditions (40°5′N; 0°10′E). Seawater was pumped ashore (open system), and water parameters were daily monitored. Oxygen content of water effluents was measured with an Oxyguard polarographic electrode (OxyGuard, Farum, Denmark) and it was always above 85% saturation. Unionized ammonia remained below 0.02 mg/L, as determined by means of a dissolved ammonia monitor Q46N (Analytical Technology, Inc., Delph, United Kingdom). During the acclimation and experimental period (May–July), water temperature increased from 18°C in May to 25°C in July.

Diets

Three extruded isoproteic and isolipidic diets were formulated by LSAqua, and produced by Research Diet Services BV (RDS, Wijk bij Duurstede, Netherlands) following current industry practices (**Table 1** and **Supplementary Table 1**). The inclusion level of FM in the control (CTRL) diet was 15%. In the other two experimental diets, FM was partially (50%, 50LSAqua) or totally replaced (100%, 100LSAqua) by LS-Aqua SusPro, a mix of PAPs (66%) and bacterial SCP (33%). Both LS-Aqua diets were conveniently supplemented with L-lysine, DL-methionine and calcium bicarbonate phosphate in order to reach the same concentrations present in the control diet and meet the nutrient species requirements.

TABLE 1 | Ingredients and chemical composition of experimental diets.

Ingredients	CTRL (%)	50LSAqua (%)	100LSAqua (%)
Fish meal, herring, 70% crude protein	15	7.45	
Gluvital[1]	10.4	11	11
Guar korma	12	12	11.9
Wheat	11	11.9	11
Corn gluten 60	12.5	10	10
DHA oil	7.5	7.86	8.2
Rapeseed oil	6.3	6.22	6
Rapeseed	7	7	7
Soycomill *R*[2]	6	5	5
Bosoy – GMO[3]	6	3.8	3
Bicaphosph 18 P/25 Ca[4]	2	2.6	3.35
PMX Fish[5]	2	2	2
Alphasoy 530 GMO[6]	2	1.5	1.1
L-lysine HCl	0.28	0.088	0.137
Limestone Ca		0.067	0.07
DL-methionine	0.007	0.105	0.193
Salt, 99% NaCl	0.013	0.11	0.25
LSAqua SusPro	–	11.3	19.8
Chemical Composition			
Crude protein, % feed	43.09	43.312	43.768
Crude fat, % feed	18.434	18.203	18.021
EPA + DHA, % feed	2.214	2.216	2.214

[1]*Wheat gluten, 75% crude protein.*
[2]*Soy protein concentrate, 63% crude protein.*
[3]*Low-soy protein concentrate, 36% crude protein.*
[4]*Dicalcium phosphate, 70% crude protein.*
[5]*Vitamins and minerals from VDS company.*
[6]*Low-soy protein concentrate.*

Feeding Trial

In May 2020, fish of 22–26 g were randomly distributed in nine 500 L tanks to establish triplicate groups of 40 fish each. All fish were tagged into the dorsal skeletal muscle with passive integrated transponders (PIT) (ID-100A 1.25 Nano Transponder, Trovan, Madrid, Spain) and were individually weighed and measured at initial, intermediate and final sampling points (every 4 weeks), using a FR-200 Fish Reader W (Trovan) for data capture and pre-processing. The trial lasted 8 weeks, and fish were fed by hand once daily (12:00 h), six days per week, near to visual satiety with CTRL or experimental diets for the entire duration of the trial. Feed intake was registered daily, and normal fish behavior was assessed routinely by camera monitoring. No mortalities were registered throughout all the experimental period.

Sample Collection

At the end of the feeding trial and following two fasting days, 9 fish per diet (3 fish/tank) were anaesthetized with 0.1 g/L of tricaine-methasulfonate (MS-222, Sigma-Aldrich) and sacrificed by cervical section. Liver, intestine (excluding the pyloric caeca) and mesenteric fat were weighed and length measured (intestine) to calculate the hepatosomatic index (HSI), mesenteric fat index (MSI), and intestine weight (IWI) and length (ILI) indices. Tissue portions (∼0.4 cm) of liver, AI (immediately after the pyloric caeca) and PI (immediately before the anal ampoule) were fixed in 10% neutral buffered formalin for subsequent histological analyses. The remaining AI was opened and gently washed with sterile Hank's balanced salt solution to remove non-adherent bacteria. Intestinal mucus was scrapped off using the blunt edge of a sterile scalpel and collected into sterile 1.5 mL tubes. Mucus samples were kept on ice and DNA extraction was performed immediately after the sampling exactly as previously described (Piazzon et al., 2020). The anterior intestine portion was selected due to its importance in fish nutrient absorption and metabolism. This procedure targets the autochthonous bacteria, because these populations are capable of colonizing the mucosal surface, directly impacting the fish physiology. The allochthonous bacteria (not resident) cannot colonize these habitats under normal conditions and have a more transient impact on the host (Hao and Lee, 2004). Additional fish (10 fish per diet) were sampled 8 h after feeding for the analysis of intestinal lactic acid and SCFA. Briefly, fish were anesthetized and sacrificed by cervical section, intestine was cut out, and the intestinal content was collected by stripping. In the two sampling days corresponding to fasting and postprandial sample collection, all

samples were obtained in a short-period lasting 2–3 h, alternating among replicates of each dietary group to avoid biases due to sampling time.

Histological Analysis

Formalin fixed pieces of liver, AI, and PI were embedded in paraffin, 4 μm-sectioned and stained with Giemsa and periodic acid-Schiff (PAS) following standard procedures. Tissue sections were analyzed under a Leitz Dialux 22 light microscope connected to an Olympus DP70 camera and representative microphotographs taken. The histological alterations observed were scored according to semiquantitative scales in order to evaluate the intensity of the inflammatory response, the intestinal epithelial lesions, and changes of the mucus secretion. Intestinal inflammatory markers included the degree of hyperplasia in the lamina propria-submucosa, scored from 0 (absence) to 3 (severe), as well as eosinophilic granular cells (EGCs) and intraepithelial lymphocytes (IELs) abundance, which were scored ranging from 0 (absence) to 3 (very abundant, meaning 25–30 cells/microscope field at 500× magnification). Intestinal epithelial lesions included the degree of epithelial necrosis and desquamation and the degree of lipid vacuolization in enterocytes, both scored from 0 (absence) to 3 (severe). Cell abundance of differentially stained goblet cells (light- or dark-stained with Giemsa) was used to assess dietary-induced changes of the mucus secretion, and scored as previously described for the other cell markers. In liver sections, the degree of lipid and glycogen storage in hepatocytes was scored from 0 (absence) to 3 (pervasive) by Giemsa or PAS staining, respectively. In addition, the abundance of melanomacrophage centers and of lymphocyte aggregates was quantified in the liver.

Lactic Acid and SCFA Determinations

Intestinal contents (200 mg) were homogenized with 0.1% peptone solution with 0.85% NaCl (500 μL) and centrifuged at 10,000 × *g* for 5 min at 4°C. The supernatant was filtered and 0.2 μL were injected on a HPLC system (Jasco, Tokyo, Japan) equipped with a UV-975 detector. SCFA were separated using a Rezex ROA Organic Acids column (Phenomenex, Macclesfield, United Kingdom) following the method described by Sanz et al. (2005). The mobile phase was a linear gradient of 0.005 M sulfuric acid in HPLC grade water, and flow rate was 0.6 mL/min. The elution profile was monitored at 210 nm and peak identification was carried out by comparing the retention times of target peaks with those of standards. Calibration curves of formic acid, acetic acid, propionic acid, butyric acid, and lactic acid were prepared in the concentration range of 1–100 mM.

Illumina MiSseq Sequencing and Bioinformatics Analysis

The V3–V4 region of the 16S rRNA gene (reference nucleotide interval 341–805 nt) was sequenced using the Illumina MiSeq system (2 × 300 paired-end run) at the Genomics Unit from the Madrid Science Park Foundation (FPCM). The details on the PCR and sequencing of amplicons were previously described (Piazzon et al., 2019). Raw sequence data from this experiment were uploaded to the Sequence Read Archive (SRA) under Bioproject accession number PRJNA713764 (BioSample accession numbers: SAMN18260336-62). Raw forward and reverse reads were quality filtered using FastQC[2] and pre-processed using Prinseq (Schmieder and Edwards, 2011). Terminal N bases were trimmed in both ends and sequences with > 5% of total N bases were discarded. Reads that were < 150 bp long, with Phred quality score < 28 in both of the sequence ends and with a Phred average quality score < 26 were excluded. Then, forward and reverse reads were merged using fastq-join (Aronesty, 2013).

Bacteria taxonomy assignment was performed using the Ribosomal Database Project (RDP) release 11 as a reference database (Cole et al., 2014). Reads were aligned with a custom-made pipeline using VSEARCH and BLAST (Altschul et al., 1990; Rognes et al., 2016). Alignment was performed establishing high stringency filters (≥90% sequence identity, ≥90% query coverage). Taxonomic assignment results were filtered and data were summarized in an operational taxonomic units (OTUs) table. Sample depths were normalized by total sum scaling and then made proportional to the total sequencing depth, following the recommendations previously described (McKnight et al., 2019).

Inferred Metagenome and Pathway Analysis

Piphillin was used to normalize the amplicon data by 16S rRNA gene copy number and to infer metagenomic contents (Iwai et al., 2016). Piphillin predicts the gene inventory of the analyzed OTUs and estimates potential expression based on OTU count values, but does not estimate gene activity. In addition, some predictions are performed at the genus level, not considering the variability among species. Thus, this analysis contains a good degree of uncertainty, but it is helpful to evaluate the potential transcriptomic capacity of a subset of bacteria. The analysis was performed with the OTUs significantly driving the separation by diets in the PLS-DA analysis (described in the Statistics section). A sequence identity cut-off of 97% was implemented, and the inferred metagenomic functions were assigned using the Kyoto Encyclopaedia of Genes and Genomes database (KEGG, October 2018 Release). Raw KEGG pathway output from Piphillin was analyzed with the R Bioconductor package DESeq2 using default parameters, after flooring fractional counts to the nearest integer (Love et al., 2014; Bledsoe et al., 2016; Piazzon et al., 2020).

Statistics

Data of the growth were analyzed by one-way ANOVA using SigmaPlot v14 (Systat Software Inc., San Jose, CA, United States). Normality of the data was verified by Shapiro-Wilk test, and Dunn's post-test was used for multiple comparisons among groups. Analysis of semiquantitative and quantitative histological data was carried out with the non-parametric Kruskal–Wallis test, followed by Dunn's post-test for multiple

[2]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

comparisons. SCFA results were analyzed by one-way ANOVA followed by Holm-Sidak post-test. Rarefaction curves (plotting the number of observed taxonomic assignations against the number of sequences), species richness estimates and alpha diversity indices were obtained using the R package phyloseq (McMurdie and Holmes, 2013). To determine the coverage for the microbial communities, the ratio between observed and expected OTUs (determined by the Chao1 index) was calculated. Differences in species richness, diversity indices, and phylum abundance were determined by Kruskal–Wallis test using the Dunn's post-test, with a significance threshold of $P < 0.05$. Beta diversity across groups was tested with permutational multivariate analysis of variance (PERMANOVA) using the non-parametric method *adonis* from the R package Vegan with 10,000 random permutations. To study the separation among groups, supervised partial least-squares discriminant analysis (PLS-DA) and hierarchical clustering of samples were sequentially applied using EZinfo v3.0 (Umetrics, Umeå, Sweden) and R package ggplot2, respectively. Values of normalized counts of OTUs present in 5 or more samples were included in the analyses. The contribution of the different genes to the group separation was determined by the minimum Variable Importance in the Projection (VIP) values achieving the complete clustering of the conditions with a VIP value of 1. Hotelling's T^2 statistic was calculated by the multivariate software package EZinfo v3.0 and points above 95% confidence limit for T^2 were excluded. The quality of the PLS-DA model was evaluated by the parameters R2Y (cum) and Q2 (cum), which indicate the fit and prediction ability, respectively. To assess whether the supervised model was being over-fitted, a validation test consisting on 500 random permutations was performed using SIMCA-P+ (v11.0, Umetrics). The inferred metagenomics pathways were considered differentially represented using a FDR-corrected significance threshold of 0.05.

RESULTS

Growth Performance

Data on the growth performance are shown in **Table 2**. All fish grew efficiently during the trial reaching an overall FCR of 1.09–1.19. However, daily specific growth rates (SGR) varied significantly from 2.31 in the CTRL fish to 2.19 in the 100LSAqua fish, with intermediate values (2.27) very close to CTRL values in fish fed the 50LSAqua diet. As a result of this, final body weight and condition factor were significantly lower ($P < 0.001$) in the 100LSAqua fish than in CTRL and 50LSAqua groups. Regarding organosomatic indices, no statistically significant differences were found in HSI, MSI and IWI, but overall the intestine of the LSAqua fish was shortened and the lowest ILI was observed in the 50LSAqua group.

TABLE 2 | Effects of dietary treatment on growth performance of gilthead sea bream juveniles fed to visual satiety from May to July (8 weeks) with CTRL, 50LSAqua and 100LSAqua diets.

	CTRL	50LSAqua	100LSAqua	P^1
Initial body weight (g)	23.83 ± 0.28	23.88 ± 0.31	23.87 ± 0.30	0.943
Final body weight (g)	81.07 ± 0.90^a	79.24 ± 0.98^a	76.07 ± 0.96^b	**<0.001**
Final condition factor[2]	2.70 ± 0.02^a	2.66 ± 0.02^a	2.60 ± 0.02^b	**<0.001**
Feed intake (g DM/fish)	62.59 ± 0.56	64.31 ± 0.56	61.96 ± 0.56	0.059
FCR[3]	1.09 ± 0.02	1.16 ± 0.01	1.19 ± 0.03	0.106
SGR (%)[4]	2.31 ± 0.02^a	2.27 ± 0.02^a	2.19 ± 0.01^b	**<0.001**
Liver weight (g)	1.14 ± 0.04	1.09 ± 0.04	1.17 ± 0.04	0.295
Mesenteric fat (g)	1.30 ± 0.14	1.10 ± 0.10	1.26 ± 0.14	0.297
Intestine weight (g)	3.23 ± 0.13	3.05 ± 0.14	3.14 ± 0.15	0.543
Intestine length (cm)	12.41 ± 0.50^a	10.78 ± 0.40^b	11.50 ± 0.39^{ab}	**0.017**
HSI (%)[5]	1.45 ± 0.04	1.44 ± 0.03	1.51 ± 0.03	0.308
MSI (%)[6]	1.62 ± 0.16	1.44 ± 0.12	1.58 ± 0.15	0.266
IWI (%)[7]	4.11 ± 0.13	4.03 ± 0.13	4.02 ± 0.14	0.892
ILI (%)[8]	86.77 ± 3.34^a	73.79 ± 3.91^b	78.40 ± 3.99^{ab}	**0.021**

[1] *Result values from one-way analysis of variance.*
[2] *CF = 100 × (body weight/standard length^3).*
[3] *Feed conversion ratio = dry feed intake/wet weight gain.*
[4] *Specific growth rate = 100 × (ln final body weight – ln initial body weight)/days.*
[5] *Hepatosomatic index = 100 × (liver weight/fish weight).*
[6] *Mesenteric fat index = 100 × (mesenteric fat weight/fish weight).*
[7] *Intestinal weight index = 100 × (intestine weight/fish weight).*
[8] *Intestinal length index = 100 × (intestine length/standard length).*
Data on body weight, feed intake, and growth indexes are the mean ± SEM of triplicate tanks. Data on organosomatic indexes are the mean ± SEM of 27 fish. Different superscript letters in each row indicate significant differences among dietary treatments (Holm-Sidak post-test, P < 0.05, bold values).

Histological Scoring

Both replacement diets had prevailing pro-inflammatory effects at histological level in the two examined intestine portions (**Figures 1**, **2**). In these fish, intestines presented inflammatory hyperplasia with intense EGC and lymphocyte recruitment at both AI and PI segments, and thus mean scoring for all inflammatory markers was lower in CTRL fish. At the AI, abundance of IELs was only significantly higher for the 100LSAqua diet, whereas submucosal hyperplasia was significantly severer for both 50LSAqua and 100LSAqua diets. No differences were found in the distribution pattern of goblet cells in the AI. In this intestinal segment, epithelial lesions were almost absent (score < 0.5), and no lipid vacuolization occurred (score 0). At the PI, the inflammatory reaction triggered by the dietary intervention was more acute. 50LSAqua and 100LSAqua groups showed a significant increase of EGC abundance as well as a significantly higher submucosal hyperplasia, compared to the CTRL fish. Furthermore, in fish fed the extreme substitution diet 100LSAqua, the scoring for submucosal hyperplasia was significantly severer than in the 50LSAqua fish. This intestinal segment presented extensive lipid vacuolization of enterocytes, which was not affected by the diet, and though not significant, epithelial lesions in the PI increased with the degree of protein replacement. Abundance of light-stained goblet cells (with Giemsa) in the PI significantly increased in the 100LSAqua fish, and this cell type presented a PAS + staining pattern indicative of neutral mucins. The inflammatory effect was also noted at hepatic level, where a significant increase of lymphocyte aggregates was observed for the 50LSAqua diet, which was concomitant with an increase in hepatic fat depots. No dietary-induced differences were found for hepatic glycogen storage, and the changes in melanomacrophage centers were not significant.

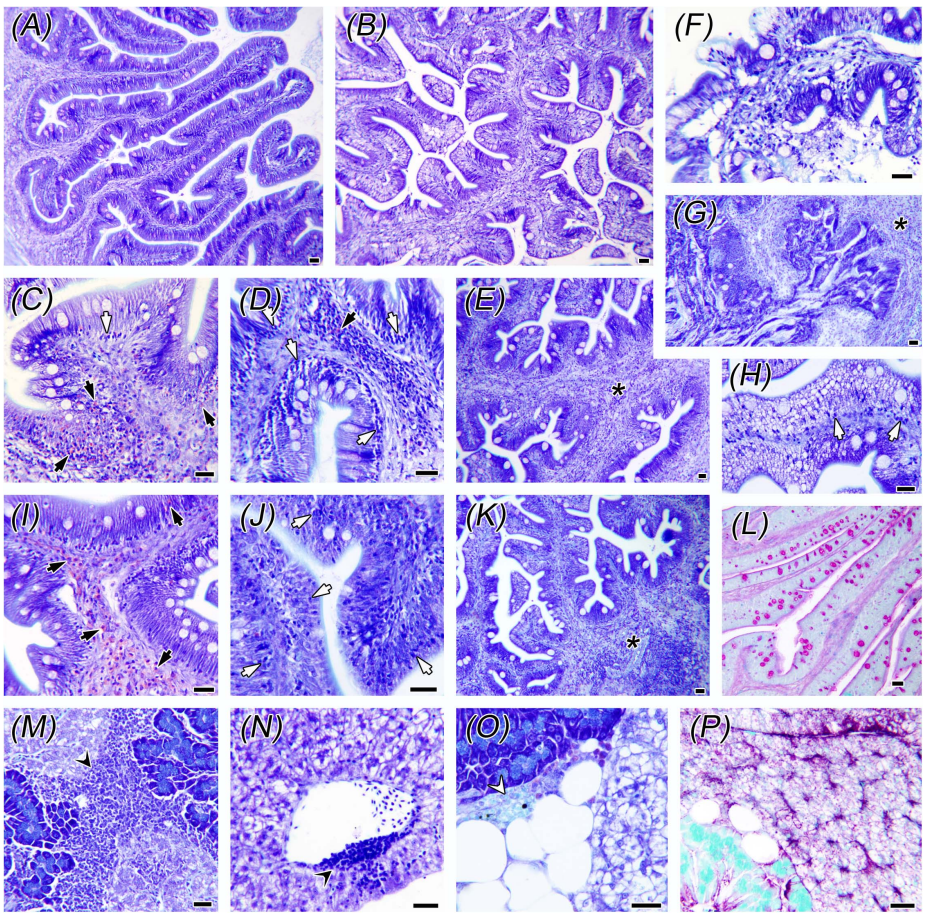

FIGURE 1 | Histological alterations in intestine and liver. Control fish anterior (AI) and posterior (PI) intestines are shown in panels **(A,B)**, respectively. Panels **(C–H,L)** correspond to PI of fish fed the 50LSAqua diet, **(I-K)** correspond to AI of the 100LSAqua diet, and **(M–P)** to livers of the 50LSAqua diet. Note the inflammatory reaction in both intestinal segments consisting of eosinophilic granular cells (EGCs) (black arrows), intraepithelial lymphocytes (IELs) (white arrows), and submucosal hyperplasia (asterisks). Epithelial necrosis and desquamation **(F–G)**, together with lipid vacuolization in enterocytes **(H)** and higher abundance of PAS + Goblet cells **(L)** were found in 100LSAqua-PI. Livers presented lymphocyte aggregates (black arrowheads) close to pancreatic acini **(M)** and blood vessels **(N)**, early melanomacrophage centers (white arrowhead) and large lipid depots **(O)**, and moderate glycogen storage **(P)**. All images are Giemsa-stained sections, except **(L)** and **(P)** PAS-stained. Scale bars = 20 μm.

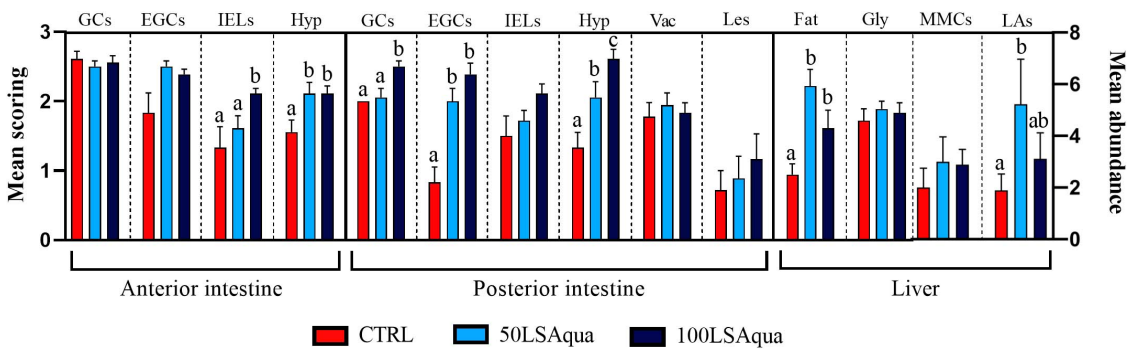

FIGURE 2 | Scoring of histological alterations in fish fed with control (CTRL), 50LSAqua and 100LSAqua diets. Mean semiquantitative scoring (+SEM) from 0 (absence) to 3 (very abundant) is shown for abundance of PAS + Goblet cells (GCs), eosinophilic granular cells (EGCs), and intraepithelial lymphocytes (IELs). Mean semiquantitative scoring (+SEM) from 0 (absence) to 3 (severe) is shown for the degree of hyperplasia in the submucosa (Hyp), vacuolization of enterocytes (Vac), epithelial lesions (Les), hepatic fat storage (Fat) and hepatic glycogen storage (Gly). Total melanomacrophage centers (MMCs) and lymphocyte aggregates (LAs) were quantified in livers (mean abundance + SEM). Different letters within each alteration indicate statistically significant differences among diets ($P < 0.05$).

Intestinal Lactic Acid and SCFA

No significant differences among groups were found for the measured concentrations of lactic, formic, propionic, and acetic acids but they all showed a trend to be decreased with high FM replacement, except for propionic acid. Thus, the total concentration of SCFA decreased significantly with the FM replacement from 18.91 μmol/g in the CTRL fish to 14.05 μmol/g in the 50LSAqua fish, and 11.3 μmol/g in the 100LSAqua fish (**Figure 3**).

Alpha Diversity and Microbial Composition

Illumina sequencing of the 27 samples yielded 3,521,879 high quality reads, with a mean of 130,439 reads per sample, ranging from 81,080 to 198,602 (**Supplementary Table 2**). The reads were assigned to 1,567 OTUs at 97% identity threshold. Almost half of these OTUs (48.3%) were classified up to the level of species, 87.2% to the level of genus, and more than 95% to the level of family (95.1%), order (97.5%), class (98.8%), and phylum (99.9%). Rarefaction analysis showed most of the curves approximating saturation (horizontal asymptote) (**Supplementary Figure 1**) but to ensure the adequateness of the samples, the coverage in terms of richness achieved in our assay was calculated. This analysis helped us to unravel two samples with fewer predicted OTUs than expected, which can be considered as potential outliers. The rest of the samples showed a good coverage of the prokaryotic community, with an average value of 67.3% (**Supplementary Table 2**).

When comparing the bacterial diversity and composition of the CTRL and the LSAqua-based diets (**Table 3**), no significant differences were found in Chao1 and ACE richness indices, but a higher ($P < 0.05$) Simpson diversity index was found in the 100LSAqua group, with the 50LSAqua fish showing intermediate values. At the phylum level (**Figure 4**), Proteobacteria were the most abundant bacteria constituting more than 50% of the total microbiota in all groups, with no significant changes. In parallel, a significant increase in the phylum Actinobacteria was found in both 50LSAqua and 100LSAqua groups (22.1–24.1%) in comparison to the CTRL fish (12.1%). The phylum Firmicutes decreased significantly in 100LSAqua fish (8.6%) in comparison to the CTRL group (23.7%), with intermediate values for the 50LSAqua fish (11.9%). Finally, the less abundant Bacteroidetes phylum accounted for ∼3–6% in all groups.

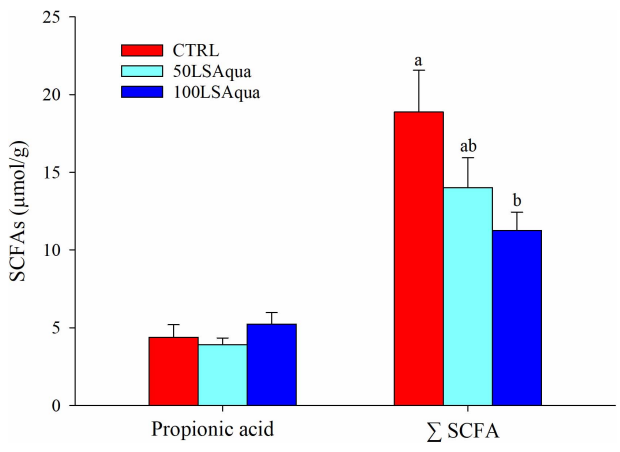

FIGURE 3 | Concentration of intestinal propionic acid and total short chain fatty acids (ΣSCFA) in fish fed CTRL (red bars), 50LSAqua (light blue bars), and 100LSAqua (dark blue bars) diets. Means + SEM are represented (n = 10 fish per group). Significant differences (one-way ANOVA, Holm-Sidak post-test, $P < 0.05$) are indicated by different letters, which correspond to pairwise comparisons among dietary groups.

TABLE 3 | Species richness estimators (Chao1 and ACE) and diversity indexes (Shannon and Simpson) of 9 fish of CTRL, 50LSAqua and 100LSAqua groups.

	CTRL	50LSAqua	100LSAqua	*P* value
Chao1	408.80 ± 63.25	355.19 ± 48.65	312.42 ± 32.71	0.705
ACE	336.95 ± 72.46	346.75 ± 27.12	292.08 ± 31.12	0.420
Shannon	2.41 ± 0.32	2.52 ± 0.20	2.81 ± 0.17	0.230
Simpson	0.82 ± 0.09[a]	0.84 ± 0.07[b]	0.90 ± 0.01[b]	**0.047***

The asterisk (*) indicates significant differences among groups (Holm-Sidak post-test, $P < 0.05$, bold value) denoted by different superscript letters.

Microbiota Discriminant Analysis

Permutational multivariate analysis of variance test was used to evaluate differences in bacterial composition by dietary group but no statistical differences were detected when comparing animals fed the three different diets ($P = 0.211$, $F = 1.1320$, $R^2 = 0.0404$). However, statistical differences were detected when comparing fish fed with CTRL and LSAqua-based diets (50/100LSAqua) ($P = 0.032$, $F = 1.1820$, $R^2 = 0.0531$). To validate and study in more detail these differences, a PLS-DA model (R2Y = 98%, Q2 = 41%) was constructed and statistically validated. During the construction of the model, a fish from the 100LSAqua group and a fish from the CTRL group were identified as outliers and discarded from the model. These outliers coincided with the samples with low coverage ratios, formerly calculated in **Supplementary Table 2**. The remaining points were used to construct a PLS-DA model (**Figure 5A**) showing a clear separation of fish fed 50/100LSAqua diets along component 1 (89.02%) from fish fed CTRL diets. The PLS-DA model was successfully validated with a permutation test discarding the possibility of over-fitting of the supervised model (**Supplementary Figure 2**). These results highlight that the diet has a significant impact on the composition of the adherent bacterial communities of the anterior intestine. To determine which groups of bacteria were driving these separations with the diet changes, a more detailed analysis of the variable importance in projection (VIP) was performed in the all-group PLS-DA throughout a heatmap representation (**Figure 5B**). Hierarchical clustering of samples was applied and the minimum VIP values significantly driving the separation of the groups in the model were calculated. The OTUs within these values were selected for further analysis. Differences in dietary groups were mainly changing 112 OTUs (VIP ≥ 1), which can be accessed in **Supplementary Table 3**.

Figure 6 shows the most abundant bacteria (>1% in at least one group; 46 OTUs) of those that exclusively drove the

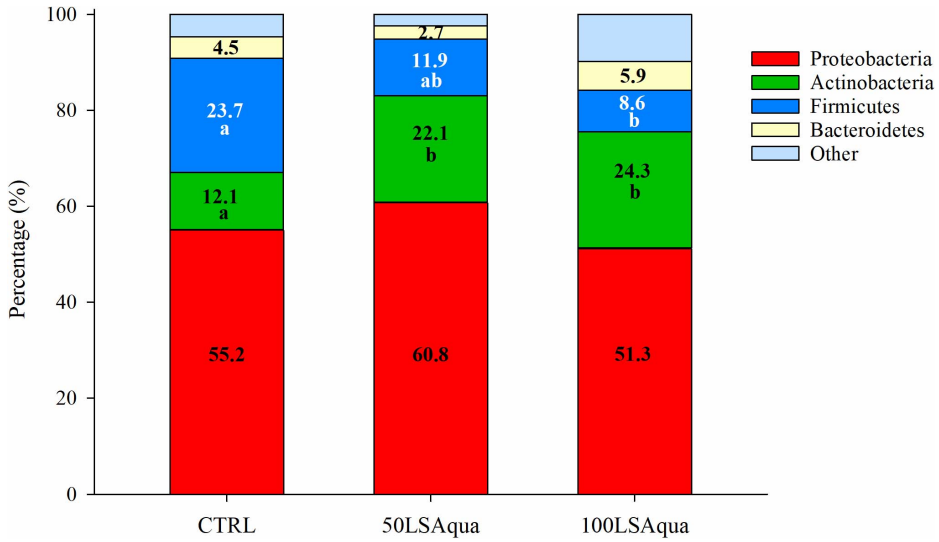

FIGURE 4 | Relative abundance of bacterial phyla in the anterior intestine of fish fed CTRL, 50LSAqua, and 100LSAqua diets. Significant differences (Kruskal–Wallis test, Dunn's post-test, $P < 0.05$) are indicated by different letters which correspond to pairwise comparisons within each phylum among dietary groups.

separation by dietary group, ordered by the corresponding type of response retrieved by the clustering. A first type of response included 29 OTUs increasing with the introduction of LSAqua SusPro. Verrucomicrobia (*Luteolibacter* genus) and Chlamydiae (Parachlamydiaceae family) phyla were exclusively found in this first type of response, as well as several species of the Betaproteobacteria class. Some species of the Rhodospirillales, Rhizobiales, Salinisphaerales, Xanthomonadales, and Enterobacteriales families were increased in LSAqua-based diets. Lastly, at a genus level, the presence of *Paracoccus*, *Omithinimicrobium*, *Tetrasphaera*, *Rubellimicrobium*, *Dietzia*, *Propioniclava*, and *Butyrivibrio* was also characteristic of this response. The second type of response grouped 17 OTUs with a significantly higher proportion in the CTRL group. Bacteroidetes of the Flavobacteriaceae family appeared in this group, as well as certain OTUs from the Chromatiales, Bacillales, and Methylococcales orders. The genera exclusively changing in this type of response were *Aggregatibacter*, *Clostridium sensu stricto*, *Acinetobacter*, *Rhodovulum*, *Novosphingobium*, *Albimonas*, and *Psychrobacter*.

Inferred Metagenome and Pathway Analysis

In an attempt to evaluate the biological significance of the differences induced by diets in the microbiota of the different groups of families, pathway analysis was performed using the inferred metagenomes of the 112 OTUs driving the separation by diet (**Figure 7**). The results showed that 27 pathways could be significantly changing, taking into account the gene inventory of the discriminant OTUs, in the comparison between fish fed 50/100LSAqua diets and fish fed CTRL diets.

Pathways related to immune response (IL-17 signaling pathway, Th17 cell differentiation and antigen processing and presentation), hormonal processes (estrogen signaling pathway and progesterone-mediated oocyte maturation) and alpha-linoleic acid metabolism were under-represented in LSAqua-based diets. The over-represented pathways in LSAqua groups were those related with calcium signaling pathway, melanogenesis, cutin, suberin, and wax biosynthesis, flavonoid biosynthesis, quorum sensing and Ras signaling pathway. Hormonal regulation was also retrieved here, with the over-representation of thyroid hormone signaling and GnRH signaling pathways. A remarkable presence of up to four pathways related with antimicrobials production (streptomycin biosynthesis, biosynthesis of ansamycins, penicillin and cephalosporin biosynthesis and nemoycin, kanamycin and gentamicin biosynthesis) was predicted. Inferred metagenome analysis also displayed a total of 17 predominant (VIP ≥ 1; $> 1\%$ in abundance) OTUs whose genomes could be associated to the expression of genes involved in the differentially represented pathways (FDR < 0.05). The list of bacteria related to each pathway can be found in **Supplementary Table 4**. Of note, we have to consider that this information was obtained from *in silico* inference and only reflects what could be potentially occurring, but it is still of value to estimate the putative metabolic capability of the bacterial populations.

DISCUSSION

Aquaculture intensification and sustainability led to new challenges since there is a need to adapt not only to the inclusion of new dietary ingredients, but also to cope with the challenges arising from intensive fish production. The use of wild fish as aquaculture feeds is becoming unsustainable and new substitution strategies with alternative and sustainable ingredients are being conducted for an efficient production of low FM or FM-free diets (Peixoto et al., 2019;

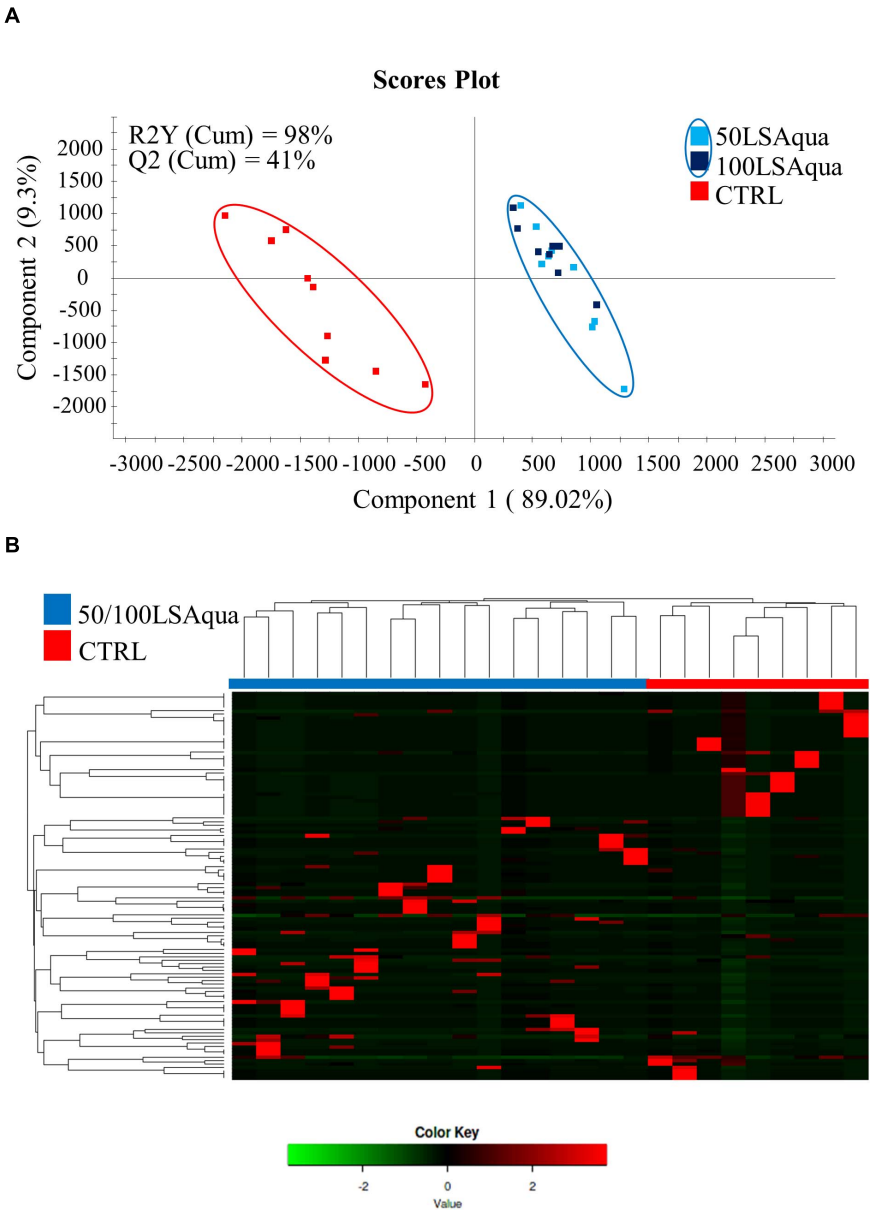

FIGURE 5 | (A) Two-dimensional PLS-DA scores plot constructed using the variable diet with all groups, representing the distribution of the samples between the first two components in the model. The validation by the permutation test can be found in **Supplementary Figure 2**. **(B)** Heatmap representing the abundance distribution (*Z*-score) of the OTUs identified to be driving the separation by diet among all groups.

Ramos-Pinto et al., 2019; Reis et al., 2021). Among these strategies, PAP and SCP are promising materials to develop future fish feeds. However, the effect of these new materials on the host physiology and its associated microbiota, and how these two systems interact, remain largely unknown. The present study suggested that the reshaping of gut microbiota is perhaps a permissive, but also necessary, process for the long-term preservation of growth performance and gut homeostasis in a model of a marine fish fed diets devoid of FM.

At a closer look, growth performance of fish fed with 50LSAqua was almost undistinguishable from the CTRL group. Yet, a slight but statistically significant decrease of SGR (5%) was found with the total replacement of FM by LSAqua SusPro. The achievement of statistically significant differences was favored by the low variability between replicate tanks through the feeding trial. Certainly, the growth performance in all dietary groups in the present study was in the upper range for the class of size and the rearing conditions given for gilthead sea bream (Simó-Mirabet et al., 2018; Martos-Sitcha et al., 2019). However, as reviewed by Glencross et al. (2020), the success of FM replacement by PAP and SCP products is highly variable in aquafeeds. Indeed, the partial

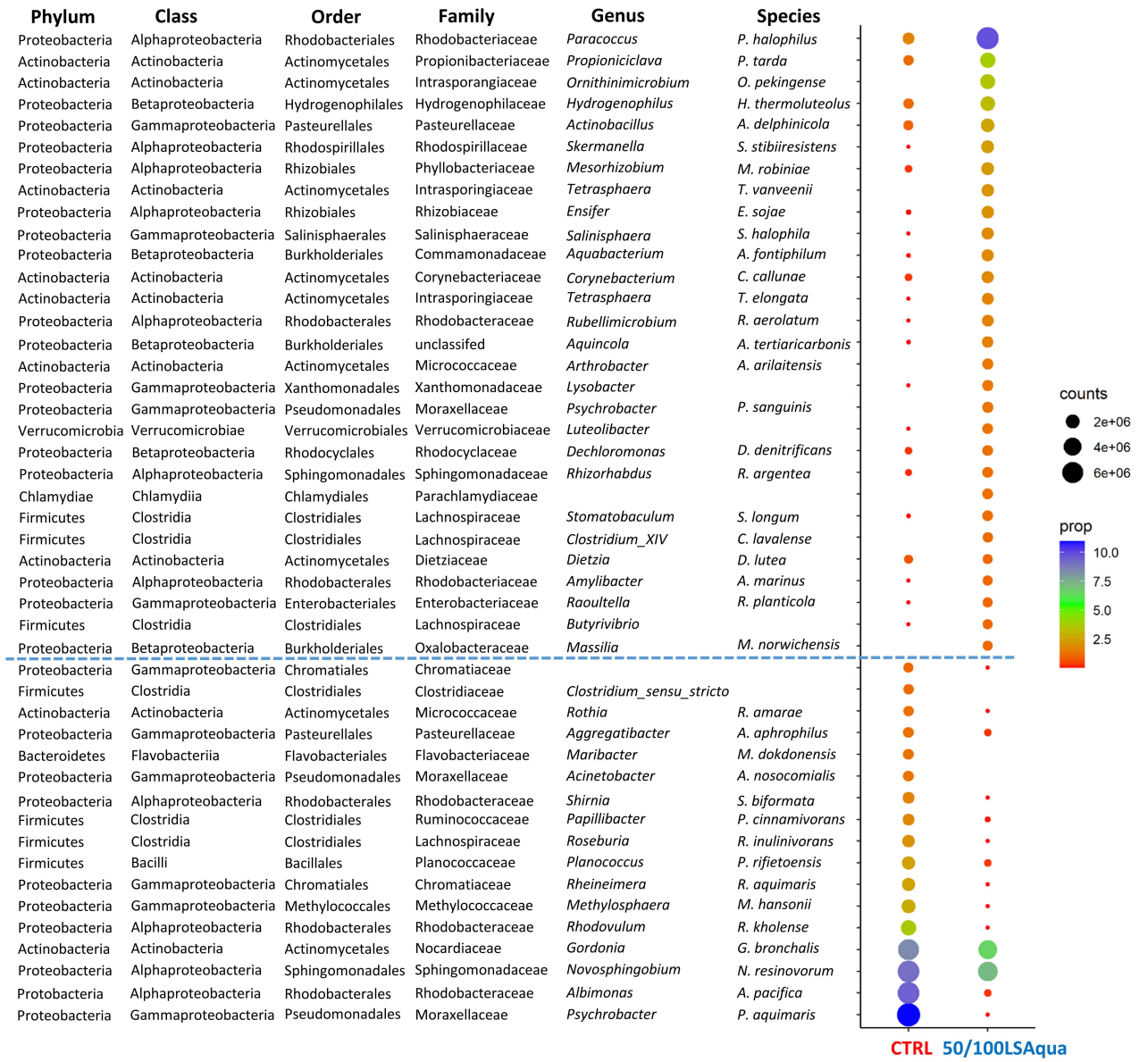

FIGURE 6 | Dotplot map depicting the OTUs with more than 1% of proportion in at least one dietary group. The size of the dots represents the normalized counts in each group. The color scale represents the mean abundance, in percentage, of each OTU within each group. CTRL and 50/100LSAqua refers to control and combined LSAqua-based diets, respectively. OTUs above the dotted blue line showed an higher abundance in 50/100LSAqua group; OTUs below the blue dotted line showed an higher abundance in CTRL group.

and total replacement of FM by yeast (Zhao et al., 2017) or bacterial proteins (Hardy et al., 2018) is highly feasible in shrimp. Likewise, up to 40–50% can be replaced without growth impairment in tilapia (*Oreochromis mossambicus*) and European sea bass (Davies and Wareham, 1988; Oliva-Teles and Gonçalves, 2001). The replacement of FM by bacterial proteins seems to be more limited in Atlantic halibut (*Hippoglossus hippoglossus*) (Aas et al., 2007). Similarly, partial but no total replacement was feasible in Asian sea bass (Delamare-Debouteville et al., 2019). Therefore, the optimal level of FM replacement needs to be assessed for each SCP, fish species, developmental stage and rearing condition. Apparently, the success of FM replacement by PAP is overall less restrictive. This is in fact extensive to gilthead sea bream, in which total or high levels of FM replacement (>75%) are feasible during short-term trials (Moutinho et al., 2017: Sabbagh et al., 2019; Fontinha et al., 2021). In agreement with this, only a slight impairment of growth performance was found herein with the use of a mix of poultry PAP and bacterial proteins. However, this will be limited by a pro-inflammatory condition that was evidenced in a dose-dependent manner by the histopathological scoring of AI and PI segments of fish fed LSAqua feeds. Indeed, inflammation is a

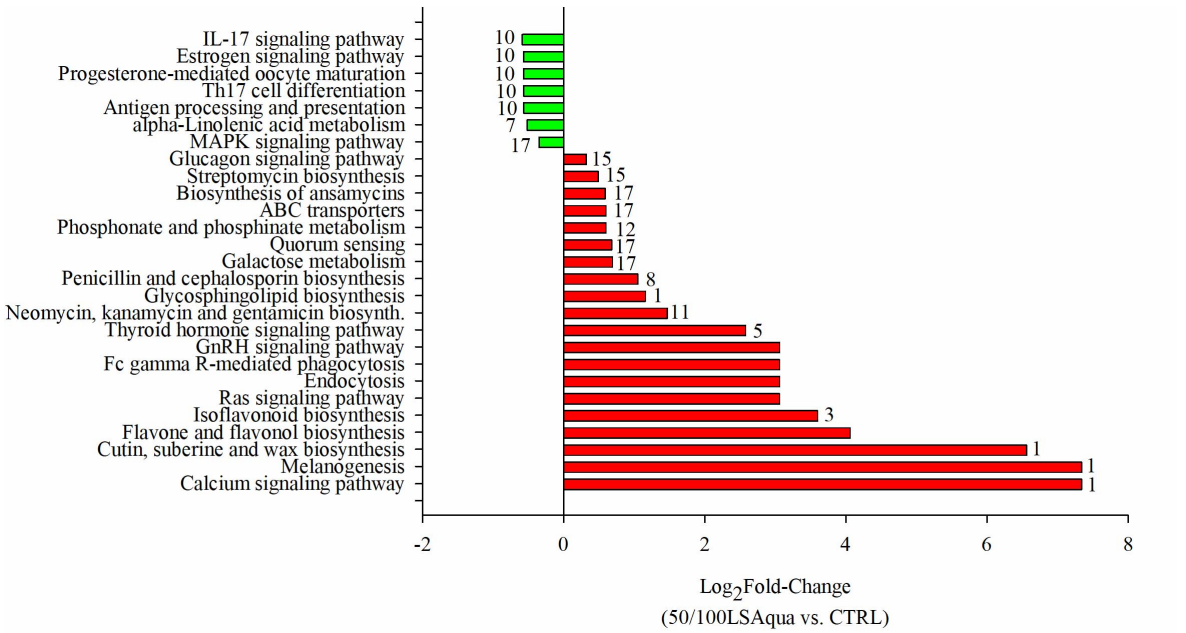

FIGURE 7 | Pathway analysis from predicted metagenome. Values represent the Log_2 fold change of the comparison between 50/100LSAqua vs. CTRL groups (FDR < 0.05). Numbers in bars indicate the number of abundant OTUs (VIP > 1; > 1%) related with the expression of genes involved in each pathway.

common sign of nutritionally mediated metabolic derangements in gilthead sea bream juveniles fed practical diets with a high level of replacement of FM/FO by plant ingredients (Ballester-Lozano et al., 2015).

Short chain fatty acids are end products of protein and fiber microbial fermentation that serve as a primary energy source for enterocytes, but also to preserve intestinal homeostasis through a complex trade-off of anti-inflammatory activities (Donohoe et al., 2011; Corrêa-Oliveira et al., 2016; Morrison and Preston, 2016; Abdel-Latif et al., 2020; Abdel-Tawwab et al., 2021). Their beneficial effects have been demonstrated in gilthead sea bream, where dietary SCFA supplementation helped to reverse the pro-inflammatory condition of fish fed plant-based diets (Robles et al., 2013; Benedito-Palos et al., 2016; Piazzon et al., 2017). Otherwise, there is now evidence that dietary heptanoate, a medium chain fatty acid, contributed to improve the feed efficiency and swimming performance in gilthead sea bream (Martos-Sitcha et al., 2018). In the current study, the intestinal content of total SCFA decreased in a dose-dependent manner with FM replacement, being this feature associated with changes in the growth and pro-inflammatory status. In the same species, the dietary supplementation with protein extracts of *Navicula* microalgae triggered inflammatory markers (Reyes-Becerril et al., 2013). Likewise, the total substitution of FM with poultry by-product meal had a clear hepatic inflammatory action in tench (*Tinca tinca*) (Panicz et al., 2017), and increased lipid deposition rates with a negative impact on the growth and immune response of largemouth bass (*Micropterus salmoides*) (Subhadra et al., 2006). Taking all this in mind, it could be hypothesized that supplementation of 100LSAqua diets with SCFA, like butyrate or propionate, could counteract some of the observed drawback effects in these fish, though future studies are needed to answer this specific question as further discussed below.

Gut microbiota regulates feeding, digestive and metabolic processes, and is crucial for immune development and training (Feng et al., 2018; Butt and Volkoff, 2019), being dietary factors one of the most important drivers of intestinal microbial diversity (Moschen et al., 2012). In fact, the core intestinal microbiota is clearly different among carnivorous, omnivorous, and herbivorous fish species (Liu et al., 2016). In our study, an increase of the Simpson diversity index was disclosed in both 50LSAqua and 100LSAqua groups (**Table 3**), which suggests that the intestine of fish fed with FM-substitution diets contained a wider range of bacteria capable of enhancing a more diverse number of host functions, as previously reported in trout upon the use of algal SCP (Lyons et al., 2017). Despite this, the typical microbiota of marine fish with a high abundance (>90%) of Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes phyla was found herein in all dietary groups in agreement with previous gilthead sea bream studies (Kormas et al., 2014; Estruch et al., 2015; Piazzon et al., 2019). However, LSAqua diets had an impact on microbiota beta-diversity, and changes in gut bacterial communities were already found at the phylum level (**Figure 4**), supporting the fact that intestinal microbiota is highly influenced by dietary nutrients. In this regard, discriminant analysis (**Figure 5**) disclosed the significant change of 46 dominant OTUs (VIP $\geq$ 1; > 1% in abundance) (**Figure 6**), which allowed discovering differences in the gut microbial communities at all taxonomic levels. These changes together with the predictions of the inferred metagenome (**Figure 7**) suggest that the microbiome structure is shifting to deal with

the alternative fish feed formulations used in the present study. This assumption is further supported by a recent study in European sea bass using PAP and SCP as FM replacers (Pérez-Pascual et al., 2020). The exact nature of the main source of variation of bacterial OTUs cannot be deduced by comparing experiments performed in different species. However, it is likely that the protein source is an important factor modulating intestinal microbial communities.

Proteobacteria, as gram-negative facultative anaerobic organisms, commonly dominate aquatic niches due to their highly flexible metabolic properties (Ikeda-Ohtsubo et al., 2018), being one of the most abundant symbionts in marine fish (Tarnecki et al., 2017). In the present study, the most abundant OTU belonging to this phylum in LSAqua-fish was *Paracoccus halophilus*, present in < 3% in the CTRL group and raising up to ∼9.5% in fish fed the experimental diets. Supplementation with *Paracoccus marcusii* was recently described to decrease intestinal inflammation by suppressing TLR4 signaling, with a positive impact on the growth performance and intestinal function of juvenile grass carp (*Ctenopharyngodon idella*) (Xue et al., 2020). However, Proteobacteria, as gram negatives, are generally related to increased intestinal inflammation and its increase is very often related with an inflammatory condition in the human gut (Mukhopadhya et al., 2012; Satokari, 2015). One of the main drivers of the inflammatory action of gram negative bacteria is the lipopolysaccharide, an endotoxin molecule that induces the release of the pro-inflammatory chemokine IL-8 at the intestinal local level (Teghanemt et al., 2005; Hiippala et al., 2020). The lipopolysaccharides of different gram-negative bacteria differ structurally, and those from the Proteobacteria class Gammaproteobacteria present specific modifications that showed to increase the inflammatory effects, at least in mammals (Teghanemt et al., 2005). Interestingly, in our experimental model, the class Gammaproteobacteria was significantly reduced from a ∼21% in the CTRL fish to 8.5% in 50/100LSAqua fish. More precisely, the decrease of Gammaproteobacteria was mainly due, to a ∼10% decrease of the *Psychrobacter* genus. Some species of this genus have been described as activators of NF-kB transcription factor, capable of inducing the transcription of several pro-inflammatory cytokine genes (Chow et al., 1999; Korneev et al., 2014). Indeed, this genus has been recently described to be phylogenetically close to pathobionts (Welter et al., 2021). In addition, *Acinetobacter*, only present in the CTRL fish in our study, is also a known enhancer of the production of reactive oxygen species (ROS), inducing oxidative stress at the cellular level (Schieber and Chandel, 2014; Ajiboye et al., 2018). Thus, the detected changes in these OTUs could have a potential anti-inflammatory role that needs to be verified by host transcriptomic analyses.

The second and the third most abundant phyla in all groups were the gram-positive bacteria Firmicutes and Actinobacteria, which shared an altered equilibrium among groups. Actinobacteria significantly increased in the LSAqua-based diets in concomitancy with a decrease of Firmicutes. This imbalance does not seem to be senseless, as Actinobacteria, and specifically Actinomycetes, are considered a source of pharmaceutically important secondary metabolites (Dholakiya et al., 2017). Marine Actinomycetes have been found to possess *in vitro* anti-oxidant and anti-inflammatory potential (Lavanyalatha et al., 2014). All the predominant Actinobacteria in our experiment were assigned to the order Actinomycetales, which represented ∼10% in the CTRL group and ∼20% in LSAqua-based diets, again suggesting a potential shift to an anti-inflammatory gut microbiota which contradicts the histologically observed inflammatory profile. It can be hypothesized that the shift toward and anti-inflammatory microbial structure is a host-driven change of the bacterial populations in an attempt to balance the diet-induced inflammation, but more studies are required to unravel the real metabolic potential of these populations and the basis of this regulation. The most abundant genus within the Actinobacteria of LSAqua-based diets was *Propioniciclava* (∼6%), previously described as propionate-producing bacteria (Sugawara et al., 2011; Zhang et al., 2017). This feature might contribute to maintain the amount of propionic acid in stripped feces of LSAqua fish relatively constant despite the overall decreased concentration of total SCFA.

Another interesting result of the inferred metagenome was the over-representation of quorum sensing and several pathways related to the biosynthesis of antimicrobials. All the 17 abundant OTUs identified by Phiphillin were related to the quorum sensing pathway, key for effective signaling among bacteria (Jiménez and Sperandio, 2019). Among other processes, the synthesis of antimicrobial substances can be controlled by quorum sensing (Duerkop et al., 2009). It is clear that antibiotics affect bacterial community composition, but a high proportion of the fish intestinal microbiome seems to be able to persist despite antibiotic intake (Kokou et al., 2020). The production of antimicrobials in complex bacterial communities, such as the ones found in the gut, are key in competition or antagonism in bacteria with overlapping niches, but are also signaling facilitators between individual strains, helping to maintain the balance of these communities (García-Gutiérrez et al., 2019). Again, these results are based on predictions of potential functions within the observed bacterial populations, thus, metatranscriptomic or metabolomic approaches must be performed to identify actual functional metabolic differences between groups. In any case, the current results seem to suggest that the observed remodeling of microbiota in LSAqua-fed fish is, at least in part, directly driven by the gut bacteria through the production of antimicrobial substances.

In summary, in terms of the growth performance, the partial and total FM replacement by LSAqua SusPro is highly feasible in short term gilthead sea bream trials. Some detrimental effects involving changes in gut health markers are found, especially with the highest level of replacement resulting in a FM-free diet. This is a common constraining feature in fishless feeds and supplementation with pro- or prebiotics emerge as key strategies to induce an adaptive response of the gut microbiota to produce robust farmed fish with a high capacity to grow efficiently with low FM/FO diets. This opens new research opportunities for fish physiologists and breeders to promote a more ethical and sustainable farmed fish production, according to the criteria of circular economy.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA713764; https://www.ncbi.nlm.nih.gov/, SAMN18260336-62.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics and Animal Welfare Committees of Institute of Aquaculture Torre de la Sal (IATS-CSIC, Castellón, Spain), CSIC (Permit number 112/2020), and "Generalitat Valenciana" (permit number 2020/VSC/PEA/0064).

AUTHOR CONTRIBUTIONS

PS-J, FN-C, MCP, IE, AS-B, and JP-S: formal analysis. PS-J, FN-C, MCP, and JP-S: writing-original manuscript. PS-J, AS-B, DV, and JP-S: conceptualization. All authors involved in experimental investigation, writing-review and editing, and read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.705041/full#supplementary-material

Supplementary Figure 1 | Rarefaction curves obtained from the sequencing data of the 27 samples included in this study.

Supplementary Figure 2 | Validations (permutation tests) of the PLS-DA model shown in this study.

Supplementary Table 1 | Amino acid profile of experimental diets.

Supplementary Table 2 | Table showing the detailed sequencing data obtained in this study.

Supplementary Table 3 | List of OTUs with minimum VIP values (VIP $\geq$ 1) responsible for the separation of samples by diet in the different dietary groups.

Supplementary Table 4 | List of abundant OTUs (VIP $\geq$ 1; $>$ 1%) with the genomic potential to express genes involved in differential (FDR $<$ 0.05) pathways.

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A Temporally Dynamic Gut Microbiome in Atlantic Salmon During Freshwater Recirculating Aquaculture System (RAS) Production and Post-seawater Transfer

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Atlantic salmon aquaculture is undergoing an expansion of land-based recirculating aquaculture systems (RAS), especially for freshwater (FW) stages of production. Juvenile salmon undergo parr-smolt transformation, also known as smoltification and become pre-adapted to tolerate seawater (SW). One aspect requiring study is the development of microbial communities during this time, especially in RAS systems. Here we analyzed temporal changes in microbiome associated with the intestine in Atlantic salmon during smolt production in a commercial RAS production facility and followed the same cohort of fish post-seawater transfer (SWT), using 16S rRNA gene sequencing. Microbial diversity and richness showed an increase over time across FW production, but declined sharply and significantly 1-week post-SWT before re-establishing itself with a completely different community structure after 4 weeks. Core microbial taxa could be assigned to three distinct categories; (1) omnipresent, (2) salinity specific, or (3) transient. By including diet and water samples in the analyses, we classified true core taxa associated with the host, those associated with the diet, and transient cores associated with microbial communities in tank water. The rising trend observed in microbial richness in the water may be a consequence of a temporal increase in organic load while dominance of *Vibrionaceae* may be attributed to the higher temperatures maintained during RAS production and above average natural water temperatures post-SWT. Functional analysis suggests modulation of metabolic pathways post-SWT, but downstream impacts on fish growth and health in a commercial setting remain to be elucidated. A deeper understanding of the interplay between microbial composition and functionality can play a role in optimizing fish performance in tightly regulated RAS production.

Keywords: aquaculture, Atlantic salmon (*Salmo salar*), intestine, microbiome, recirculating aquaculture systems, temporal

INTRODUCTION

The continued growth of the global population coupled with declining wild fish stocks continues to drive expansion of the global aquaculture industry worldwide, with production growing at 7.5% per year since 1970, providing 52% of global fish produced for human consumption in 2018 and generating 250 billion USD first sale value (FAO, 2020). A key stage in the life history of the anadromous Atlantic salmon is the process of smoltification, where the juvenile changes from a FW-adapted parr to SW-adapted smolt and is characterized by a myriad of physiological, morphological and behavioral changes (Björnsson et al., 2011; McCormick, 2012). The parr-smolt transformation (PST) is critical for the long-term health and performance of the stocks, and freshwater environmental conditions (e.g., light, temperature, microbiota, and water quality) and manipulations (e.g., smoltification regimes and vaccination) can impact on the robustness of smolts.

The production of smolts in land-based recirculating aquaculture systems (RAS) is expanding rapidly and globally as a means to provide a controlled stable environment for optimal growth, reduced water usage, biosecurity and minimize the impact on ecosystems (d'Orbcastel et al., 2009; Attramadal et al., 2014). However, fish farmed in RAS experience very different conditions than open water systems including microbial populations which are regulated by water physiochemical factors as well as available nutrients and space (De Schryver and Vadstein, 2014; Fossmark et al., 2020). Microbial communities in RAS play a vital role in converting waste nutrients from uneaten feed and feces to maintain high water quality, which in turn is critical to fish health (Sullam et al., 2012; Blancheton et al., 2013). Established biofilters in the RAS loop are critical to operational success and contain communities of microbes including nitrifying and denitrifying bacteria which convert potentially toxic by-products of nutrient metabolism such as ammonia into non-toxic forms (Blancheton et al., 2013; Fossmark et al., 2021). In addition, fish mucosal surfaces including skin, gill and gut are always in contact with microbes living in the surrounding water and, in the case of the gut, with feed-associated taxa, presenting opportunities for colonization.

The intestinal microbial community of fish consists of both autochthonous species which are attached to the intestinal mucosa as well as allochthonous species which do not attach due to inability or out-competition (Navarrete et al., 2012; Llewellyn et al., 2014; Givens et al., 2015). Microbial communities show extensive plasticity in response to environmental change, but may also reflect environmental or physiological history where the sequence of arrival of microbes into a community is important in determining microbiome composition, even under identical conditions (Vellend, 2016; Sprockett et al., 2018). Gut microbes also play a key role in the priming, protection and development of the host immune system and provide the hosts with exogenous nutrients and extracellular fatty acids and vitamins (Dhanasiri et al., 2011).

Host physiology and external environment provide niche environments that are colonized by microbes and form specialized microbial communities which may change in composition over time, for example across host development, or during a change in host environment. The gut microbiome of Atlantic salmon is strongly influenced by environmental factors including rearing system (Minich et al., 2020), diet (Schmidt et al., 2016; Jaramillo-Torres et al., 2019), seasonality (Zarkasi et al., 2014, 2016), and also by physiological factors such as developmental stage (Lokesh et al., 2019; Heys et al., 2020). In particular, a shift in the microbiome of Atlantic salmon has been observed following transition from FW to SW, often with a set of core microbes displaying stability across this transition (Llewellyn et al., 2016; Dehler et al., 2017b; Rudi et al., 2018; Fossmark et al., 2021). Water and diet are likely key environmental sources of microbes, with this being controlled to an extent by the fish retaining or expelling specific bacteria, ensuring that gut microbial communities are not a passive reflection of seeding communities (Sullam et al., 2012; Heys et al., 2020). The transfer of smolts to the hypertonic SW means these fish require to increase their drinking rates, as well as overall intestinal fluid re-absorption rates (McCormick, 2012), which is likely to impact microbial dynamics in the intestine dependent upon the surrounding environment.

While land-based RAS facilities are becoming the norm for Atlantic salmon smolt production, our understanding of gut microbiome, temporal changes throughout smoltification, and the associated water microbial composition is limited. Incidence of bacterial diseases are associated with seawater transfer (SWT) of smolts (Johansson et al., 2016) and for anadromous fish, a change in salinity means exposure to substantially different microbial communities in the water to which fish must be able to adapt (Schmidt et al., 2015). To this end, adaptive shifts in host-associated microbiota may be hypothesized to accompany the well-characterized and extensive physiological, morphological and behavioral adaptations of salmon undergoing PST. In addition, the RAS environment itself may also directly impact the colonization and succession of the FW gut microbial community with potential consequences post-SWT. In this study, we investigated the microbiome of Atlantic salmon hindgut reared in a commercial FW RAS facility, and following transfer to open seawater cages. Deep sequencing of the V3-V4 hypervariable region of the microbial 16S rRNA gene was performed to analyze the temporal stability of the gut microbiome pre- (FW) and post- (SW) PST.

MATERIALS AND METHODS

Fish Maintenance and Sampling Schedule

Mixed sex juvenile Atlantic salmon were followed from parr to smolt stage in a single stream of a commercial RAS in Scotland. Water in the RAS was maintained at an average temperature of 15.4 ± 0.7°C, pH 7.0 ± 0.16 and oxygen saturation of 99.5 ± 5.5%. Fish were then transferred to a sea cage site. Fish were fed to satiation using automatic feeding systems in both FW and SW units (standard FW RAS diet, Skretting; standard SW diet, Mowi).

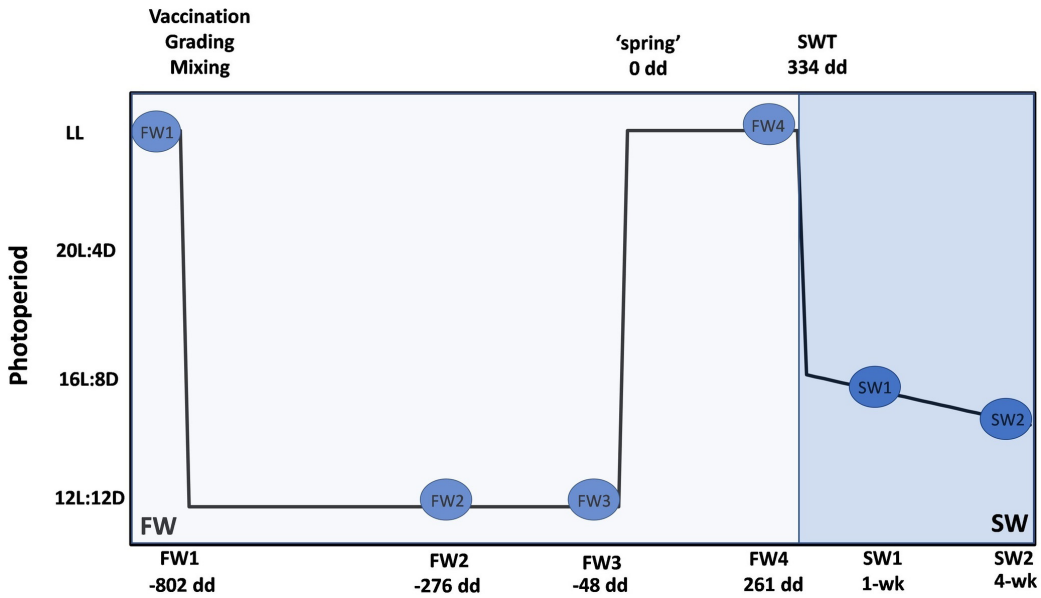

FIGURE 1 | Experimental design for microbiome sampling at FW RAS and open SW cage site. Blue circles represent sampling points; four in FW and two in SW. Degree days is a measure used to determine smolt windows. All dd are calculated relative to application of spring photoperiod (0 dd) and represent mean dd across replicate tanks. At FW1 (–802 dd), unvaccinated fish were sampled from duplicate tanks (n = 2, 12 fish in total). Fish were then vaccinated (ALPHA HECT micro® 6, Pharmaq), graded and mixed, and three experimental tanks of medium grade fish were established. Post-grading, six fish from triplicate tanks were sampled at each FW point (n = 3, 18 fish in total). Fish were exposed to a "winter" photoperiod for a mean of 714 dd and sampled at a mean of –276 dd (FW2) and –48 dd (FW3). "Spring" photoperiod (24 h light – LL) was then applied until SWT and fish sampled at 261 dd (FW4) prior to SWT at a mean of 334 dd. Fish in duplicate open SW cages (n = 2, 12 fish in total) were sampled from duplicate open seawater cages at approximately 1 and 4 weeks post-SWT.

The sampling program is described in **Figure 1**. The first FW sampling (parr stage, FW1 – 14.05.2019, pre-winter photoperiod) was carried out from two replicate tanks under constant (24-h) artificial light (LL). Fish were then vaccinated (ALPHA JECT micro® 6, Pharmaq) and graded. Medium grade fish from the two tanks were then mixed and three experimental tanks were established. Fish were then exposed to a "winter" photoperiod (12L:12D) for 6–7 weeks and sampled after 4 weeks of winter (FW2 – 18.06.2019) and just prior to the onset of the spring photoperiod (FW3 – 02.07.2019). A "spring" signal in the form of LL was then applied until transfer to SW and fish were sampled prior to SWT (smolt stage, FW4 – 18.07.2019 to 25.07.2019). Details of degree days (dd – cumulative temperature over a number of days) associated with sampling points are described in **Figure 1**. At each sampling point post-grading, six fish were sampled from triplicate tanks (n = 3 tanks, 18 fish in total). Individuals from different tanks were mixed at the time of SWT. Fish in the open SW cages were exposed to natural ambient temperature and photoperiod. Fish (n = 2 cages, 12 fish in total) were sampled approximately 1 week and 4 weeks post-SW transfer.

At each sampling point, fish were killed by anesthesia overdose (MS222) followed by a blow to the head. Individual weight (g) and fork length (cm) were recorded. Condition factor was calculated using the formula CF = 100 × [weight (g)/fork length $(cm)^{-3}$]. Specific growth rates between sampling points were calculated using the formula SGR = 100*((ln(final weight (g)) – ln(initial weight (g)))/days (Houde, 1981). Smolt index was recorded on all fish culled at each sampling point and from all fish at the time of transfer to sea using the following scale: 1, parr; 2, some silvering, parr marks visible; 3, fully silvered but parr marks visible; 4, smolt, no parr marks visible (Sigholt et al., 1995).

Gut samples were taken by aseptically dissecting out the hindgut with any digesta (100–150 mg of tissue) and adding to a 2 ml sample collection tube containing 1.5 ml RNAlater™ (Ambion Inc., United States). Samples were stored at 4°C for 24 h followed by longer term storage at −80°C. For water analyses, 4 × 50 ml of tank or cage water were collected at each sample point. Water samples were transported at room temperature then stored at −20°C prior to filtration through 0.2 μM Whatman Cyclopore polycarbonate membrane filters (Sigma-Aldrich; WHA70634702) using a vacuum pump. Filters were stored at −80°C until extraction. Samples of each diet were also collected at each sampling point in FW. Diet samples were transported to the lab at room temperature and stored at −80°C prior to DNA extraction.

DNA Extraction

Hindgut samples in RNAlater containing digesta were thawed on ice, sliced open lengthwise and a scalpel used to scrape approximately 50 mg of digesta and mucosal layers from the interior of the gut to ensure collection of both adherent and allochthonous bacteria. Excess RNA later was removed by gently squeezing between tissue to remove residual salt from the storage solution before transferring to a 2 ml Eppendorf tube for extraction. The QIAamp Fast DNA Stool Mini Kit (Qiagen)

was used for DNA extraction according to the manufacturers protocol with modifications described by Dehler et al. (2017b) and described briefly here. InhibitEx buffer was added to the sample tube along with two 3 mm tungsten carbide beads (Qiagen). The samples were then pre-treated with mechanical lysis using a TissueLyser for 4 min to avoid biases against tough-walled Gram-positive bacteria. Lysis temperature was 95°C to allow for cell-wall break-down of difficult to lyse bacteria. DNA was eluted in a final volume of 30 μl. Each batch of DNA extractions were randomized and ensured samples from all sampling timepoints were in each batch to mitigate against technical artifacts. A negative extraction control was carried out alongside each extraction batch. DNA was extracted from water filters and diets using this same protocol. Diet extractions were carried out in triplicate using 200 mg of feed pellets in each replicate. DNA quantity and purity were determined by NanoDrop spectrometry.

PCR Amplification and Sequencing

For primary PCR reactions, variable regions 3 and 4 of the 16S rRNA gene were targeted with the 341F/785R primer pair (Klindworth et al., 2013). Illumina adapter overhang sequences were added to the 5′ end of each primer. The forward primer (341F) had the sequence 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CCTACGGGNGGGCWGCAG**, and the reverse primer (785R) 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GACTACHVGGGTATCTAATCC** with the bold underlined sequence being the locus-specific V3–V4 primers. Triplicate PCR reactions were performed for each sample and pooled post-amplification to avoid PCR efficiency-related biases. PCR reactions were performed in a 10 μl reaction including 2 μl of each forward and reverse primer (1 μM stock, Sigma), 5 μl of 2x KAPA HiFi HotStart ReadyMix including high-fidelity polymerase (KAPA Biosystems Ltd., United Kingdom) and 1 μl of DNA. PCR conditions included an initial denaturation at 95°C for 3 min, followed by 26 cycles of 30 s at 98°C, 30 s at 57°C, and 30 s at 72°C after which a final extension of 72°C for 5 min was applied. Diet samples were diluted to 200 ngμl^{-1} prior to PCR amplification and only 22 cycles of initial amplification were utilized for water and diet samples. A subset of resulting PCR products was run on an Agilent 2200 TapeStation (Agilent Technologies, Italy) to verify amplification. Overall, DNA extracts from 90 fish hindgut samples were amplified for sequencing along with 12 water extracts, 12 diet extracts, 16 extraction negatives, three PCR negatives, and three positive controls consisting of a mock community (136 samples).

PCR products were cleaned with Agencourt AMPure XP beads on a BioMek 4000 Liquid handling machine (Beckman Coulter Genomics, Italy). The NextEra XT Index Kit (Illumina, San Diego, CA, United States) was used to attached dual indices and Illumina sequencing adapters (P5 and P7) by PCR to the amplicons to produce the final libraries. The index PCRs were carried out in 50 μl reactions containing 5 μl of DNA, 5 μl of NextEra XT Index Primer 1, 5 μl of NextEra XT Index Primer 2, 25 μl of 2x KAPA HiFi HotStart Ready Mix (Kapa Biosystems Ltd., United Kingdom), and 10 μl of nuclease-free water. The PCR conditions were as follows: 95°C for 3 min, 8 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 5 min. Prior to quantification, libraries were cleaned using AMPure XP Beads (Beckman Coulter Genomics, Italy) and the size of the amplicons were verified on an Agilent 4200 TapeStation (Agilent Technologies, Italy). The expected size of the final library was ∼630 bp. Libraries were quantified using a Quant-iT High-Sensitivity dsDNA Assay (Thermo Fisher Scientific, United States) using an Omega FLUOstar plate reader (BMG Labtech, United Kingdom). Final libraries were pooled equimolarly and quantification of pooled libraries confirmed by both qPCR using a KAPA Library Quantification Kit (Roche Sequencing Solutions, United States) and by fluorescence using Qubit dsDNA high-sensitivity (HS) assay (Invitrogen, United States). The final library was denatured and diluted to 1.2 nM prior to loading onto a MiSeq flow cell and sequencing on the Illumina MiSeq platform (Illumina, San Diego, CA, United States). 10% of PhiX Control library was spiked into the amplicon library. MiSeq reagent Kit v3 (600 cycles) (Illumina, San Diego, CA, United States) was used for library denaturing and for MiSeq sample loading. Sequencing was performed on an Illumina MiSeq platform using a 2 × 300 bp paired end protocol.

Sequencing Data Bioinformatics

Analysis of sequence data were carried out using DADA2 (Callahan et al., 2016) and phyloseq (McMurdie and Holmes, 2013) in RStudio v1.1.456 using R v3.6.1 (R Core Team, 2020). DADA2 infers an Illumina sequencing error profile to resolve true sequences from noise and quantifies the number of each actual sequence variant (ASV). Briefly, adapters and primers were removed using TrimGalore![1] and reads with an overall Phred quality score less than 30 were discarded. Forward reads were truncated to 250 bp and reverse reads to 200 bp. Remaining reads were denoised, merged, screened for chimeric sequences which were subsequently removed, and assigned as distinct actual sequence variants (ASVs) using DADA2. In total, 10,502,559 raw reads were obtained for both forward and reverse reads with a mean read depth of 77,225 ± 6512 (SE). After quality filtering, denoising and chimera removal in DADA2, 5,380,123 reads with a mean of 39,560 ± 3736 (SE) per sample were retained. Samples with less than 1000 reads were excluded from further analysis. Taxonomic classification of ASVs was carried out within phyloSeq using the Silva reference taxonomy v132 (Quast et al., 2013). Assignment of species was also conducted using the Silva species assignment v132, allowing for assignment of multiple species. Known contaminants including mitochondrial, eukaryotic, cyanobacteria and chloroplast sequences were removed along with singletons. Samples with less than 500 reads following removal of contaminants were excluded from further analysis. Two further samples were excluded as outliers. Of the 90 hindgut samples sequenced, 83 were retained for downstream analysis. Taxonomic composition of triplicate positive controls was in agreement with the mock community reference (**Supplementary Figure 1**).

[1]https://github.com/FelixKrueger/TrimGalore

TABLE 1 | Length, weight, condition, and specific growth rate in Atlantic salmon smolts during smolting (n = 2 or 3, 12–18 fish, ±SD).

	FW1 14th May	FW2 18th June	FW3 2nd July	FW4 25th July	SW1 2nd August	SW2 22nd August
Water	FW RAS	FW RAS	FW RAS	FW RAS	SW LOCH	SW LOCH
Temperature	14.8°C	16.5°C	14.8°C	16.3°C	Ambient	Ambient
Photoperiod	LL	SD	SD	LL	Ambient	Ambient
Degree days	−802	−276	−48	261	–	–
Length (cm)	14.6 (0.7)	17.9 (0.62)	19.8 (0.2)	21.9 (1.4)	22.2 (0.3)	23.0 (0.8)
Weight (g)	41.5 (4.8)	77.1 (8.9)	98.8 (2.5)	134.1 (21.6)	123.6 (4.65)	128.3 (19.1)
CF	1.31 (0.02)	1.34 (0.03)	1.27 (0.03)	1.27 (0.06)	1.12 (0.00)	1.03 (0.04)
SGR (%)	–	+1.8 (0.3)	+1.8 (0.6)	+1.4 (0.7)	−1.1 (1.7)	+0.2 (0.6)

Fork length was measured in centimeters and weight in grams.

Statistical Analysis

Statistical analysis was carried out in RStudio v1.1.456 using R v3.6.1 and the package phyloseq (McMurdie and Holmes, 2013). Growth parameters were analyzed by one-way ANOVA with Tukey's HSD *post hoc* test. All samples were subsampled to an equal depth of 2,622 reads before calculation of alpha and beta diversity. Differences in alpha diversity across sampling points was determined by Kruskal–Wallis comparisons of Shannon (Shannon, 1948) and Chao1 measurements (Chao, 1984) followed by pairwise testing using the Wilcoxon rank sum test.

Community structure (beta diversity) determined by Bray-Curtis dissimilarity distance (Bray and Curtis, 1957) was visualized using non-metric multidimensional scaling (NMDS) ordination plots, implemented using the Vegan package (Oksanen et al., 2020) and plotted using ggplot2 (Wickham, 2016). Data ellipses based upon an assumed multivariate t-distribution were drawn at a level of 0.75 with stat-ellipse in ggplot2 to provide a visual summary. PERMANOVA (permutational multivariate statistical analysis of community separation) was carried out using the Adonis function in the Vegan package and pairwise comparisons computed using adonis.pair in the EcolUtils package (Salazar, 2020). Core microbiota were identified using the microbiome R package (Lahti and Shetty, 2017) with a prevalence cut-off of 80% and a lower relative abundance limit of 0.1%. Log2 relative abundances of core ASVs across samples were presented in heatmaps drawn with Pheatmap (Kolde, 2012) within R, using Euclidean distance clustering of ASVs.

In order to identify functional pathways based upon 16S communities, Piphillin was used to normalize the non-rarefied amplicon data by 16S rRNA gene copy number and to infer metagenomic contents (Iwai et al., 2016; Narayan et al., 2020). A sequence identity cut-off of 99% was implemented. The inferred metagenomic functions were assigned using the Kyoto Encyclopedia of Genes and Genomes database (KEGG; May 2020 Release) and KEGGREST (Tenenbaum, 2019) was utilized to obtain KEGG pathway names and BRITE hierarchies from pathway identifiers. STAMP v2.1.3 (Parks et al., 2014) was used to test for statistically significant differences in pathway contributions to parent terms using Welch's t-test corrected for multiple-testing by Benjamini-Hochberg false discovery rate (FDR). Differences were considered significant at $q < 0.05$.

RESULTS

Fish Growth and Smolt Indicators

Length and weight increased significantly throughout FW ($p < 0.001$), but no further significant increases were observed in the first 4 weeks post-SWT, considered to be due to initial loss of appetite upon transfer (**Table 1**). Condition factor showed a decline throughout the study period in line with smoltification ($p < 0.001$), and smolt index was 3.48 ± 0.5 at the time of SWT. Specific growth rate was positive throughout FW sampling points, but significantly declined upon initial SWT ($p < 0.05$). SGR across the entire study equated to 1.12% body weight per day.

Alpha Diversity

To maintain sample numbers across sub-groups, samples were limited to 2,622 sequences/samples prior to alpha and beta diversity comparisons, to account for different read depths between samples. Alpha diversity in the hindgut was generally higher in FW compared to SW and showed significant temporal changes (Shannon $F = 35.341$, $p < 0.001$), presented in **Figure 2**. Multiple comparisons revealed significant differences in Shannon diversity between FW3 and FW4 and both SW timepoints ($p < 0.001$ for all) although no significant differences were observed within FW or SW sampling points. Chao1 species richness was also significantly different between timepoints (Chao1 $F = 22.252$, $p < 0.001$), and multiple comparisons revealed that richness at SW1 was significantly lower than at FW3 ($p < 0.001$) and FW4 ($p = 0.002$). No significant differences in alpha diversity or richness were observed with sampling point in water or diet samples. Temporal trends in alpha diversity in hindgut samples did not mirror those found in water or diet samples, but a rising trend was observed in species richness in water samples from FW2 to FW4.

Beta Diversity

PERMANOVA analysis revealed significant differences in the microbiome structure of the hindgut across sampling points

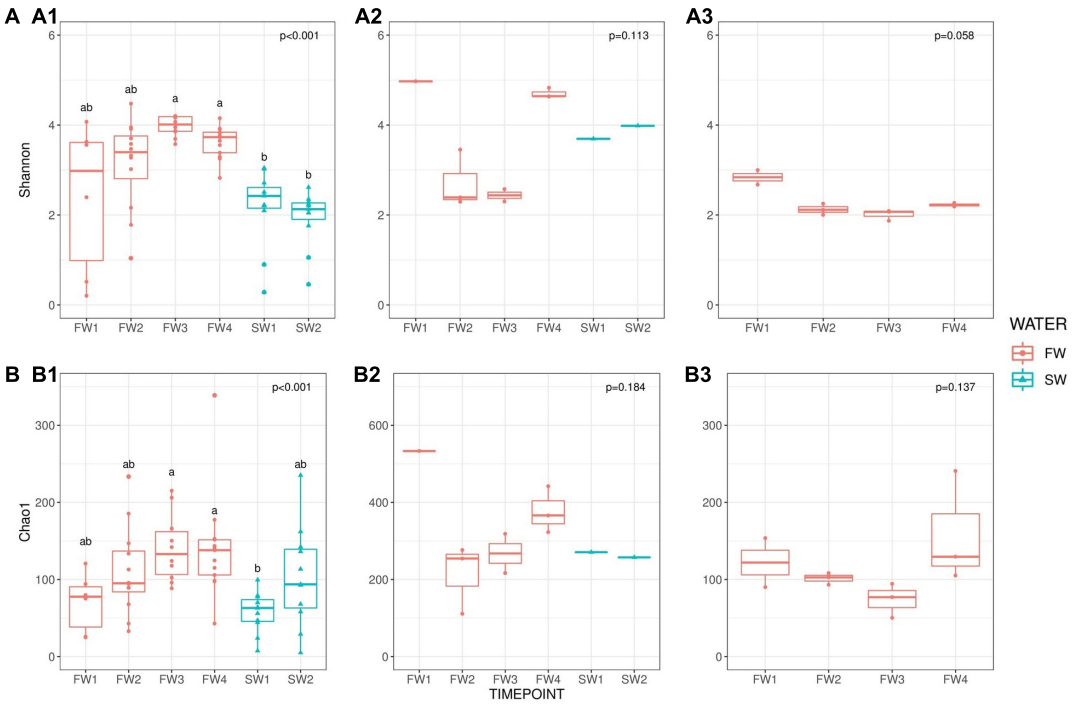

FIGURE 2 | Alpha diversity [**(A)**: Shannon] and richness [**(B)**: Chao1] comparisons. **(A1)** Shannon: hindgut, **(A2)** Shannon: water and **(A3)** Shannon diet across sampling points. **(B1)** Chao1: hindgut, **(B2)** Chao1: water and **(B3)** Chao1: diet. Red circles indicate individual samples for FW RAS and blue triangles are individual samples from SW cages. Superscripts indicate significant differences between sampling points derived from pairwise testing.

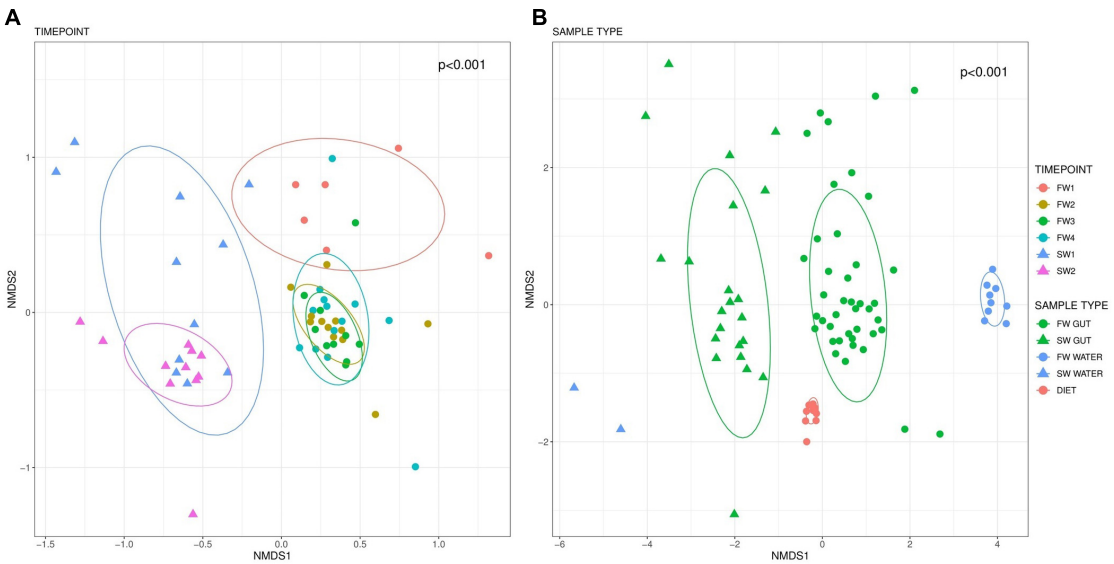

FIGURE 3 | Beta-diversity based on Bray-Curtis distances visualized in an NMDS plot. Different colors indicate sampling points while circle markers indicate FW and triangles SW samples. Beta diversity within hindgut samples by sampling point is presented in **(A)**. **(B)** Depicts beta diversity in different sample types. Data ellipses based upon an assumed multivariate *t*-distribution are drawn at a level of 0.75 to provide a visual summary.

($F_{5,57}$ = 4.13, R^2 = 0.266, p < 0.001; **Figure 3A**). Pairwise comparisons identified significant differences in beta diversity between all sampling point contrasts (BH FDR < 0.01) with the exceptions of FW2 vs. FW3 (p = 0.070) and FW2 vs. FW4 (p = 0.070). Microbiome community structure in water ($F_{5,4}$ = 10.6, R^2 = 0.919, p < 0.001) and diet ($F_{3,7}$ = 14.4, R^2 = 0.851, p < 0.001) samples were also impacted by sampling point, but no significant pairwise differences were identified.

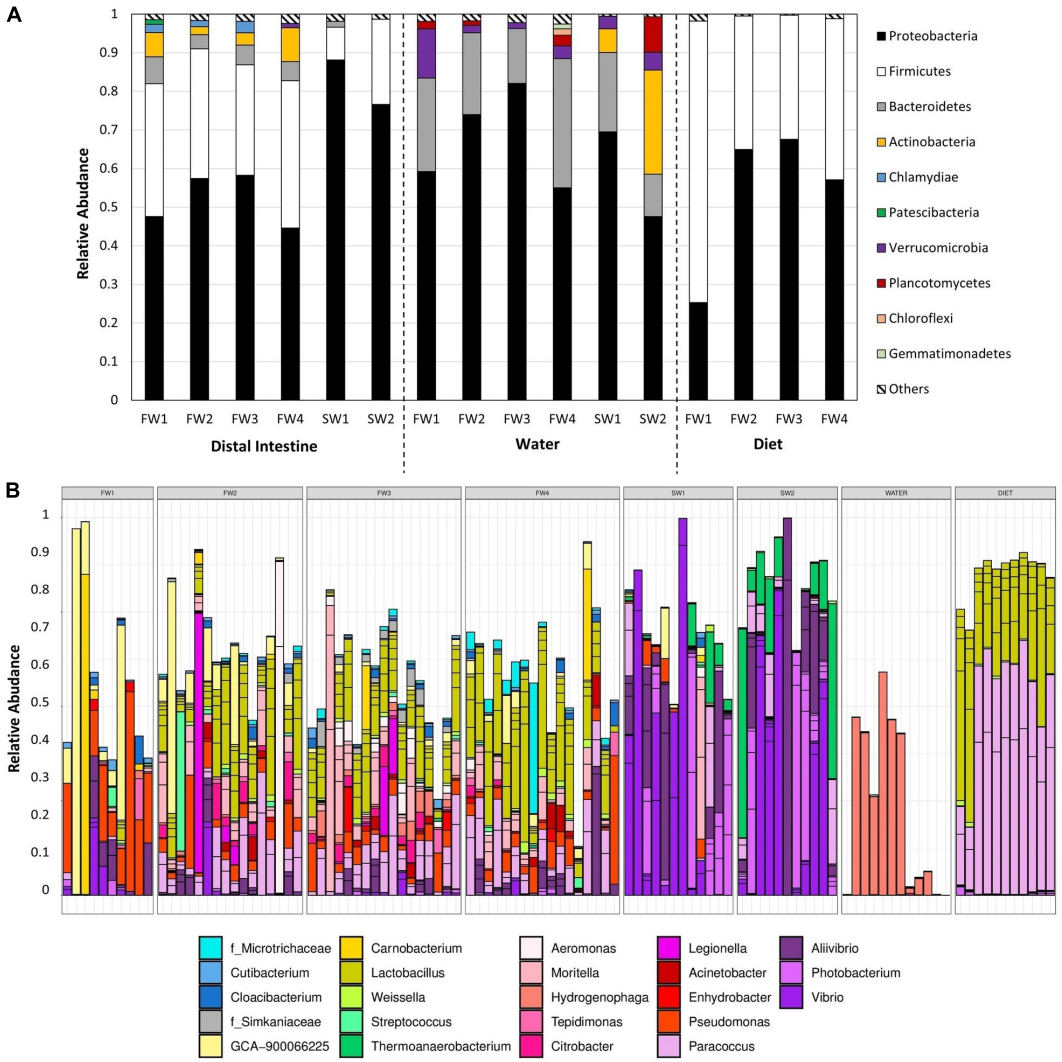

FIGURE 4 | (A) Total relative abundance of each phyla across sampling points in hindgut, water, and diet samples. Only phyla constituting >1% of total relative abundance at each timepoint are presented individually, with those at lower abundances grouped together in "Others." **(B)** Relative abundance of the 40 most abundant taxa in hindgut across all samples, colored by genus.

Clear separation in communities was apparent between all sample types ($F_{2,82} = 8.61$, $R^2 = 0.174$, $p < 0.001$; **Figure 3B**).

Community Composition in Hindgut, Water, and Diet

Community composition was examined at the phylum (**Figure 4A**) and ASV level (**Supplementary Table 1**). Nineteen phyla were observed in hindgut samples. At all FW sampling points, communities were dominated by *Proteobacteria* and *Firmicutes*, supplemented by members of *Bacteroidetes* and *Actinobacteria* while at SW sampling points, *Proteobacteria* was the dominating genus, with *Firmicutes* still showing a strong presence. Relative abundances of the dominant taxa in hindgut at the ASV level are presented in **Figure 4B**. At FW1, ASV8, a *Firmicutes* from the family *Ruminococcaceae,* made up 17.9% and was also the most abundant ASV at FW2. ASV20 (*Lactobacillus sp.*) was the most abundant ASV at FW3 (5.9%) and FW4 (8.5%). At 1-week post-SWT (SW1), a single *Proteobacteria* of the genus *Vibrio* (ASV5) made up 19.4% of abundance, and after 4 weeks in SW (SW2), ASV7 (*Vibrio sp.*) constituted 24.9% of total relative abundance.

In water samples, 19 phyla were also present, 15 of which overlapped with those observed in the hindgut (**Figure 4A**). The microbial community in water in FW RAS tanks was dominated by *Proteobacteria*, *Bacteroidetes* and *Verrucomicrobia* while at both SW1 and SW2, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Verrucomicrobia* dominated and *Planctomycetes* also became dominant at SW2 only. Relative abundances of the dominant taxa in water at the ASV level are presented in **Supplementary Figure 2**. At FW2, ASV13, and ASV24 (*Hydrogenophaga*) constituted 58.4% of relative abundance in total and by FW3,

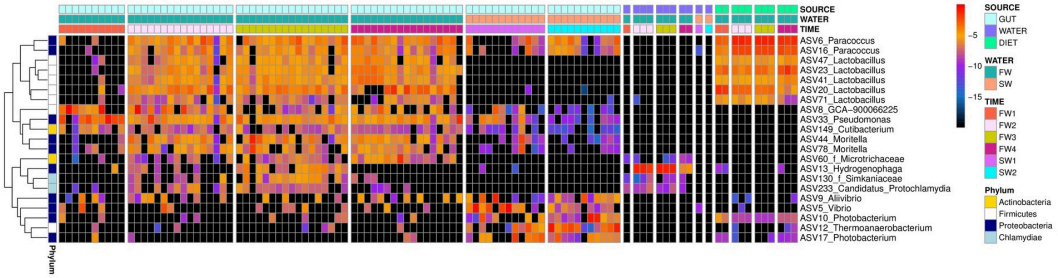

FIGURE 5 | Heatmap depicting log2 relative abundance of core taxa identified in hindgut across timepoints in hindgut, water and diet samples. Colored blocks at the top of the figure depict different sample types (SOURCE), salinities (WATER) and timepoints (TIME) as detailed in the legend. Colored blocks at the left of the figure depict different phyla. ASV and genus names are presented for each row and where genus was undetermined, family is indicated with f prefix. Black squares indicate the absence of a taxon in that sample.

these two taxa made up 72.5% of total relative abundance. At SW1, *Planktomarina* (ASV65, *Proteobacteria*) was the most abundant at 17.6%, but at SW2, *Candidatus_Actinomarina* (ASV63, *Actinobacteria*) dominated.

In diet samples, 10 phyla were detected, all of which were also observed in the hindgut, with dominance of *Proteobacteria* and *Firmicutes* throughout (**Figure 4A**). Relative abundances of the dominant taxa in diet at the ASV level are presented in **Supplementary Figure 3**. At FW1, *Firmicutes* out-weighed *Proteobacteria* as the most dominant phylum, but this was reversed in all other sampling points. At FW1, ASV20 (*Lactobacillus sp.*) constituted 32.1% of total relative abundance. At all other FW sampling points, ASV6 (*Paracoccus sp.*) dominated constituting 40.2%, 42.1%, and 34.1%, respectively.

"Core" Microbiota Throughout Smoltification

A single "core ASV" was identified across all samples from the hindgut (ASV33; *Pseudomonas* sp., present at 0.1% or more, in ≥80% of all individuals). Considering FW samples alone, three additional core ASVs were observed: ASV6 (*Paracoccus* sp.), ASV23 (*Lactobacillus* sp.) and ASV41 (*Lactobacillus* sp.). Excluding FW1, a sampling point prior to vaccination, an additional 5 FW cores were observed: ASV16 (*Paracoccus* sp.), ASV20 (*Lactobacillus* sp.), ASV44 (*Moritella* sp.), ASV47 (*Lactobacillus* sp.) and ASV149 (*Cutibacterium* sp.). In SW individuals, no additional core taxa were identified at an 80% threshold, but ASV5 (*Vibrio* sp.) was present in 78% of SW samples. Considering timepoints individually, seven additional FW core ASVs were identified at FW2, six at FW3, six at FW4 and four additional SW cores were observed at SW2 (**Supplementary Table 2**). No additional cores were identified at FW1 or SW5, and the SW core ASV5 was not observed at SW1 alone. Patterns of abundance of all core taxa across sampling points are summarized in **Figure 5**. Core ASVs could be broadly separated into three groups; (1) omnipresent, (2) transient, and (3) salinity specific cores.

Functional Annotation

Piphillin inferred 374 KEGG pathways from 3730 ASVs present in the Atlantic salmon gut at an identity cut-off of 99%. A total of 970 ASVs had identity of 99% or more with a 16S sequence. Removing human diseases and top-level terms, 294 pathways remained and 156 (53.1%) of these pathways were related to metabolism (**Figure 6A**). Statistical analysis using STAMP revealed differing contributions of Metabolism level 2 categories pre- and post-seawater transfer (SWT). "Xenobiotics biodegradation and metabolism," "Amino acid metabolism," "Metabolism of other amino acids," and "Metabolism of terpenoids and polyketides" had a higher contribution at FW4 while "Metabolism of cofactors and vitamins" and "Glycan biosynthesis and metabolism" a higher contribution at SW1 (**Figure 6B**).

A total of 39 metabolic pathways showed significant differential contributions between pre- and post-SW transfer samples. Pathways with effect size >1% between pre- and post-SWT samples are shown in **Table 2** (n = 24). These belonged to the level 1 metabolic categories which showed overall differential abundance (**Figure 6B**) as well as "Biosynthesis of other secondary metabolites," "Lipid metabolism," "Energy metabolism," and "Carbohydrate metabolism."

DISCUSSION

This study aimed to investigate the temporal stability of the gut microbiome in Atlantic salmon reared in FW RAS followed by transfer to open marine sea cages. A total of 6,999,913 quality filtered reads were classified into 3,730 ASVs from 16S sequencing of the Atlantic salmon hindgut. A rising trend in microbial richness was observed in the hindgut of Atlantic salmon parr undergoing smoltification in a FW RAS. Following transfer to SW, microbial diversity and richness declined and a distinct, less diverse, community structure was established, dominated by the *Vibrionaceae* family. Previous studies have reported a general decline in microbial diversity in the gut of Atlantic salmon as life history proceeds (Llewellyn et al., 2016; Lokesh et al., 2019; Heys et al., 2020). However, these previous studies were not conducted in RAS and covered more broadly a wide range of life history stages, while sampling intervals in the current study were designed to target the parr-smolt transformation window specifically, and the trend may be influenced by cycling of organic matter within RAS tanks.

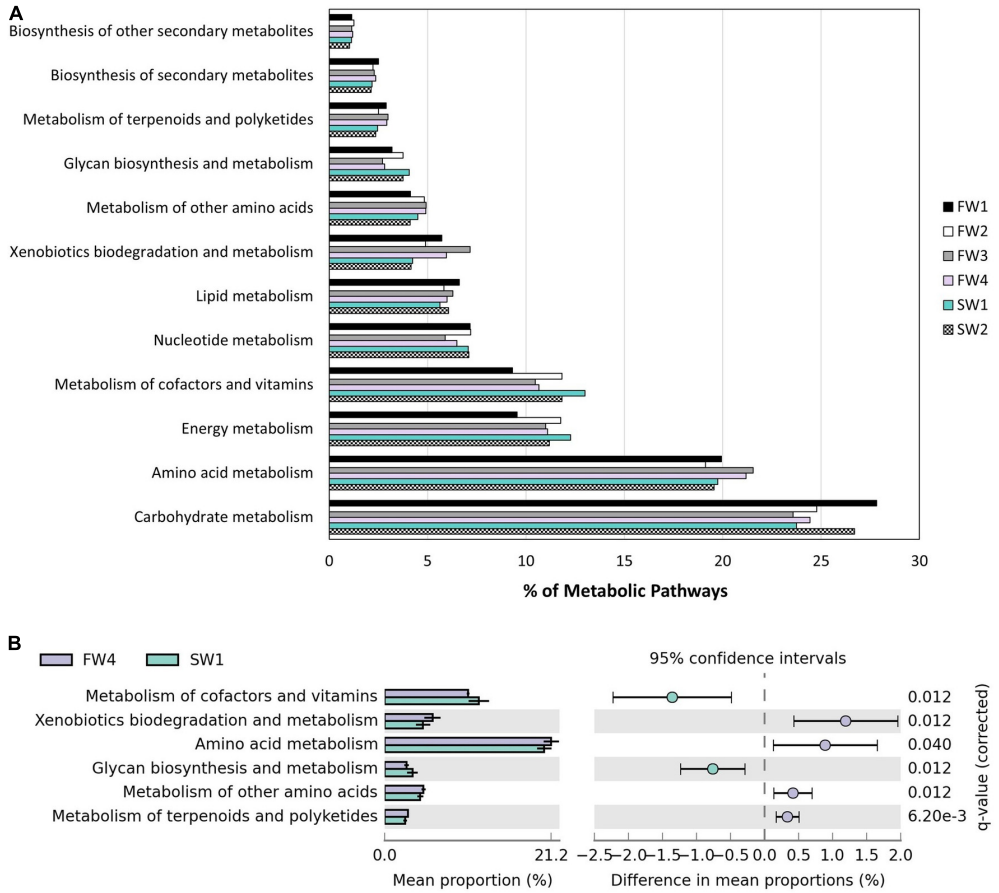

FIGURE 6 | Functional annotation inferred by Piphillin. **(A)** Level 2 metabolic pathway abundance as a proportion of parent term "Metabolism" at all 6 at sampling points. **(B)** Significant differences in "Metabolism" level 2 terms as proportion of parent terms pre- and post-SWT (FW4 and SW1). Benjamini-Hochberg adjusted *p*-values are presented (*q*).

Bacterial Composition and Core Taxa

Bacterial richness and diversity in the hindgut in the current study was similar to that observed in RAS-reared Chinook salmon (Steiner et al., 2020) and the most abundant bacterial phyla identified in digesta from all FW sampling points in RAS (i.e., *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria*) were also in agreement with previous studies performed in FW RAS (Minich et al., 2020; Steiner et al., 2020). A single *Pseudomonas* sp. was identified as the only overall core taxon in the gut in the current study, as was also the case in Uren Webster et al. (2018), although additional cores were also observed when considering sampling points or FW and SW independently. Most core ASVs identified in the hindgut were also present in water and/or diet samples, with the exceptions of the omnipresent cores *Pseudomonas* ASV33, *Ruminococcaceae* ASV8, *Moritella* ASV44, *Moritella* ASV78, and *Cutibacterium* ASV149. Previous studies suggested little impact of the rearing water microbiota on the microbiome of gastrointestinal tract in fish (Gupta et al., 2019a). Core taxa identified transiently in the hindgut were generally also identified in tank water, highlighting a passing role, but suggest little to no persistent colonization.

The core taxa identified are in agreement with other studies covering FW juvenile stage, but these also reported more extensive core taxa from the phyla *Firmicutes*, *Proteobacteria*, *Actinobacteria,* and *Tenericutes* (*Mycoplasma* sp.) (Llewellyn et al., 2016; Dehler et al., 2017a,b; Jin et al., 2019). *Mycoplasma* sp. of the genus *Tenericutes* were notably absent in the current study, possibly as our studies focus on RAS in the FW stages, however, *Tenericutes* were also absent in flow-through FW systems investigated in a previous study (Jaramillo-Torres et al., 2019; Egerton et al., 2020), suggesting this observation is not specific to RAS-reared fish. *Mycoplasma* sp. colonization of the gut has been determined to be non-neutral, i.e., dependent upon the intra-host environment (Heys et al., 2020) and presence or absence may reflect exposure to *Mycoplasma* in early life. The low number of core taxa identified in the current study is likely a consequence of utilizing ASVs rather than OTUs, increasing stringency in taxon identification, but may be related also to a variety of additional factors including dietary regimes, sampling strategy, environmental factors or host genetic variation (Tarnecki et al., 2017). Distinct variability in the microbiota at the level of individual OTUs in a cohort of fish has been

TABLE 2 | Pathways showing significant differences in contribution to "Metabolism" level 3 pathways as proportion of level 2 parent terms pre- and post-SWT (FW4 and SW1).

Pathway name	FW4 (%)	SW1 (%)	Diff. (%)	*q*
Biosynthesis of other secondary metabolites				
Prodigiosin biosynthesis	**12.7**	9.70	+2.96	0.018
Streptomycin biosynthesis	**17.0**	15.0	+2.00	0.029
Phenylpropanoid biosynthesis	3.92	**4.95**	−1.03	0.042
Metabolism of terpenoids and polyketides				
Biosynthesis of ansamycins	4.20	**7.70**	−3.49	0.019
Biosynthesis of siderophore group non-ribosomal peptides	**4.67**	3.66	+1.02	0.047
Lipid metabolism				
Synthesis and degradation of ketone bodies	**9.05**	5.62	+3.43	0.036
Glycerophospholipid metabolism	20.1	**22.8**	−2.76	0.030
Biosynthesis of unsaturated fatty acids	2.68	**3.72**	−1.03	0.024
Xenobiotics biodegradation and metabolism				
Benzoate degradation	**19.3**	16.7	+2.59	0.024
Chlorocyclohexane and chlorobenzene degradation	**4.35**	2.38	+1.96	0.017
Naphthalene degradation	2.89	**4.56**	−1.67	0.016
Atrazine degradation	**3.18**	1.81	+1.37	0.027
Xylene degradation	**2.66**	1.36	+1.30	0.017
Amino acid metabolism				
Valine, leucine, and isoleucine degradation	**9.28**	6.73	+2.55	0.018
Cysteine and methionine metabolism	11.6	**13.1**	−1.49	0.029
Phenylalanine metabolism	**6.07**	4.65	+1.41	0.029
Glycine, serine and threonine metabolism	12.0	**13.4**	−1.37	0.041
Tryptophan metabolism	**6.03**	4.78	+1.26	0.026
Lysine degradation	**5.52**	4.40	+1.11	0.027
Glycan biosynthesis and metabolism				
Glycosphingolipid biosynthesis – ganglio series	0.46	**2.27**	−1.81	0.030
Various types of N-glycan biosynthesis	0.56	**2.27**	−1.72	0.037
Other glycan degradation	3.40	**5.02**	−1.62	0.017
Energy metabolism				
Nitrogen metabolism	8.92	**10.4**	−1.49	0.016
Carbohydrate metabolism				
Pentose phosphate pathway	7.31	**8.38**	−1.07	0.016

Benjamini-Hochberg adjusted p-values are presented (q). The highest proportion for each pathway between the two sampling points is shown in bold.

observed (Ciric et al., 2019). This suggests that a small number of core taxa is not surprising as the conservation of intestinal microbiota occurs primarily at the level of metabolic function, while the specific bacterial species fulfilling that function within an individual animal can vary significantly (Shafquat et al., 2014). Furthermore, fish with distinct microbiomes are often indistinguishable in terms of phenotype in a farm environment (Schmidt et al., 2016), which is interesting in the context of host-microbiome interactions, which are often considered species specific and of longstanding coevolutionary origin (Rosenberg and Zilber-Rosenberg, 2011; Franzenburg et al., 2013).

Taxa Associated With Nitrification Process in RAS

Temporal accumulation of organic matter and nitrogenous compounds are a consequence of the closed-nature of RAS, and environmental microbes play a key role in maintaining RAS water quality. Indeed, temporal increases in CO_2 and nitrogenous compounds were observed in the current study (**Supplementary Figure 4**). When organic matter accumulates in a RAS, heterotrophic blooms can occur, outcompeting nitrifying microbes as heterotrophs obtain carbon and energy from organic matter (Leonard et al., 2000). Primary heterotrophic microbes associated with denitrification in RAS include *Pseudomonas* and *Paracoccus*, two genera identified as core in the current study, and primary autotrophs include *Rhodobacter* and *Hydrogenophaga* (Rurangwa and Verdegem, 2015). Dominance by two ASVs assigned to the facultative autotrophic denitrifiers *Hydrogenophaga* (Xing et al., 2018) in water samples from the RAS tank was observed at FW2 and FW3 sampling points and this was accompanied by a delayed significant increase in abundance in the hindgut. These results suggest that the water microbiome does have the potential to alter the microbial community in the gut, although it appears to have a lesser effect than diet (Uren Webster et al., 2018; Gupta et al., 2019a; Lokesh et al., 2019). Conversely, the gut microbiome itself may

impact the microbial community of the water via excreted waste and it is challenging to determine the initial source of colonization and subsequent succession (Heys et al., 2020; Steiner et al., 2020). Primary hetero- and autotrophs characteristic of nitrogen cycling in RAS continued to be detected in the hindgut following SWT, albeit at lower relative abundance than in FW RAS, suggesting sustained colonization. Distinct microbial communities have been observed in biofilters, tank biofilms, tank water and mucosal samples (gut, skin and gill) but, as was also the case in this study, various biofilter-associated microbes were also detectable in mucus (Schmidt et al., 2016; Minich et al., 2020). The transient nature of such observations indicates the potential for temporary disturbances in water chemistry to impact upon the microbiomes of fish in the system via alterations to the biofilter and tank water communities.

Temporal Variation in Microbial Communities

A major question in this study was to determine if there were temporal changes of the microbiome during FW RAS followed by transfer to SW. We observed a rising trend in alpha diversity between sampling points in FW RAS as fish developed from parr to smolt, but no difference in overall community composition. Sampling point explained 27% of the variance in the hindgut microbiota suggesting that additional unidentified factors also play significant roles. In wild Atlantic salmon populations, microbiome signature within fresh and saltwater ecotypes (i.e., smolt vs. parr in FW) was not impacted by life-cycle stage (Llewellyn et al., 2016), in agreement with our findings. However, we also observed an increase in Chao1 richness between parr and smolt stages, and this temporal increase in FW was confirmed in a second RAS stream in the same facility despite differing smoltification regimes (unpublished data). The contrasting results in relation to richness during FW stages may arise from RAS vs. natural environment of the fish, as husbandry processes in RAS facilities such as disinfection may disrupt or steer microbial colonization and succession (Gupta et al., 2019b; Uren Webster et al., 2020). Furthermore, the closed-nature of RAS may support the accumulation of organic matter, providing additional substrate which may promote bacterial proliferation.

The gut microbiome of teleosts declines in diversity over time, becoming more stable and specialized, suggesting an increasingly important role for host-specific regulation, interaction between microbes and active dispersal (Burns et al., 2016; Stephens et al., 2016). Salinity is a restrictive environmental barrier for microbes (Logares et al., 2009, 2013) and a number of studies, including this one, have observed a significant decline in OTU richness and diversity, and re-structuring of the microbial community during the transition from FW to SW (Llewellyn et al., 2016; Schmidt et al., 2016; Dehler et al., 2017b). This was also the case in fish reared under a different smoltification regime in the same facility as those in the current study (Lorgen-Ritchie et al., in preparation). The final FW (FW4) and two post-SWT samples showed reciprocal patterns of change in abundance between *Lactobacillus* sp. (higher in FW4) and *Clostridia* sp. (higher in SW1). A number of lactic acid bacteria (LAB) genera previously showed differential distribution between FW and SW (Dehler et al., 2017b). *Lactobacillus* sp. can be added to commercial diets as a probiotic (Martínez Cruz et al., 2012) and have been observed to promote overdominance of the *Lactobacillaceae* family in the hindgut (Gupta et al., 2019a).

The intestinal microbial community in SW was dominated by the *Vibrionaceae* family. Dominance by *Vibrionaceae* has been observed in previous studies in salmonids in Tasmania and New Zealand where water temperatures exceeded 16°C for 4 months or spiked above average at the time of sampling (Zarkasi et al., 2014, 2016; Ciric et al., 2019). Water temperature was identified as a key factor in the prevalence and persistence of both *Vibrio* species in the hindgut of Atlantic salmon in Tasmania (Hatje et al., 2014). The salmon in this experiment were transferred to sea during the summer (August) and we may be echoing these previously reported findings as a spike in temperature compared to the 10-year average at a nearby climate monitoring site (15.2°C vs. 13.8°C at SW1) was observed, perhaps allowing *Vibrionaceae* taxa to out-compete other commonly observed SW taxa such as *Mycoplasma* sp. Temperature in the FW RAS itself was held at an average of 15.4°C, as the optimal temperature for growth, but higher than the 12°C generally observed in relevant studies which mostly examined fish from flow-through or aquarium systems (Dehler et al., 2017a; Lokesh et al., 2019). Furthermore, fluctuations saw water temperatures reach a maximum of 17.2°C in the RAS. The intestinal microbiome developed during FW RAS may act as a barrier to other species such as *Mycoplasma* sp. following SWT, however, this remains to be studied and confirmed. Interestingly, *Vibrionaceae* was still the dominant bacteria family at 4-weeks-post-SWT which suggests potential long-term consequences arising from the early post-transfer environment. Coincident with initial SWT and re-establishment of microbial communities, a reduction in food consumption can occur following SWT with as little as 10% of individuals feeding normally 1-week post-SWT (Stradmeyer, 1994) and we observed a decline in fish growth initially post-SWT. Additionally, the transfer of smolts to hypertonic SW results in increased drinking and overall intestinal fluid re-absorption rates (McCormick, 2012), which could impact microbial dynamics in the intestine dependent upon the surrounding environment, however, *Vibrionaceae* were present at very low relative abundance in water samples.

Role of the Hindgut Microbiome in Metabolic Function

Microbiome communities determined by 16S sequencing can be used to infer metagenomes and downstream functionality and the majority of identified pathways were related to metabolism. Pathways which showed the greatest magnitude of changes included biosynthesis of ansamycins and glycerophospholipid metabolism, which were more prevalent in SW compared to FW, while prodigiosin biosynthesis, synthesis and degradation of ketone bodies and "valine, leucine, and isoleucine" amino acid degradation were more prevalent in FW. Ansamycins are naturally occurring antimicrobial compounds which provide

protection against a number of fish pathogens, and this pathway may be activated upon exposure to a new environment as protection against bacterial pathogens (Austin and Austin, 2012). In further support of a role in antimicrobial defense mechanisms, monobactams, which are beta-lactam antibiotics with the ability to inhibit peptidoglycan synthesis (Sykes et al., 1981; Allison and Nolan, 1994) were also more prevalent than in FW by SW2. Red-pigmented prodigiosins are also naturally occurring antibiotics (Darshan and Manonmani, 2015) which were more prominent at FW4. The branched amino acids valine, leucine and isoleucine are essential amino acids for fish (Halver et al., 1957) and play a role in energy metabolism (Roques et al., 2020) while ketone bodies produce an energy substrate which plays a role in maintaining energy homeostasis via the regulation of lipogenesis (Cabrera-Mulero et al., 2019). Increased contributions of glycerophospholipid, glycosphingolipid and glycan metabolism in SW are indicative of post-SWT structural modifications in gut mucosa.

Conclusion

Land-based salmon production has been increasing dramatically over recent years leading to new challenges in fish health, water chemistry and potential impacts on later life performance. The temporal dynamics of gut-associated microbial communities in RAS-reared fish will lead to a more comprehensive understanding of the dynamics of the RAS biological system and differences observed in performance and robustness of RAS versus loch-reared fish post-SWT. Microbial richness showed a rising temporal trend in FW RAS stages before declining and forming a distinct, less diverse, community structure post-SWT, dominated by the *Vibrionaceae* family. The identification of a temporally dynamic gut microbiome in RAS highlights the need to understand the impact of the RAS environment throughout a production cycle and comparative analyses in loch-reared fish are required to further understand the interplay between microbial dynamics in the FW rearing environment and performance of fish post-SWT.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA729215.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the sampling of fish was carried out under established protocols for routine health assessments in accordance with RSPCA Assured Welfare Standards for farmed Atlantic salmon.

AUTHOR CONTRIBUTIONS

SM, JT, and HM designed the experiment. MC, JT, and LC were responsible for management of fish parameters and smolt indicator analyses. ML-R, LC, and MC conducted the sample collection. ML-R performed the DNA extraction and preparation of samples for 16S sequencing and downstream bioinformatic data analyses, and wrote the initial manuscript draft which was reviewed and edited by SM, HM, and JT. JT and MC contributed smolt indicator analyses. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.711797/full#supplementary-material

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How Does Pikeperch *Sander lucioperca* Respond to Dietary Insect Meal *Hermetia illucens*? Investigation on Gut Microbiota, Histomorphology, and Antioxidant Biomarkers

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Effects of feeding dietary defatted black soldier fly (*Hermetia illucens*) larvae meal (HI) on intestine microbiota, and on histomorphology, oxidative enzyme activities in liver and intestine of pikeperch (*Sander lucioperca*) were investigated. Four isoproteic (45% crude protein) and isolipidic (18% ether extract) diets were formulated to include 0% (CO), 9% (HI9), 18% (HI18) and 36% (HI36) of HI as replacement for fishmeal at 0, 25, 50, and 100%, respectively, and were fed to triplicate groups of juvenile pikeperch (initial body weight, 68.7 ± 7.1 g) for 84 days. No adverse effects were detected on the intestine of pikeperch fed diet groups, in terms of histomorphology ($P > 0.05$), while fish fed free or low levels of HI (≤ 9% in diet) showed significant liver degeneration ($P < 0.05$). Dietary HI significantly affected the oxidative enzyme activities of catalase and glutathione peroxidase in the liver, and glutathione S-transferase in the intestine ($P < 0.05$), while activity of superoxide dismutase in both liver and intestine was HI-dose independent ($P > 0.05$). Feeding HI-containing diets positively modulated the richness and diversity of intestinal microbiota, especially for HI18 group ($P < 0.05$). Inclusion HI up to 18% (50% fishmeal replacement) in pikeperch diets increased abundance of *Clostridium*, *Oceanobacillus, Bacteroides,* and *Faecalibacterium* genera, whereas the predominant bacterium, *Cetobacterium* was found in control and HI36 groups. This study reveals the potential of HI as an immune and health booster for juvenile pikeperch.

Keywords: pikeperch, alternative ingredient, *Hermetia illucens*, microbiota, histomorphology, antioxidative

INTRODUCTION

Aquaculture is the largest global consumer of fishmeal production, accounting for 68–73% (Shepherd and Jackson, 2013; Tacon and Metian, 2015). Fishmeal is mainly derived from marine capture fisheries (70% in 2018) (FAO, 2020a), which has reached a plateau since the 2000s (Shepherd and Jackson, 2013) and has been projected that the ecological limits of stock will be

reached by 2037 (Froehlich et al., 2018a). Therefore, the current fastest growth of aquaculture in food-producing sectors (FAO, 2020a) and the continuous increasing trend, requires the development of novel aquafeed ingredients. Terrestrial crops have been used in aquafeeds more than other alternatives until recent (Tacon et al., 2011; Tacon and Metian, 2015) and, by 2050, the use of these feedstuffs in aquaculture will rise to twice the current level in a business-as-usual scenario, reaching 91 million tonnes (Froehlich et al., 2018b). However, crop-based feeds for aquatic animals introduce concerns regarding their nutritional properties and environmental consequences. An unbalanced essential amino acid profile, low palatability, and the presence of anti-nutritional substances could impair their inclusion in aquafeeds (Gatlin et al., 2007). Moreover, the expansion and intensification of the production of terrestrial crops will lead to tremendous environmental burdens pertaining to climate change, biodiversity loss, and increasing demand for arable land and water. Among such burdens, land use is considered the one that entails the greatest pressures on the planet (Foley et al., 2005, 2011; Boissy et al., 2011). Beyond terrestrial plant ingredients, fishery by-products and insect meals have shown the greatest potential to be protein-supplied to aquafeeds in the coming years (Hua et al., 2019; Gasco et al., 2020a). Although approximately 34% of the world's fishmeal production will be derived from fish by-products by 2030 (FAO, 2020a), this potential protein source will still not be able to meet the projected aquafeed demand by 2050 (Froehlich et al., 2018a). The efficiency of insect meal as a future aquafeed ingredient has already been identified, especially concerning the feasibility of costs, scalability, and processing technology (Hua et al., 2019). Globally, insect production is on the rise, and will reach approximately 1.2 million tonnes by 2025 and become price-competitive with fishmeal by 2023 (Hua et al., 2019; Gasco et al., 2020a). In addition, the development of production facilities and processing techniques would help to improve the environmental performance of insect meal as a sustainable aquafeed ingredient (van Huis and Oonincx, 2017). The use of seven insect species (two flies, two mealworms, and three cricket species) in fish diets has been authorised by the European Commission (Regulation No. 2017/893). Among these species, black soldier fly (*Hemertia illucens*), which belongs to the Diptera order, has received the most research interest (Hua, 2021). *Hemertia illucens* larvae meal possesses important nutritional profiles, especially amino acid profile which is close to that of fishmeal (Nogales-Mérida et al., 2019). As far as environmental impact is concerned, *H. illucens* production, if obtained using non-valorised substrates, entails significantly less arable land and water use than soybean meal (Smetana et al., 2019; Gasco et al., 2020b). Moreover, *H. illucens* meal-containing diets have shown lower environmental impacts associated with abiotic depletion, acidification potential, eutrophication potential, climate change, human toxicity potential, and marine aquatic ecotoxicity potential for Arctic char (*Salvelinus alpinus*) (Smárason et al., 2017) and lower water use for European perch (*Perca fluviatilis*) (Stejskal et al., 2020) than insect-free diets.

The substitution of fishmeal with *H. illucens* meal in aquafeeds for the largest fishmeal consumers has already been investigated, and substitution levels have been achieved that do not delay growth production of the tested species, including, white leg shrimp (*Litopenaeus vannamei*) (60% plausible substitution) (Cummins et al., 2017), Atlantic salmon (*Salmon salar*) (85–100%) (Lock et al., 2016; Belghit et al., 2018, 2019), European seabass (*Dicentrarchus labrax*) (45%) (Magalhães et al., 2017), barramundi (*Lates calcarifer*) (50%) (Katya et al., 2017), and rainbow trout (*Oncorhynchus mykiss*) (45%) (Sealey et al., 2011; Renna et al., 2017; Dumas et al., 2018). In addition, dietary *H. illucens* meal has been proved to modulate bacterial diversity and richness, which play essential roles in nutrition, immunology, and health status of fish, such as rainbow trout (*O. mykiss*) (Bruni et al., 2018; Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019; Rimoldi et al., 2021), and zebrafish (*Danio rerio*) (Zarantoniello et al., 2020b). The gut health benefit of insect-fed fish has been confirmed to be suitable for species that naturally feed on insect (Antonopoulou et al., 2019; Gasco et al., 2020c).

Pikeperch (*Sander lucioperca*) is one of the main percid species that has drawn a great deal of attention in aquaculture (Schulz et al., 2006). Aquaculture production of pikeperch reached 1557 tonnes in 2018, which was doubled that of 2009 (750 tonnes) (FAO, 2020b), and has mainly been established in intensive recirculation systems (Dalsgaard et al., 2013). However, pikeperch and other percid fish have so far received very little attention from feed manufacturers (Bochert, 2020). Although some commercial aquafeeds for percids have become available, salmonids-targeted feeds are more widely used in practice (Stejskal et al., 2016). Since European pikeperch aquaculture is moving toward an established freshwater aquaculture sector (Policar et al., 2019), it will be necessary to develop suitable and sustainable feeds for aforementioned sector. Dietary protein requirements of at least 43% have been reported for appropriate growth performance and feed utilization of pikeperch fingerling (Nyina-Wamwiza et al., 2005). In the nature, aquatic insects, i.e., larvae of lake flies (*Chironomidae*) (Diptera order), play an important role as food sources for the early ontogenetic stages of pikeperch (Vinni et al., 2009; Ginter et al., 2011; Kashinskaya et al., 2018; Huuskonen et al., 2019). Therefore, the use of *H. illuces* larvae meal has been hypothesised to be suitable for pikeperch aquaculture. The aim of present study is to investigate the effects of dietary defatted black soldier fly (*H. illucens*) (HI) on the diets of juvenile pikeperch (*S. lucioperca*) on intestinal microbiota, histomorphology, and oxidative enzyme activities. The outputs could provide information in the choice of an alternative aquafeed ingredient for the emerging percid farming industry in Europe.

MATERIALS AND METHODS

Ethics Statement

The experimental procedures were performed under European Communities Directive (No. 2010/63/EU) on the protection of animals used for scientific purposes and have been approved by the Czech Ministry of Health (MSMT-6744/2018-2).

Experimental Diets, Rearing Facilities, and Feeding Procedures

The feeding trial was conducted at the wet laboratory of the Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Czech Republic. Defatted HI was obtained from a commercial source (Hermetia Geschäftsführungs GmbH, Baruth/Mark, Germany). Four isoproteic (approximately 45% crude protein) and isolipidic (approximately 18% ether extract) diets were formulated, comprising one fishmeal-based diet (CO) and three other diets, where HI was included at 9% (HI9), 18% (HI18), and 36% (HI36) to replace fishmeal at 25, 50, and 100%, respectively (**Table 1**). Experimental diets were prepared by a commercial feed producer (Exot Hobby s.r.o., Černá v Pošumaví, Czech Republic) using a dual-screw extruder (Saibainuo, China). Chemical composition of HI and experimental diets as well fatty acid (FA) composition of experimental diets are reported in **Tables 1**, **2**, respectively.

TABLE 1 | Ingredients and proximate composition of experimental diets.

Ingredients (g/kg, as it)	HI[a]	CO	HI9	HI18	HI36
Fishmeal[b]		300	225	150	0
HI		-	90	180	360
Soybean protein concentrate		75	75	75	75
Corn gluten meal		170	170	170	170
Soybean meal		150	150	150	150
Wheat meal		80	65	50	20
Merigel		60	60	60	60
Fish oil		60	60	60	60
Soybean oil		60	60	60	60
Vitamin mixture[c]		10	10	10	10
Mineral misture[d]		10	10	10	10
DL-Methionine		7	7	7	7
L-Lysine		8	8	8	8
Celite®		10	10	10	10
Proximate composition					
Dry matter (g/100g)	91.0	94.3	94.9	94.5	94.8
Crude protein (g/100g)	54.5	44.8	45.2	44.7	45.1
Ether extract (g/100g)	8.5	18.9	18.2	18.9	17.4
Ash (g/100g)	7.6	8.7	8.6	8.1	7.4
Chitin (g/100g)[e]	5.34	-	0.47	0.97	1.93
Nitrogen-free extract (g/100g)[f]	24.06	27.60	27.53	27.33	28.17
Gross energy (MJ/kg)	20.20	21.05	20.36	20.32	21.06

[a]Defatted Hermetia illucens larvae meal; [b]Purchased from Corpesca S.A. (Santiago, Chile). Proximate composition (g/100g, as fed basis): 91.3 dry matter; 65.8 crude protein; 9.4 ether extract; and 15.5 ash; [c]Vitamin mixture (IU or mg kg^{-1} diet): DL-α tocopherol acetate, 60 IU; sodium menadione bisulphate, 5 mg; retinyl acetate, 15,000 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B_{12}, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg (purchased from Granda Zootecnici S.r.l., Cuneo, Italy); [d]Mineral mixture (g or mg kg^{-1} diet): dicalcium phosphate, 500 g; calcium carbonate, 215 g; sodium salt 40, g; potassium chloride, 90 g; magnesium chloride, 124 g; magnesium carbonate, 124 g; iron sulphate, 20 g; zinc sulphate, 4 g; copper sulphate, 3 g; potassium iodide, 4 mg; cobalt sulphate, 20 mg; manganese sulphate, 3 g; sodium fluoride, 1 g (purchased from Granda Zootecnici S.r.l., Cuneo, Italy); [e]Estimated as described by Finke (2007); [f]Calculated as 100 - (CP + EE + Ash + Chitin).

The feeding experiment was conducted in a recirculation aquaculture system (total volume 11400 L), consisting of fifteen 250-L round conical plastic tanks (black walls, white bottom) connected to a mechanical drum filter (AEM 15, AEM-Products V.O.F., Lienden, Netherlands), sedimentation tanks (total volume 2600 l), a series of filtration sections (Bioakvacit PPI10), and a moving bed bio-filter (volume 4700 l, media BT10 Ratz Aqua & Polymer Technik, Remscheid, Germany), under controlled rearing conditions, with water temperature of 23.1 ± 1.0°C, photoperiod of 12h light – 12h dark, light intensity of 20–35 Lux, oxygen saturation of 98.4 ± 15.2%, and pH of 6.98 ± 0.28. Moreover, the concentration of nitrite-N, nitrate-N, and ammonia-N concentration were maintained at 0.42 ± 0.24, 48.8 ± 21.3, and 1.89 ± 0.58 mg/l, respectively.

The prepared diets were fed to triplicate groups of juvenile pikeperch (initial body weight 68.7 ± 7.1 g, with 50 individuals per tank) for 84 days. A combined feeding protocol of four meals per day, provided at 07.00, 09.00, 11.00, 13.00, by automatic feeders (EHEIM Twins, Deizisau, Germany), and one hand feeding, at 15.00 was adopted during the trial. Any unconsumed feeds were collected by siphoning and dried in an oven to calculate the exact feed intake.

Sampling Procedures

Fish Biometry

At the start and the end of the feeding trial, fish were individually weighed to calculate weight gain (WG) and feed conversion ratio (FCR):

WG (g) = final body weight-initial body weight

FCR = total feed supplied (g, Dry Matter)/WG

Antioxidative Enzyme and Histo-Morphological Analysis

After 84 days of the experiment, a total of 45 fish (3 individuals/tank) were randomly sampled, after 24 h of feed deprivation, and were euthanised by means of overdose anaesthesia (MS222, 125 mg/l).

Dissected livers and intestines from 15 fish/group were stored at −80°C for further antioxidative enzyme analysis. A similar number of samples, taken from another 15 fish/group, were fixed by immersion in a 10% buffered formalin solution for histo-morphological analysis.

Intestinal Microbiota

At the end of the experiment, three fish were randomly taken from each tank and euthanised by means of overdose anaesthesia (MS222, 125 mg/l). In order to ensure that all sampled fish had digesta throughout the intestinal tract, fish were deprived of feeds 12 h prior to sampling time. Fish exterior was wiped with 70% ethanol before abdomen was opened, whole intestine from each fish was removed from the abdominal cavity and digesta from proximal to distal intestine was squeezed gently into a 1.5 ml aseptic Eppendorf and immediately stored at −80°C for further analysis.

Analytical Methods

Diet Chemical Composition

Analysis of HI defatted meal and experimental diets for dry matter, crude protein, crude lipid, ash, and fatty acids (FAs) were performed as described elsewhere (Tran et al., 2021). Gross energy was determined by mean of a calorimetric bomb (IKA C7000, Stufen, Germany).

Oxidative Stress in Livers and Intestines

Oxidative stress biomarkers were evaluated in liver and intestine of each fish sample by means of spectrophotometer analysis (Varian Cary spectrophotometer, Santa Clara, CA, United States) as previously described by Elia et al. (2018). Briefly, superoxide dismutase (SOD) activity was measured in 50 mM Na_2CO_3, pH 10, 0.1 mM EDTA, 500 mM cytochrome C, and 1 mM hypoxanthine and xanthine oxidase. Reduction of cytochrome C by the xanthine/hypoxanthine system was measured versus a standard curve of SOD units at 550 nm. Catalase (CAT) activity was measured as the decrease in absorbance at 240 nm due to the consumption of H_2O_2. The assay was performed in an NaH_2PO_4 + Na_2HPO_4 buffer (100 mM, pH 7) and 12 mM H_2O_2. Glutathione peroxidase (SeGPx's) activities were measured by following the oxidation of NADPH at 340 nm and using 0.6 mM H_2O_2 or 0.8 mM cumene hydroperoxides (tot GPx) as substrates. Glutathione S-transferase (GST) was measured at 340 nm using as a substrate 1-chloro-2,4-dinitrobenzene (CDNB).

Histo-Morphological Analysis of Intestine and Liver

Samples of the anterior intestine were excised and flushed with a 0.9% saline solution to remove all the content. The collected samples were fixed in a 10% buffered formalin solution, routinely embedded in paraffin wax blocks, sectioned at a 5 μm thickness, mounted onto glass slides and stained with Haematoxylin & Eosin (HE). One slide per intestinal segment was examined by means of light microscopy and captured with a Nikon DS-Fi1 digital camera, coupled to a Zeiss Axiophot microscope, using a 2.5× objective lens. NIS-Elements F software was used to capture images.

Morphometric analysis was performed using Image®-Pro Plus software on ten well-oriented and intact villi. The evaluated morphometric indices were villi height (from the villus tip to submucosa) and villi width (across the base of the villus, but not including the brush border).

The observed histopathological findings were evaluated in all the organs, using a semi-quantitative scoring system as follows: absent (score = 0), mild (score = 1), moderate (score = 2), and severe (score = 3). Histopathological findings in intestine were assessed separately for each segment for mucosa (inflammatory infiltrates) and submucosa [inflammatory infiltrates and Gut-Associated Lymphoid Tissue (GALT) activation]. The total score of each gut segment was obtained by adding to the mucosa and submucosa scores. All the slides were blind assessed by two independent observers, and any discordant cases were re-examined, using a multi-head microscope, until unanimous consensus was reached.

Microbiome Analysis

DNA Extraction and 16S rRNA Amplicon Target Sequencing

Nucleic acid was extracted from the intestine content (500 mg as starting materials). Total DNA from the samples was extracted using a RNeasy Power Microbiome KIT (Qiagen, Milan, Italy), according to the manufacturer's instructions. One microlitre of RNase (Illumina Inc, San Diego, CA, United States) was added to

TABLE 2 | Fatty acid (FA) composition (as mg/g total FAs) of experimental diets.

*FAs	Experimental diets			
	CO	HI9	HI18	HI36
C12:0	0.4 ± 0^{a}	16.1 ± 0.3^{b}	25.7 ± 0.7^{c}	61.8 ± 3.4^{d}
C14:0	17.2 ± 0.1^{a}	20.1 ± 0.1^{b}	21.2 ± 0.1^{c}	27.5 ± 0.7^{d}
C16:0	102.7 ± 0.5^{a}	106.8 ± 0.3^{b}	105.2 ± 1.6^{b}	106.2 ± 0.9^{b}
C16:1	23.7 ± 0^{a}	23.9 ± 0^{ab}	24.0 ± 0.1^{b}	24.1 ± 0.1^{b}
C18:0	29.9 ± 0.2	30.2 ± 0.3	30.3 ± 1.7	28.1 ± 0.2
C18:1n9	201.3 ± 0.8^{c}	196 ± 0.2^{b}	195.6 ± 0.3^{b}	188.5 ± 0.9^{a}
C18:1n7	206.2 ± 3.5^{b}	196 ± 0.2^{a}	197.9 ± 3.8^{a}	194.5 ± 0.9^{a}
C18:2n6	257.6 ± 0.9^{d}	254.1 ± 0.4^{c}	251 ± 1.8^{b}	241.8 ± 1.0^{a}
C18:3n3	38.9 ± 0.2^{c}	37.3 ± 0^{b}	37 ± 0.2^{b}	34.3 ± 0.2^{a}
C20:1n9	33.0 ± 0.3^{c}	31.2 ± 0.1^{b}	31.0 ± 0.2^{b}	27.5 ± 0.1^{a}
C20:5n3 (EPA)	3.20 ± 0.01^{d}	3.10 ± 0.01^{c}	3.00 ± 0.01^{b}	2.60 ± 0.01^{a}
C22:6n3 (DHA)	48.2 ± 0.5^{d}	45.5 ± 0.2^{c}	39.1 ± 0.2^{b}	26.7 ± 0.5^{a}
∑n-3	91.4 ± 0.7^{d}	86.9 ± 0.3^{c}	80.1 ± 0.4^{b}	64.4 ± 0.6^{a}
∑n-6	268.1 ± 1.0^{d}	264 ± 0.5^{c}	259.8 ± 1.8^{b}	248.3 ± 1.0^{a}
∑SFA	164.6 ± 0.9^{a}	190.6 ± 0.7^{b}	200 ± 4.4^{c}	239.5 ± 4.4^{d}
∑MUFA	470.9 ± 2.5^{c}	453.6 ± 0.3^{b}	454.8 ± 3.6^{b}	440.2 ± 1.9^{a}
∑PUFA	360 ± 1.7^{d}	351.4 ± 0.7^{c}	340.4 ± 2.2^{b}	316.0 ± 4.9^{a}

**Only FAs > 10 mg/g total FAs (except for EPA) are presented; Different letters denote significant differences among the experimental groups (P < 0.05).*

digest the RNA in the DNA samples for an incubation period of 1 h at 37°C. DNA was quantified using Qubit ds and standardised at 5 ng/μ l.

DNA extracted directly from digesta samples was used to assess the microbiota, through amplification of the V3–V4 region of the 16S rRNA gene (Klindworth et al., 2012). The PCR products were purified according to the Illumina metagenomic standard procedure (Illumina Inc, San Diego, CA, United States). Sequencing was performed with an MiSeq Illumina instrument, with V3 chemistry, and 250 bp paired-end reads were generated according to the manufacturer's instructions.

Statistical Analysis

All data for antioxidative enzyme activities were tested for homogeneity of variance using Cochran, Hartley, Bartlett test. The effects of diet on oxidative stress in different organs were analysed separately, by means of one-way ANOVA, followed by Tukey test. Statistical analyses were performed using STATISTICA 12.0, with P-value < 0.05 as the significant difference.

Raw reads of microbiota were first joined, after sequencing, using FLASH software (Magoč and Salzberg, 2011), with default parameters, and were filtered, using QIIME 1.9.0 software and the pipeline as recently described (Biasato et al., 2018). Briefly, shorter reads (<300 bp) were discarded, using Prinseq. USEARCH software (version 8.1) was used for chimera filtering, and the Operational Taxonomic Units (OTUs) were picked, at a threshold of 97% similarity, using UCLUST algorithms. Taxonomy was assigned against 16S rRNA from Greengenes. The OTU table was rarefied at 10,144 sequences/sample. The OTU table displays the highest taxonomy resolution that was reached. When the taxonomy assignment was not able to reach the genus level, the family or phyla were displayed. R software was used to calculate the alpha diversity, while Weighted and Unweighted UniFrac distance matrix and OTUs table were used to find differences between samples, using permutational multivariate analysis of variance (Anosim) and analysis of similarity (Adonis) statistical test, considering the same function in R environment. Pairwise Wilcoxon test were used to determine any significant differences in alpha diversity or OTU abundance as a function of dietary insect meal. Principal component analysis (PCA) were plotted, using the *dudi.pca* function, through the *made4* package of R environment. Non-normally distributed variables were presented as median values (interquartile range, IR), and box plots represented the interquartile range between the first and the third quartile, with the error bars showing the lowest and the highest value. Pairwise Kruskal-Wallis tests were used to find any significant differences in microbial taxa abundance according to the dietary treatment. P-values were adjusted for multiple testing, and a false discovery rate (FDR) < 0.05 was considered as significant. The data generated from sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under the BioProject Accession Number PRJNA704237.

GraphPad Prism® software (version 8.0) was used to perform statistical analysis, for histo-morphometrical investigations. The Shapiro–Wilk test was used to test the normality of the data distribution before statistical analyses. Data were described by mean and standard deviation (SD), or median and IR depending on data distribution. Bivariate analysis was performed, by means of one way-ANOVA or Kruskall Wallis tests, to compare the intestine morphology and organs histopathology among different diet groups. P-values < 0.05 were considered statistically significant.

RESULTS

Diet Composition and Growth Production of Pikeperch

Formulated diets had a similar proximate composition, except for chitin which increased with the increase of HI inclusion (**Table 1**). The inclusion of dietary HI significantly altered the FA profile of experimental diets. As regards saturated FAs (SFA), lauric (C12:0), myristic (C14:0), and palmitic acid (C16:0) significantly increased with the increase of HI inclusion ($P < 0.05$). Monounsaturated FAs (MUFA), dominated by palmitoleic acid (C16:1), C18:1n9 and C18:1n7, were found to be significantly higher in CO than H36 ($P < 0.05$), while MUFAs

TABLE 3 | Growth performances and histopathological traits divided by diet groups.

	Experimental diets				
	CO	**HI9**	**HI18**	**HI36**	***P-value***
Growth performances					
Weight gain (g), *mean (SD)*	85.3^{a} (24.1)	84.8^{a} (23.7)	83.2^{a} (26.4)	62.8^{b} (18.3)	<0.001
FCR, *mean (SD)*	1.27^{b} (0.06)	1.28^{b} (0.07)	1.29^{b} (0.03)	1.81^{a} (0.15)	<0.001
Anterior gut					
Villi height (mm), *mean (SD)*	0.31 (0.07)	0.32 (0.07)	0.29 (0.05)	0.28 (0.07)	0.979
Villi width (mm), *mean (SD)*	0.03 (0.005)	0.03 (0.006)	0.03 (0.008)	0.11 (0.34)	0.065
Inflammation, *median (IR)*	0.00 (0.0–0.5)	0.00 (0.0–0.3)	0.00 (0.0–0.5)	0.00 (0.0–0.5)	0.967
Liver					
Degeneration, *median (IR)*	3.00^{a} (3.0–3.0)	3.00^{a} (2.0–3.0)	2.50^{b} (1.0–3.0)	2.50^{b} (1.0–3.0)	0.015
Inflammation	Absence of alterations				

SD, standard deviation; FCR, feed conversion ratio; IR, interquartile range. Values in the same row not sharing common superscript letter are significantly different.

in H9 and H18 remained comparable ($P > 0.05$). Increasing inclusion level of HI significantly reduced polyunsaturated FAs (PUFA) ($P < 0.05$). A similar trend was observed for EPA, DHA, linoleic acid, alpha-linolenic acid ($P < 0.05$) (**Table 2**).

At the end of the feeding trial, WG in fish fed HI36 (62.8, mean value) was significantly lower than the control group (85.3 g) ($P < 0.05$), whereas pikeperch fed HI9 (84.8 g) and HI18 (83.2 g) did not show significant difference with CO ($P > 0.05$). FCR of the CO group (1.27) was comparable with that of HI9 (1.28) and HI18 (1.29) ($P > 0.05$), but significantly lower than HI36 (1.81) ($P < 0.05$) (**Table 3**).

Oxidative Stress in Liver and Intestine

The results of oxidative biomarkers, SOD, CAT, SeGPx, and GST, in liver and intestine of pikeperch fed experimental diets are depicted in **Figure 1**. Dietary HI did not alter the SOD activities in either liver or intestine, CAT activities in liver, SeGPx activities in intestine, or GST activities in liver of pikeperch ($P > 0.05$). No significant difference was observed across experimental groups ($P > 0.05$) for liver, as regards CAT activities, whereas this biomarker was significantly lower in HI18 and HI36 than in HI9 ($P < 0.05$), but remained similar to CO ($P > 0.05$) in intestine. Even if did not differ from the CO group, among fish fed HI-containing diets, HI9 produced highest SeGPx activity in liver ($P < 0.05$), while the lowest activity was found in HI36 group ($P < 0.05$). A significant increase in the GST concentration was observed in intestine of pikeperch fed HI-containing diets, compared to CO ($P < 0.05$). Of the different insect-fed groups, HI9 showed a higher GST than HI18 ($P < 0.05$), while HI36 was remained intermediate position.

Histo-Morphology

Data regarding histopathological evaluation are reported in **Table 3**. Only few differences were observed for morphometry and histopathology of intestine among diet groups. Although there was no significant difference, a trend could be observed ($P = 0.065$) with HI36 group recording wider villi than the other groups. Thus, dietary HI inclusion did not induce any significant morphological changes in the pikeperch intestine, thereby suggesting no negative influence of such dietary HI on the physiological development of intestine.

Mild to severe multifocal to diffuse liver vacuolar degeneration was recorded in all treatments, and it was found to be greater in CO and HI9 group than in the HI18 and HI36 ones. Dietary HI did not show any evidence of inflammation of the liver of pikeperch (**Table 3** and **Figure 2**).

Microbiota

The total number of high-quality paired-end sequences obtained from 16S rRNA sequencing reached 1.916.822 raw reads. After the filtering, 1.295.693 reads passed the filters applied by QIIME, with a median value of 37.559 ± 15.565 reads/sample, and a mean sequence length of 443 bp. The rarefaction analysis and Good's coverage, expressed as a median percentage (97%), also indicated satisfactory coverage of all samples.

The result of the OTUs analysis showed that there was no significant difference in Shannon index ($P > 0.05$) among diet groups, while alpha-diversity of intestinal bacteria, associated with Chao1 and observed OTUs, in fish fed HI18 significantly increased relative to CO diet ($P < 0.05$) (**Figure 3**).

Adonis and Anosim statistical tests, based on weighted and on unweighted UniFrac distance matrix using the OTUs table, showed significant differences between diet groups as a administration of HI ($P < 0.002$). These differences were also observed when the PCA plot was produced at a genus level (**Figure 4**). It was also possible to observe a certain degree of separation, following diet groups. Microbiota of CO diet was near to the insect meal inclusion of 9%, while the microbiota of fish fed with 18 and 36% of HI was well separated (**Figure 4**).

The dominant OTUs, at the phyla level, were *Firmicutes* (mean values, 45–75%), regardless to dietary HI. Perch fed CO diet was enriched with *Proteobacteria* (26%), while *Bacteroidetes* (7–13%) was the prevalent phyla in fish fed HI-containing diets. As a result, *Clostridiaceae, Enterococcaceae,* and *Bacillaceae* were found to be the predominant families across fish fed diet groups. *Clostridium*, *Acetobacter*, *Cetobacterium*, *Plesiomonas*, *Acetobacter*, *Peptostreptococcaceae*, *Bacteroides,* and *Oceanobacillus* were, at the genus level, the most abundant genera found in intestine of perch considered in our study (**Figure 5**).

Dietary HI positively affected relative abundance of almost OTUs, compared with CO (FDR < 0.05), excepted for *Bacillus*, *Burkholderia,* and *Sporosarcina,* which were dominant in the CO group (**Figure 6**).

DISCUSSION

Oxidative Enzymes

Reactive oxygen species (ROS) is the production of aerobic metabolism processes, including superoxide, hydrogen peroxide, and lipid peroxides (Buetler et al., 2004). Excessive ROS compounds cause cellular and tissue damages (Rosa et al., 2008). The balance of ROS production ensures the normal physical function of any organism and is regulated by antioxidant systems (Rosa et al., 2008) involving two mechanisms, (i) enzymes that remove ROS, including SOD, CAT, and SeGPx; and (ii) antioxidative compounds, i.e., ascorbate, glutathione, scavenge free radicals (Passi et al., 2002). Antioxidative enzyme activities were documented to be tissue-specific in pikeperch, and liver was the most sensitive organ to the diet manipulation under recirculating aquaculture system (Policar et al., 2016). In the case of detoxification in the intestine, however, certain enzymes such as SOD were known to play a vital role (Tang et al., 2013). This study indicates that in liver of pikeperch dietary HI did not alter the SOD, CAT, or GST oxidative enzymes, while significantly reduced SeGPx activity, a result that is in agreement with those of previous study (Elia et al., 2018), who performed a trial on rainbow trout fed dietary HI. The significant reduction in the catalytic SeGPx efficiency in liver of pikeperch fed dietary HI could be explained by the presence of chitin (Elia et al., 2018). Indeed, increasing inclusion levels of HI increased chitin levels in diets (**Table 1**). In addition, declining in SeGPx activities, as a result of increasing dietary HI, could be attributed to different dietary PUFA levels

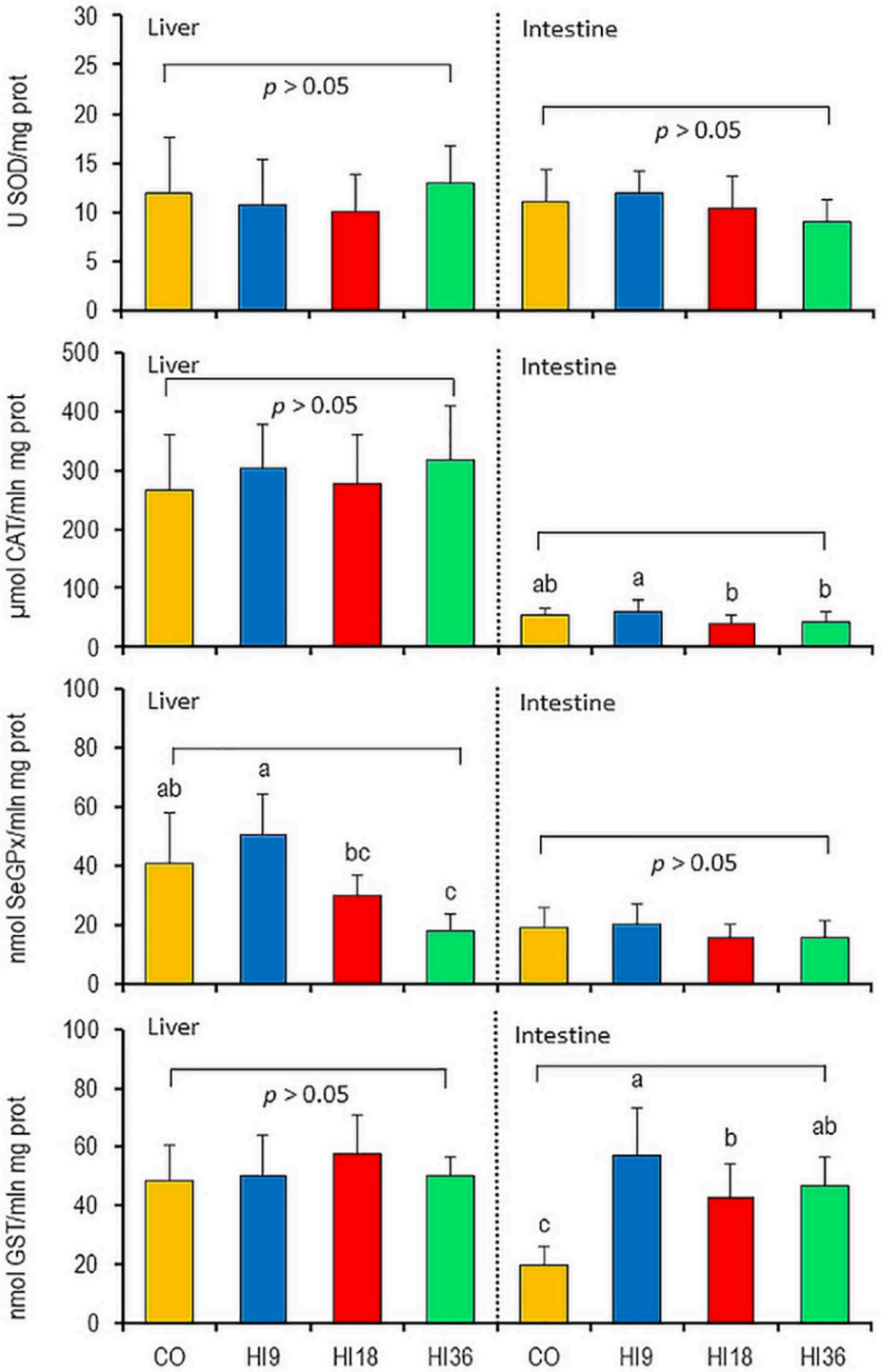

FIGURE 1 | Oxidative biomarkers in liver and intestine of pikeperch (*Sander lucioperca*) fed experimental diets. Values are presented as means ± standard deviation. Means with different letters are significantly different ($P < 0.05$) from each other.

(**Table 2**), which are highly susceptible to oxidation. In fact, Tocher et al. (2002) reported that a high dietary PUFA content increased lipid peroxidation in fish tissues, and consequently the SeGPx enzyme activity involved in reducing peroxides, including FA hydroperoxides and hydrogen peroxide, will be also high (Passi et al., 2002).

The present study indicates that the CAT activity in intestine of pikeperch was significantly higher for HI9 than

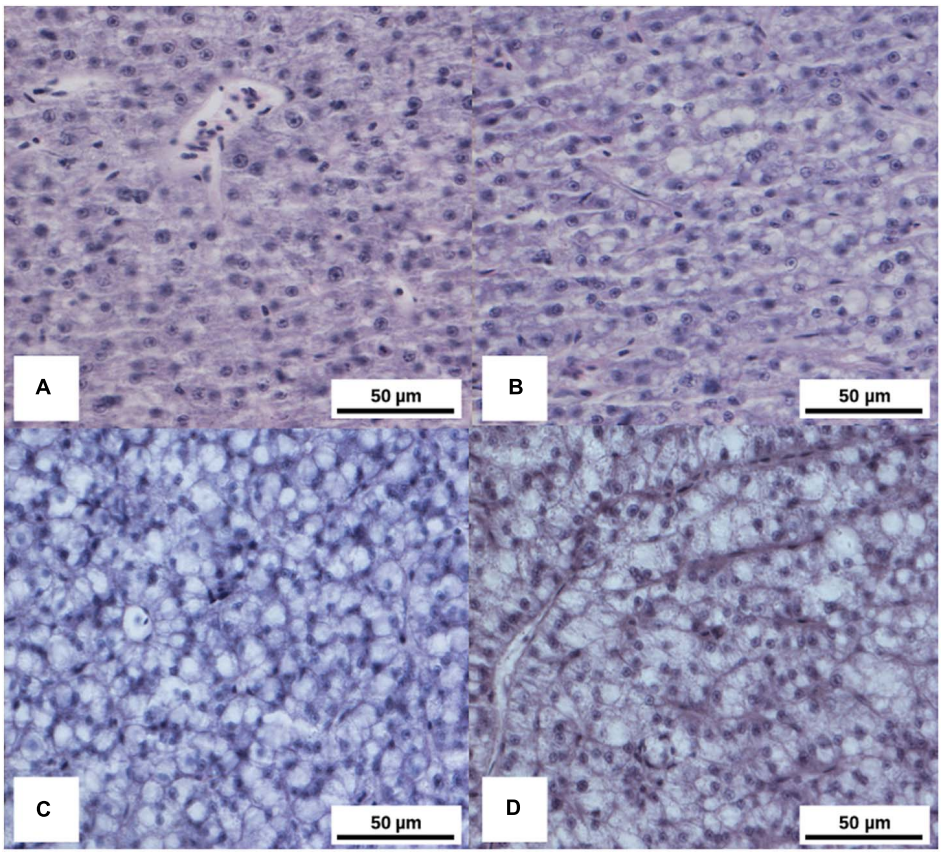

FIGURE 2 | Histopathological alteration of liver of pikeperch considered in the present study. **(A)** Normal liver, Haematoxylin & Eosin (H-e) stain, 40× magnification, for HI36 diet. **(B)** Mild and multifocal vacuolar degeneration (grade 1), H-e, 40× magnification, for HI18 diet. **(C)** Moderate and multifocal vacuolar degeneration (grade 2), H-e, 40× magnification, for the HI9 diet. **(D)** Severe and diffuse vacuolar degeneration (grade 3), H-e, 40× magnification, for CO diet.

for HI18 and HI36 groups. A similar phenomenon was reported for CAT activity in the intestine of rainbow trout fed insect meal (*T. molitor*), where a substitution level of 25% fishmeal displayed higher activity than the 50% level (Henry et al., 2018a). The CAT and SeGPx activities in the present study were similar for CO and HI9, and lower than for HI18, HI36 groups. This result indicates that substantial substitution of fishmeal with HI reduced antioxidant enzyme activities in pikeperch. This is in line with a previous finding pertaining to rainbow trout (*O. mykiss*) (Elia et al., 2018). The decline of these biomarkers in HI18 and HI36 groups could be related to an imbalance between ROS production and antioxidant capacity. A suitable concentration of antioxidants, such as chitin and other bioactive compounds (Ngo and Kim, 2014), may support antioxidant enzyme activities in HI9 compared to the other HI-contained diets (Henry et al., 2018a).

Glutathione S-transferase plays an essential role in scavenging free radicals and xenobiotics detoxification (Aksnes and Njaa, 1981; Li et al., 2010). Increased glutathione S-transferase activity in intestine, but not liver, was observed across diet groups in the present study (**Figure 1**), thus implicating that some of the compounds in HI may have stimulated the biotransformation pathway in intestine of pikeperch, which was also found in liver of tilapia (*Oreochromis niloticus*) fed cricket-based feeds (Ogunji et al., 2007). In fact, insect meals may contain harmful substances, i.e., heavy metals and pesticides (van der Spiegel et al., 2013). The absence of an alteration of the hepatic GST activities after administration of HI could be the result of factors other than xenobiotics (Collier and Varanasi, 1991) or tissue-specific response (Martínez-Álvarez et al., 2005).

We also observed numerically higher oxidative biomarkers in liver of pikeperch than in intestine (**Figure 1**), which was in agreement with recent findings (Policar et al., 2016), reporting that liver was one of the most susceptible tissue in response to artificial nutrition and controlled conditions.

Histo-Morphology

Dietary HI in our study did not induce any morphological or inflammatory changes in the intestine of pikeperch, a result that is in agreement with previous studies conducted on different fish species fed dietary insect meals (Elia et al., 2018; Zarantoniello et al., 2019; Zarantoniello et al., 2020a). The absence of intestinal and hepatic inflammation could be linked to anti-inflammatory properties regulated by dietary saturated fatty acids content, especially lauric acid (C12:0) and chitin

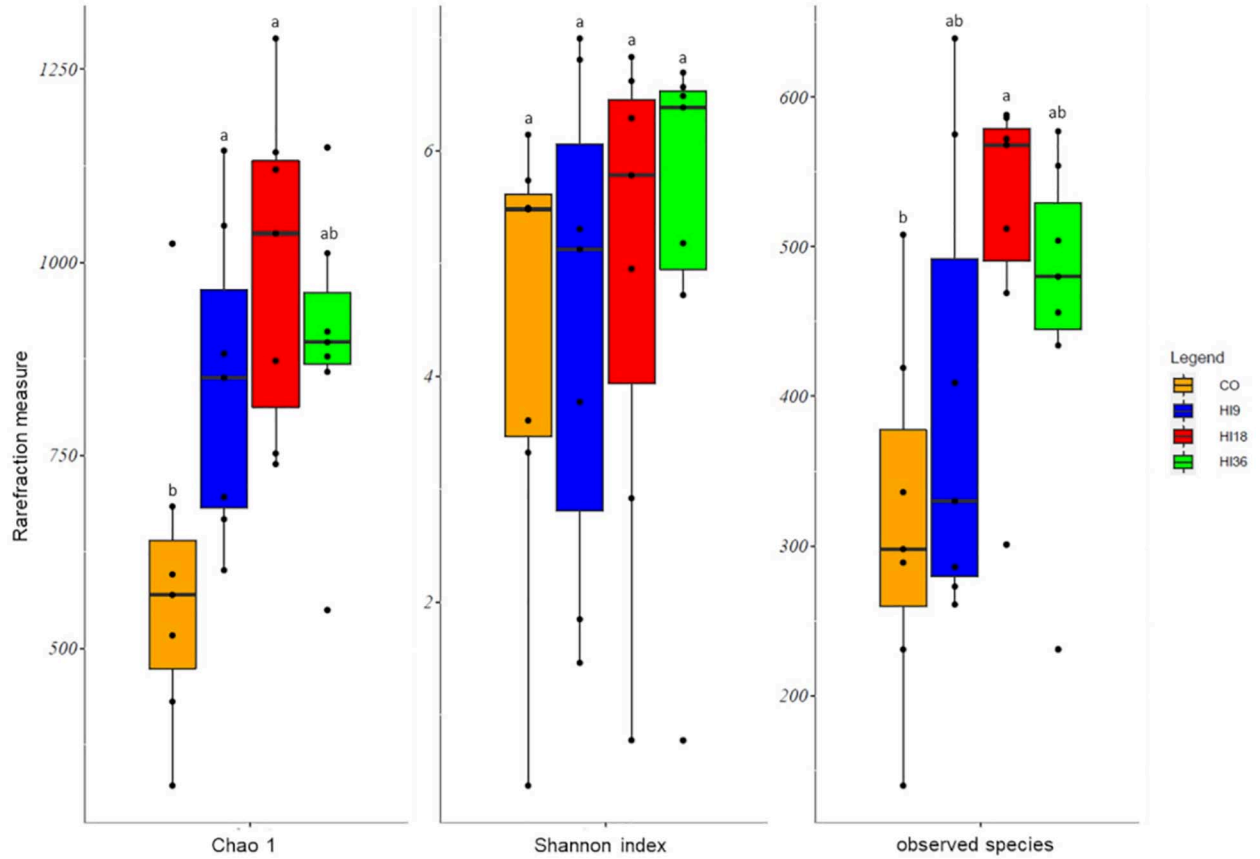

FIGURE 3 | Boxplots showing the alpha diversity rarefaction index across experimental diets. The boxes represent the interquartile range (IR) between the first and third quartiles, and the line inside the boxes represents the median. Whiskers denote the lowest and the highest values within 1.56 IR from the first and third quartiles, respectively. Circles represent outliers beyond the whiskers. Different superscripts within boxplot indicate significant differences.

component (Henry et al., 2018b; Vargas-Abundez et al., 2019; Zarantoniello et al., 2019; Gasco et al., 2020b,c) which were found to be particularly high in HI and HI-containing diets in the present study. Although there were no significant differences (at P-value < 0.05), the villi were more expanded in the HI36 group than in the other groups (**Table 3**), and this was attributed to the presence of chitin. Chitin could stimulate the growth of villi thickness in tilapia (*O. niloticus*), probably due to its viscosity and water holding capacity (Kihara and Sakata, 1997). Chitin also induced the production of short-chain fatty acids, such as acetate, propionate and n-butyrate, and n-butyrate in particular was observed in intestine of tilapia (Kihara and Sakata, 1997), thereby increasing intestinal histo-morphology of fish, e.g., villi length and weight (Dawood, 2021). The large quantity of *Paenibacillus* genus in intestinal digesta of fish fed HI36 (**Figure 6**) could act as a probiotic for aquatic animal species (Midhun et al., 2017; Chen et al., 2019; Amoah et al., 2020), consequently enhancing intestinal health indices, including histomorphology (Dawood, 2021).

In contrast to recent findings, which reported that an increasing inclusion of insect meals induced a higher degree of hepatic vacuolization degeneration in fish (Li et al., 2017; Zarantoniello et al., 2019), the present study indicates that feeding pikeperch with $\leq 9\%$ HI caused more severe hepatic degeneration than 18 or 36% did (**Table 3**), which could be related to a fatty liver status. Schulz et al. (2005) reported that a low level of palmitic acid (C16:0) yielded a higher hepatic lipid content. In the present study, the significantly lower palmitic acid in the control group than in the HI-containing groups could partly explain the hepatocellular vacuolization phenomenon. The mechanism to which palmitic acid affecting hepatic tissues remained to be elucidated. However, this FA promotes hepatocyte proliferation (Wang et al., 2011) and possess anti-inflammatory and antiviral effects (Librán-Pérez et al., 2019). On the other hand, the high content of dietary lauric acid (C12:0), high oxidation and low tissue deposition, was found to decrease liver lipid storage in freshwater Atlantic salmon (Belghit et al., 2019). This could explain the reduction in the adipose liver in pikeperch fed HI18 and HI36, compared to the control and HI9 diets (**Table 3**). Two FAs, linoleic and oleic acids, were confirmed to induce the occurrence of hepatic steatosis in sea bream (*Sparus aurata*) (Caballero et al., 2004). Moreover, owing to large molecular weight, oleic acid could produce a large lipid droplet while inrush hepatocyte (Bradbury, 2006).These FAs were found to

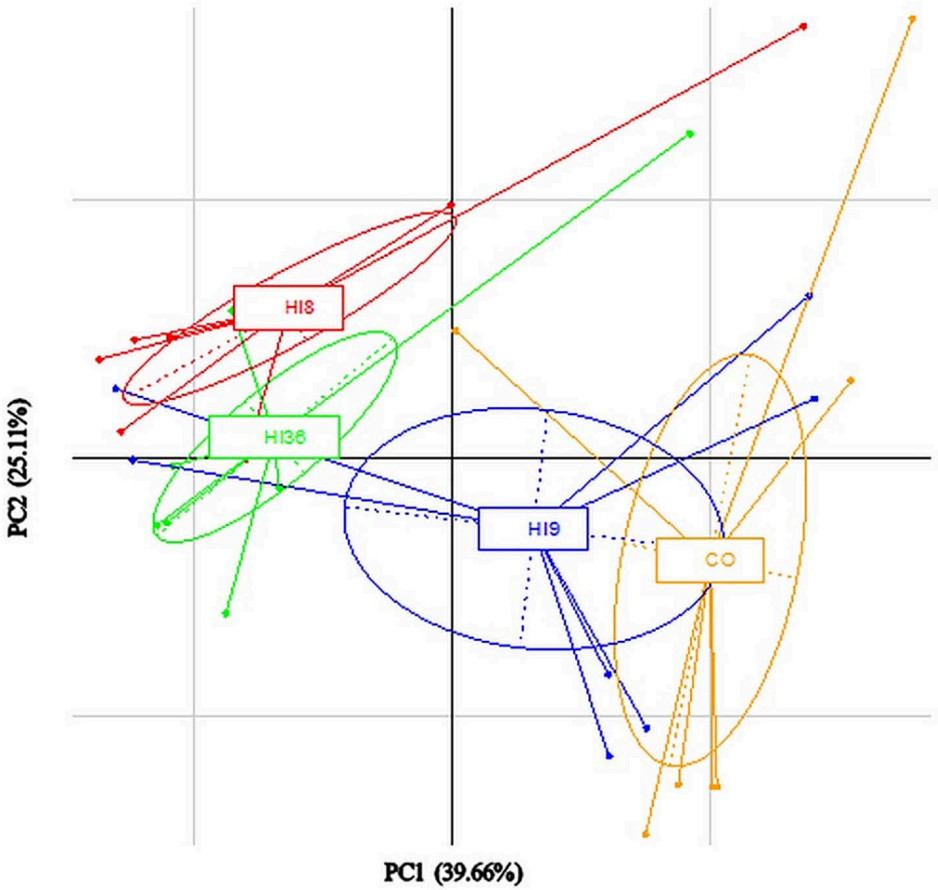

FIGURE 4 | Microbiota composition (PCA plots) in intestine of pikeperch (*Sander lucioperca*) fed experimental diets.

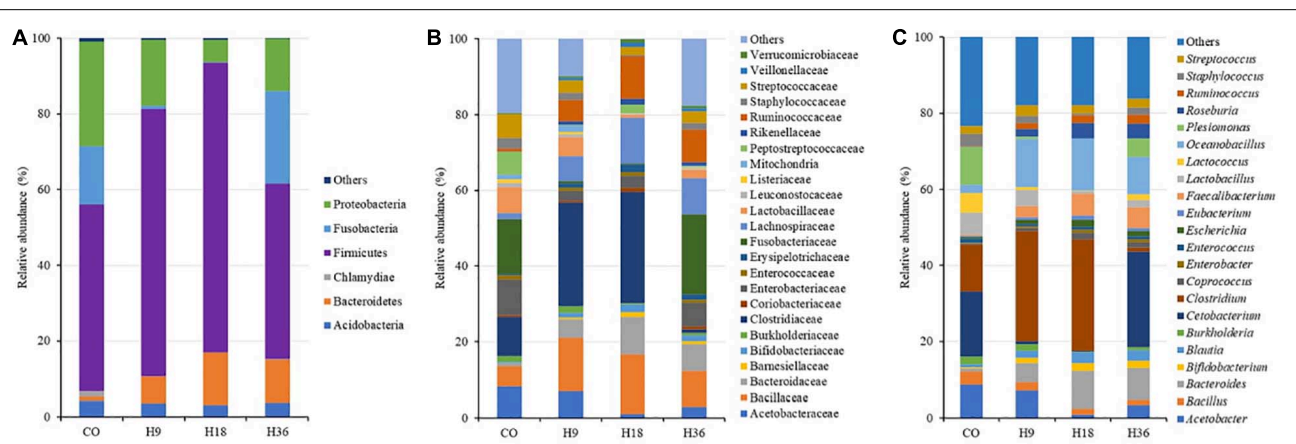

FIGURE 5 | Relative abundance (%) of the OTUs in the intestine of pikeperch fed experimental diets at phyla **(A)**, family **(B)**, and genus **(C)** level. Only bacteria with an overall abundance of ≥ 1% and ≥ 0.5% at phylum and family/genus level, respectively, were presented. The bacteria were pool as "Others," when lower than aforementioned abundance.

be significantly higher in CO than in HI18, HI36 (**Table 2**), which could indicate severe steatosis in livers of the former group (**Table 3**). High intakes of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are known to an inhibitor of lipid accumulation in livers of sea bream (*S. aurata*) (Caballero et al., 2004). Therefore, the change in the percentage of the different

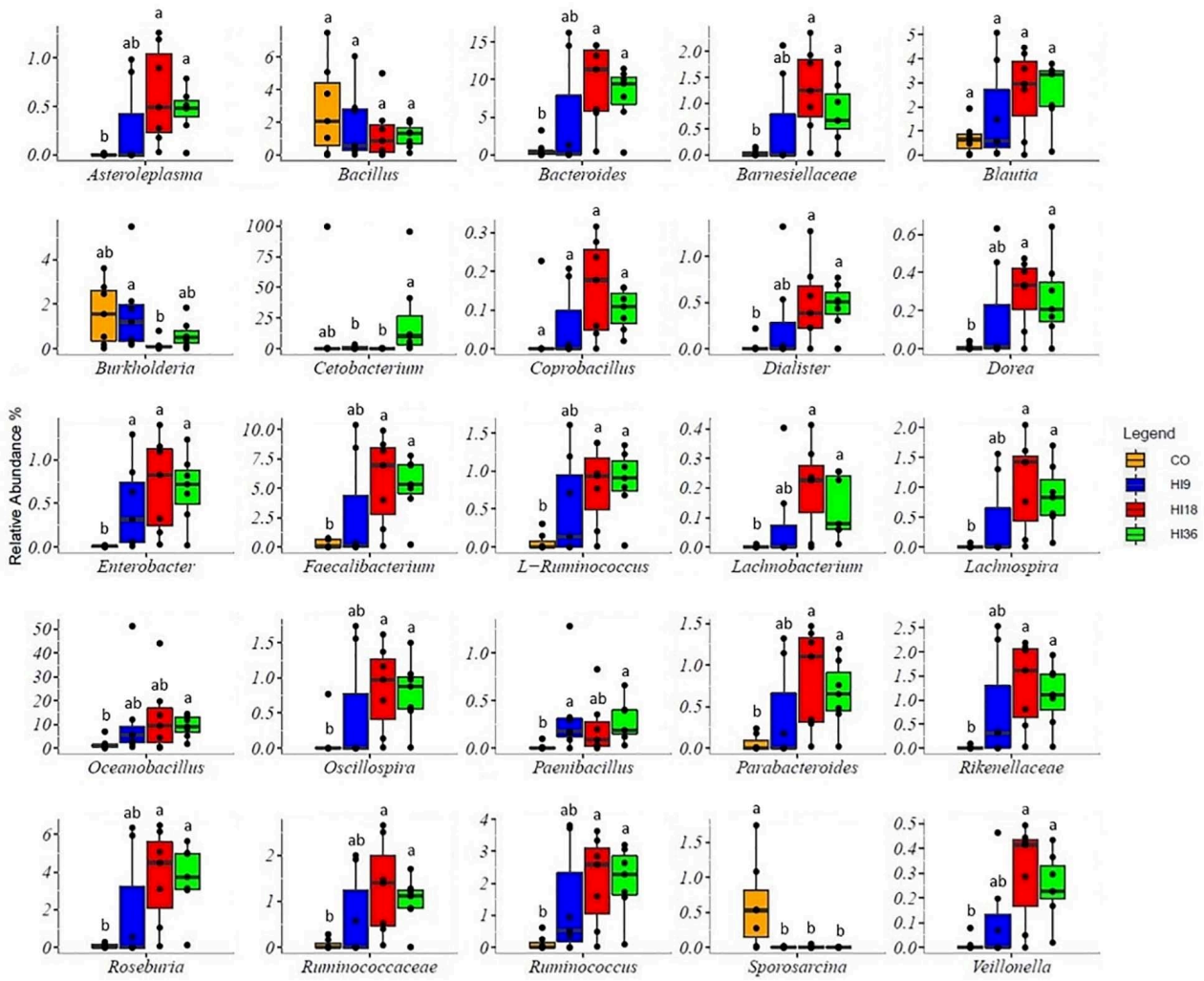

FIGURE 6 | Boxplots showing the relative abundance at the genus level of the OTUs in the intestine of perch fed experimental diets. Means with different letters are significantly different (FDR < 0.05) from each other.

FAs in the experimental diets, due to the inclusion of HI, could further explain the severity of hepatic vacuolization degeneration observed in perch fed CO and H9 diets.

Microbiota

The present study reveals that dietary HI enhanced microbial biodiversity indices in intestine of pikeperch, compared with insect-free diet, a result that is in line with recent findings on rainbow trout (Bruni et al., 2018; Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019), thereby contributing to gut health and health status of the host.

In agreement with previous studies on intestinal microbiota of percid fish and freshwater species, the present study reveals that *Firmicutes*, *Proteobacteria*, *Bacteroidetes* were the most dominant phyla in the intestine of pikeperch, regardless of the HI inclusion level (Li et al., 2014; Kashinskaya et al., 2018; Terova et al., 2019).

Our results show an abundance of *Clostridium* genus in fish fed HI9 and HI18, which was even greater than those fed CO and HI36. Members of the *Clostridium* genus are common effective microorganism used as probiotics in aquaculture (Nayak, 2010a,b). *Clostridium butyricum* has been shown to possess a pathogenic inhibition capacity in farmed fishes (Pan et al., 2008a,b; Gao et al., 2013), improve feed efficiency in shrimp (Duan et al., 2017; Li et al., 2019), and to be suitable for use as probiotics in farmed fish (Hai, 2015; Zorriehzahra et al., 2016). The greater prevalence of *Clostridium* and other probiotic-used bacteria in HI9, such as *Lactobaccillus* and *Bacillus* genera, than in HI36, could explain the difference in feed conversion ratio between these diets in present study. The *Bacteroides* and *Clostridium* genera are known to be the main taxa involved in production of fatty acids and vitamins (Balcázar et al., 2006). The abundant presence of these taxa could partially compensate for nutritional insufficiencies in HI-containing diets, and consequently resulted in a comparable growth rate among control, HI9 and HI18 diets, yet the offset may be not efficient for HI36 group.

It is worth noting that *Cetobacterium,* the most predominant bacterium in intestine of natural pikeperch (Kashinskaya et al., 2018) and other freshwater fish (Larsen et al., 2014), was detected in our captive pikeperch fed dietary HI. Similar findings were also observed in rainbow trout (Etyemez and Balcázar, 2015), common carp (van Kessel et al., 2011), and giant arapaima (Ramírez et al., 2018) fed commercial aquafeeds. It seems relevant that *Cetobacterium* is among the core bacteria in pikeperch.

Insect meal, in general, is a chitin-rich ingredient. The degradation and digestion of this substance require binary enzymes, including chitinase and β−N−acetylglucosaminidase, and involve various microbacteria derived from digestive tract of fish with a chitinase-produced capacity (Ray et al., 2012; Ringø et al., 2012). Among these chitin-degraded bacteria, the *Plesiomonas* and *Bacillus* genus were detected across treatment groups at a particularly low abundance (**Figure 4**). This finding implicates that pikeperch may not be able to degrade chitin. A limited presence of chitinase-producing bacteria was also observed in rainbow trout (Bruni et al., 2018; Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019) and this may help to explain the low or absent chitin digestibility in this species (St-Hilaire et al., 2007; Henry et al., 2015; Renna et al., 2017; Caimi et al., 2020).

In conclusion, HI, fed as a partial or total replacement of fishmeal did not induce any inflammation of liver or intestine, or any intestine degeneration, but did show signs of severe hepatic steatosis of pikeperch fed CO and HI9 groups. Dietary HI promotes antioxidative enzyme activities of CAT, GPx and GST, but not of SOD, in liver and, to a lesser extent, in intestine of pikeperch. The inclusion of HI up to 18% or 50% fishmeal replacement in pikeperch diets increased abundance of *Clostridium*, *Oceanobacillus, Bacteroides,* and *Faecalibacterium,* whereas the predominant bacterium, *Cetobacterium* was found in the control and HI36 groups. Because of the absence of inflammation in tissues, the evolution of antioxidative enzyme, and modification of the favourable microbiota observed in the present study, it is possible to assume that defatted HI could have an immunological effect on juvenile pikeperch. Further study on immune response and disease resistance of pikeperch fed insect meal could help to explore this efficiency.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Czech Ministry of Health (MSMT-6744/2018-2).

AUTHOR CONTRIBUTIONS

LG and VS: planning the experiment and editing the manuscript. HT: data analysis, writing, and editing of the manuscript. MP, MZ, and TG: wrote the manuscript. AE, EC, IF, CC, and FG: analysis and the first draft. All authors contributed to the article and approved the submitted version.

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Dietary Filamentous Fungi and Duration of Feeding Modulates Gut Microbial Composition in Rainbow Trout (*Oncorhynchus mykiss*)

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Changes in gut microbial composition over time in rainbow trout fed differentially processed diets supplemented with the filamentous fungi *Neurospora intermedia* were investigated in a 30-day feeding trial. Fish were fed a reference diet, non-preconditioned diet (NPD), or preconditioned (heat-treated) diet (PD), with the same inclusion level of *N. intermedia* in diets NPD and PD. Gut microbiota were analyzed on day 0, 10, 20, and 30. Gut microbial composition was similar for all diets on day 0, but was significantly different at day 10 and day 20. On day 30, the gut again contained similar communities irrespective of diet. The overall gut microbiota for each diet changed over time. Abundance of *Peptostreptococcus* and *Streptococcus* was higher in the initial days of feeding in fish fed on commercial diet, while a significant increase in lactic acid bacteria (*Lactococcus lactis*) was observed on day 30. Feed processing (preconditioning) did not contribute largely in shaping the gut microbiome. These results indicate that dietary manipulation and duration of feeding should be considered when evaluating gut microbial composition in cultured fish. A minimum 30-day feeding trial is suggested for gut microbiome, host and diet interaction studies.

Keywords: rainbow trout, filamentous fungi, duration of feeding, gut microbiome, fish, *Lactococcus*, amplicon sequencing

INTRODUCTION

Single-cell proteins such as microalgae, bacteria, and fungi are microbial protein sources that represent potential alternatives as fish feed ingredients (Nalage et al., 2016). In particular, filamentous fungi are versatile microorganisms that can grow on a wide range of wastes, industrial by-products, and side-streams. The nutritional value of filamentous fungal biomass derives from its high protein content, fatty acid composition, and presence of other nutrients such as vitamins, minerals, anti-oxidants, and immune stimulant components (Karimi et al., 2019a). Despite these attractive nutritional properties of filamentous fungal biomass, few studies have explored its use as a fish feed ingredient. Using nuclear magnetic resonance (NMR) spectroscopy, Abro et al. (2014) investigated changes in the metabolism of Arctic charr (*Salvelinus alpinus*) fed with filamentous fungal species *Rhizopus oryzae*. In another study, Vidakovic et al. (2016) used intact and extracted baker's yeast (*Saccharomyces cerevisiae*) and *Rhizopus oryzae* as separate diet ingredients and evaluated the effects on digestibility and intestinal barrier function in Arctic charr.

Neurospora intermedia is a food-grade filamentous fungus isolated from traditional fermented food in Indonesia, and is therefore among the filamentous fungi species recognized as safe. Its nutritional properties and cultivation conditions have been extensively explored by our research group (University of Borås) and reported in previous studies (Ferreira et al., 2014, 2015; Gmoser et al., 2018; Karimi et al., 2019b). The high nutritional value of *N. intermedia* and its categorization as a dietary safe microorganism make it an ideal alternative ingredient for fish feed.

The gut microbiota is critical to fish nutrition as it produces several enzymes which help in digestion, transport of nutrients, direct protection from pathogens, and enhanced immunity (Austin, 2006; Merrifield et al., 2010; Camp et al., 2012; de Bruijn et al., 2018). Several studies have found that environmental (abiotic) and host (biotic) factors play important roles in shaping the gut community in fish. Gut microbial composition and diversity are influenced by genetics, sex, weight, age, rearing conditions, diet, and feeding habits (Hovda et al., 2012; Ingerslev et al., 2014a; Li et al., 2016; Ringø et al., 2016; Yan et al., 2016; Sun et al., 2020). High-throughput sequencing has been used previously to explore dietary effects on the gut microbiota of several fish species, such as rainbow trout, Atlantic salmon (*Salmo salar*), Arctic charr, sea bream (*Sparus auratus*), and channel catfish (*Ictalurus punctatus*) (Navarrete et al., 2013; Gajardo et al., 2017; Huyben et al., 2017; Nyman et al., 2017; Wang et al., 2019). Most of these studies have investigated the short- or long-term effect of diet on gut microbiota but, to our knowledge, none has investigated gradual changes in microbial communities over time. Diet can adversely modulate gut microbial composition in fish, leading to inflammation of the distal intestine, as demonstrated for Atlantic salmon fed high levels of soy protein (Gajardo et al., 2017). It has also been shown that Arctic charr fed filamentous fungi (*Rhizopus oryzae*) display higher frequency of diarrhea, despite high apparent digestibility coefficient (Langeland et al., 2016). Knowledge of the interactions between host, gut microbiota, diet, and feeding strategy is important when developing novel diets, in order to ensure better fish health and welfare. The present study sought to extend this knowledge by examining the role of novel filamentous fungi in modulating the intestinal microbiota of rainbow trout over successive 10-day feeding intervals and its efficiency as a fish feed ingredient.

MATERIALS AND METHODS

Fish Husbandry

Juvenile rainbow trout were purchased from Vilstena Fiskodling AB, Fjärdhundra, Sweden, and the experiment was carried out in the Aquatic Facility, Center of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences, Uppsala, Sweden. A total of 300 fish (average weight 127.8 ± 19.8 g) were randomly and evenly distributed between 15 oval experimental tanks (200 L) and reared in a 12-h light cycle (08.00–20.00 h). The experimental tanks were equipped with a partial recirculation system and supplied with fresh tap water at 3 L min^{-1}. All fish were judged to be healthy, with no visible signs of injuries detected on skin, gills, or fins. Each experimental tank was connected to a waste feed and feces collection system. Temperature during the whole experiment was 11 ± 1°C and oxygen level was 8 ± 2 mg/L (HQ40D Portable Multi Meter, Hach, Loveland, CO, United States). The fish were acclimatized for 10 days on a commercial diet (Biomar EFICO ENVIRO 920 ADVANCE, 2% of body weight once a day prior to the experiment. The experiment was performed in compliance with laws and regulations on procedures and experiments on live animals in Sweden, which are overseen by the Swedish Board of Agriculture (diary number: 5.8.18-16347/2017).

Production of *Neurospora intermedia* Biomass

Fungal biomass of *N. intermedia* CBS 131.92 (Centraalbureau voor Schimmelcultures, Netherlands) was produced under semi-continuous cultivation condition at the Swedish Center for Resource Recovery, University of Borås. The fungus was cultivated on complex medium containing 30 g/L glucose and 5 g/L yeast extract as the major carbon and nitrogen source, respectively. Trace elements in the form of $(NH_4)_2SO_4$, KH_2PO_4, $CaCl_2.2H_2O$, and $MgSO_4$ $x7H_2O$ in concentrations of 7.5, 3.5, 1.0, and 0.75 g/L were added to the cultivation medium to support filamentous fungi growth, using a 26 L capacity bubble column bioreactor (airlift bioreactor converted to bubble column bioreactor by removing the internal loop tube) (Bioengineering, Switzerland). Cultivation was carried out at 35°C and 1 vvm (volume of air per volume of medium per minute). Cultivation condition parameters and sterilization method were according to Ferreira et al. (2015). To harvest biomass, 75% of the working volume of the reactor (15 L) was harvested twice per day, at 11.00 and 23.00 h. Fresh sterilized cultivation media was added to top up the cultivation broth after harvesting. Harvested broth, containing post-cultivation medium and biomass, was transferred to a cold room and stored at 4°C. After termination of cultivation, biomass was quickly separated from the culture media using a sieve, washed with distilled water, and dried in an air oven at 70°C.

Diets and Feeding

Feed preparation was carried out at the Swedish University of Agricultural Sciences, Uppsala, Sweden. Three experimental diets were prepared, a reference diet (RD), a non-preconditioned diet (NPD), and a preconditioned diet (PD). Diet RD was prepared with fishmeal as the major protein source. Diets NPD and PD were prepared by mixing 30% (by weight) of *N. intermedia* biomass with 70% of diet RD according to Cho (1979). The ingredients were mixed in a kitchen mixer, gelatin dissolved in hot water was added as a binder, and the ingredients were mixed again and pelleted through a meat grinder, using a 3.5 mm die (Nima Maskinteknik AB, Örebro, Sweden). The strings produced were dried in an air oven at 50°C for 12 h and cut into pellets with a twin blade blender (Kneubühler, Luzern, Germany).

Diets PD and NPD were formulated in the same way, but diet PD was preconditioned by heat-processing in a convection

oven (Electrolux Professional, FCE061) at 105°C for 5 min, in order to increase the degree of gelatinization of starch and emulate temperature treatment during extrusion conditions. The prepared feed was stored at −20°C until it was fed to the fish (approximately 2 weeks). Data on feed composition and proximate analysis are presented in **Tables 1**, **2**, respectively. Rainbow trout were fed twice a day throughout the 30-day feeding trial, using automatic belt feeders (Hølland teknologi, Sandnes, Norway). Feed was initially provided in excess (starting with a ration equal to 1.5% of initial body weight) and the ration was adjusted according to the feed waste in the tank.

Sample Collection

Fish were anesthetized with 80 mg/L tricaine methanesulfonate (MS-222, Western Chemical Inc., Ferndale, WA, United States) and weighed at the start and end of the trial, and growth performance was recorded. Sampling for gut microbiota was performed on five fish per treatment at 0, 10, 20, and 30 days of feeding. For this, euthanized fish were aseptically dissected from the ventral side after swabbing with ethanol (70% solution). The hindgut was dissected from the ileocecal valve to 0.5 cm above the anus, and digesta samples and mucosal scrapings were taken. These were snap-frozen in liquid nitrogen and stored at −80°C until DNA extraction.

TABLE 1 | Dietary composition (g kg^{-1} on dry matter basis) of the reference diet (RD), non-preconditioned diet (NPD), and preconditioned (heat-processed) diet (PD).

Ingredients (g kg^{-1})	Diets		
	RD	NPD	PD
Neurospora intermedia	-	298.5	298.5
Fishmeal	420	294	294
Soy protein concentrate	100	70	70
Wheat meal	220	154	154
Fish oil	100	70	70
Rapeseed oil	70	49	49
Carboxymethyl cellulose	10	7	7
Gelatin	60	42	42
Titanium dioxide	05	05	05
Vitamin mineral premix	15	10.5	10.5

TABLE 2 | Proximate composition [g kg^{-1} dry matter (DM)] and energy content (MJ kg^{-1} DM) of the reference diet (RD), non-preconditioned diet (NPD), preconditioned (heat-processed) diet (PD), and *Neurospora intermedia* fungal biomass.

Dietary component	RD	NPD	PD	*N. intermedia*
Dry matter (%)	95.7	95.9	96	–
Crude protein	484	514	516	609
Crude fat	194	171	171	64.5
Neutral detergent fiber	37.7	83.7	87.9	249.9
Ash	85.7	77.8	74.5	83.1
Gross energy	23.4	22.9	22.9	–

Chemical Analysis

Experimental feeds were freeze-dried, milled, and stored at −20°C until analysis. In order to determine the dry matter content, the samples were dried in an oven for 16 h at 103°C and then cooled in a desiccator before weighing. Crude protein content (N × 6.25) (Nordic Committee on Food Analysis, 1976) was determined by the Kjeldahl method, using a 2020 Kjeltec digester and a 2400 Kjeltec Analyser unit (FOSS Analytical A/S, Hilleröd, Denmark). Crude lipid content was analyzed according to the Official Journal of the European Union (2009), using an extraction unit (1047 Hydrolysing Unit and a Soxtec System HT 1043; FOSS Analytical A/S). Neutral detergent fiber (NDF) was measured based on the method described by Chai and Udén (1998) using 100% neutral detergent solution, while amylase and sulphite were used for reduction of starch and protein. Gross energy (GE) content was determined in an isoperibol bomb calorimeter (Parr 6300, Parr Instrument Company, Moline, IL, United States). Dry matter, gross energy, and ash content were analyzed according to standard methods (AOAC, 1995).

Extraction of DNA

Intestinal samples (200 mg) were transferred to sterile cryotubes containing 1 mL InhibitEX buffer and 0.5 g of 0.1 mm silica beads, and homogenized at room temperature in a bead beater (Precellys Evolution, Bertin Technologies) for 2 × 1 min at 6,000 rpm, with a 5 min rest. DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen Gmbh, Hilden, Germany) according to the manufacturer's instructions.

Library Preparation and Sequencing

The V4 region of the 16S rRNA gene was amplified from the extracted DNA using the primers 515F (5-GTGCCAGCMGCCGCGGTAA-3) and 805R (5-ACTACHVGGGTATCTAATCC-3). Polymerase chain reactions (PCR) were carried out using Phusion® High-Fidelity PCR Master Mix (New England Biolabs). PCR products were confirmed by gel electrophoresis and were purified with the Qiagen Gel Extraction Kit (Qiagen, Germany) and quantified by Qubit®3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific). Final libraries including barcodes and adaptors were generated with the NEBNext® Ultra™ DNA Library Prep Kit, and the amplicons were then sequenced using Illumina sequencing (NovaSeq 6000) at Novogene (Beijing, China). The BioProject accession number is PRJNA743247.

Bioinformatics Analysis

Paired-end reads were assigned to samples based on their unique barcode. These reads were merged after truncating off the barcode and primer sequence using FLASH (v1.2.7[1]) (Magoč and Salzberg, 2011). Quality filtering on the raw sequence tags was performed using QIIME (v1.7.0[2]) (Caporaso et al., 2010; Bokulich et al., 2013). Sequence analysis by clustering of operational taxonomic units (OTUs) was performed using Uparse software

[1]http://ccb.jhu.edu/software/FLASH/

[2]http://qiime.org/scripts/split_libraries_fastq.html

(Uparse v7.0.1001[3]) (Edgar, 2013). Sequences with ≥ 97% homology were assigned to the same OTUs. Representative sequences for each OTU were screened for further annotation. For each representative sequence, Mothur software was applied to the SSU rRNA data in the SILVA Database[4] for species annotation at each taxonomic rank (Wang et al., 2007; Quast et al., 2012).

Statistical Analysis

A linear mixed effect (LME) model ("nlme" package) was used to test for statistically significant differences between relative proportions of OTUs and diet, sampling day, and diet × day interactions. The LME model results were analyzed using R statistical software version 3.6 (Pinheiro et al., 2014; R Core Team, 2015), considering diet and day as fixed factors and tank as random factor. Data on bacterial OTUs were normalized by log transformation. LME comparison was conducted on OTUs with average abundance > 1%, followed by *post hoc* analysis of emmeans ("emmeans" package) with Tukey adjustment for multiple pairwise comparison. Similarity percentage analysis (SIMPER), analysis of similarity (ANOSIM), principal coordinate analysis (PCoA), principal component analysis (PCA), and Spearman correlation analysis

[3]http://drive5.com/uparse/
[4]http://www.arb-silva.de/

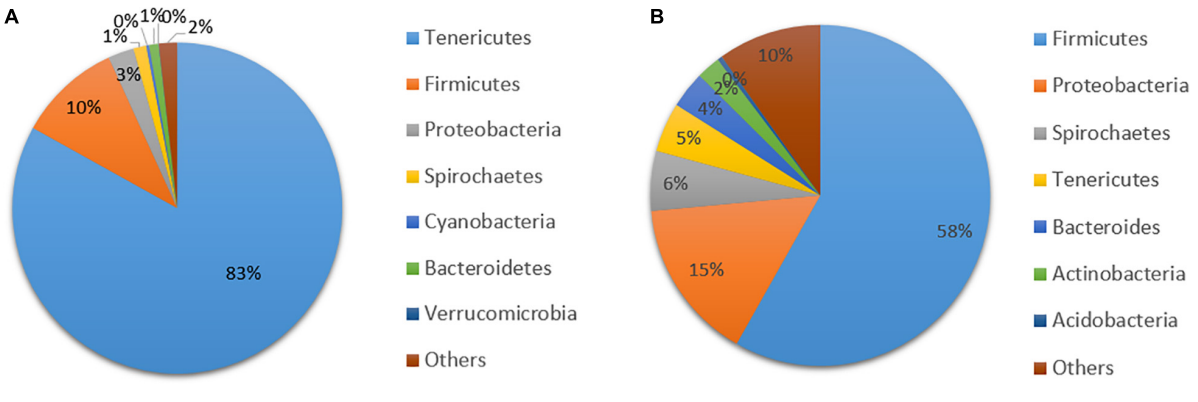

FIGURE 1 | Mean relative abundance of bacterial taxa (phylum level) in the gut of rainbow trout fed the diets from 0 to day 30 (n = 60 samples), calculated with **(A)** with dominant *Mycoplasma* operational taxonomic units (OTUs) included and **(B)** without dominant Mycoplasma OTUs.

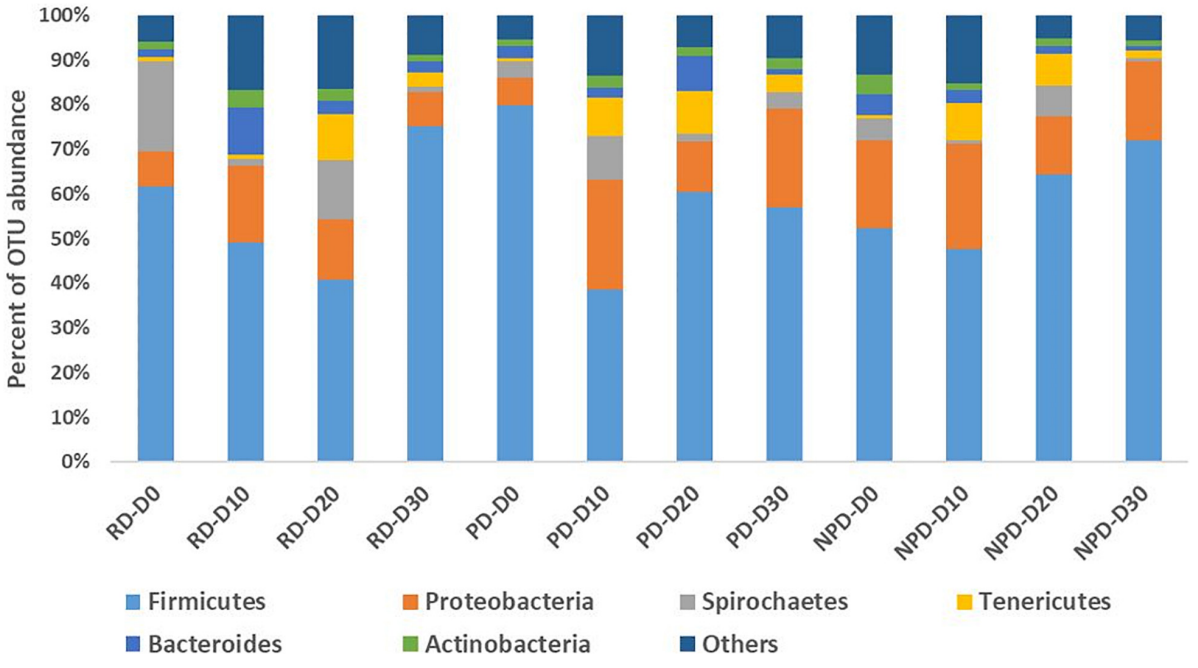

FIGURE 2 | Relative abundance of bacterial taxa in the gut of rainbow trout at different time intervals (after excluding operational taxonomic unit OTU1 and OTU2, belonging to *Mycoplasma*) (n = 5). RD: reference diet, PD: preconditioned (heat-processed) diet, NPD: non-preconditioned diet. D0, D10, D20, and D30 refer to the 10-day time interval. Until day 0, all fish had been fed a commercial diet for 10 days.

were performed using Paleontological Statistics Software version 4.03 (PAST). Two-way ANOSIM was performed to investigate the effect of diet and day interval on beta diversity of gut microbial composition. The ANOSIM and SIMPER analyses were both based on Bray Curtis index, and Bonferroni correction was used to adjust for multiple pairwise comparisons to determine differences in gut microbial composition within and between time intervals for each diet. PCoA based on Bray Curtis and Jaccard dissimilarity was used to assess the overall clustering of samples according to microbial community composition based on diet and time interval.

RESULTS

Gut Microbial Composition of Rainbow Trout

The overall gut microbial composition after 30 days of feeding showed high dominance of Tenericutes (84%), followed by Firmicutes (10%), and only very low relative abundance of other bacterial phyla. The high dominance of the Tenericutes phylum was due to two dominant *Mycoplasma* OTUs (**Figure 1A**). Assessment of the data did not reveal logical patterns, however, mainly since the two dominant *Mycoplasma* OTUs were not correlated to any of the parameters evaluated. Therefore, in further analyses on microbial composition the two dominant OTUs of *Mycoplasma* were excluded and relative abundance was recalculated, to discern effects on other bacterial taxa. In total, 4.8 million sequence reads of bacteria were obtained. The average number of sequence reads per sample without *Mycoplasma* was 13,244 and the lowest number obtained was 1,398. A total of 5,961 OTUs were obtained after excluding *Mycoplasma* OTUs, and bacterial OTU abundance was then dominated by two phyla, Firmicutes (58%) and Proteobacteria (15%) (**Figure 1B**). The overall trend in bacterial community composition from day 0 to day 30 was that Firmicutes ranged from 38 to 79% and Proteobacteria ranged from 8 to 24% for the different diets (**Figure 2**). Of the top 10 OTUs with abundance > 1% (**Figure 3**), *Peptostreptococcus* (9%), *Lactococcus* (*L. lactis*, 7%), *Brevinema* (6%), *Streptococcus* (5%), *Deefgea* (5%), and *Anaerotruncus* (4%) were the most abundant over the 30-day period.

Shift in Gut Microbial Composition of Rainbow Trout With Diet and Days of Feeding

Principal coordinate analysis was performed to graphically explore the shift in community structure for different diets after different time intervals (**Figures 4A,B**). The percentage variation (PoV) explained for axis 1 and 2 when using Bray Curtis index was 25.5 and 10.3%, respectively. For the analysis based on Jaccard's dissimilarity, PoV explained by axis 1 was 13% and by axis 2 was 6.68%. Differences in gut microbial composition at each day (within interval) were analyzed with one-way ANOSIM. The results confirmed that gut microbial composition for the different diets was similar at day 0 and day 30, but dissimilar at day 10 and day 20 (**Supplementary Table 1**).

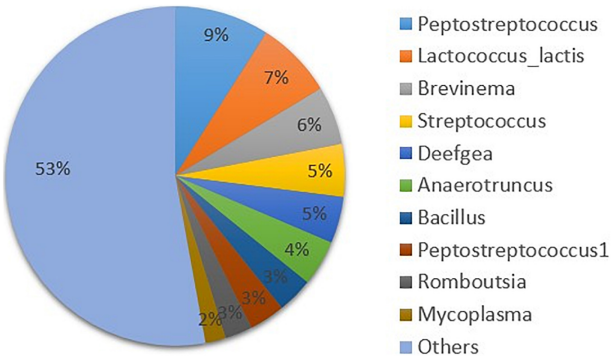

FIGURE 3 | Mean relative abundance of the top 10 bacterial operational taxonomic units (OTUs) in the gut microbiota of rainbow trout from 0 to 30 days on the commercial and experimental diets (n = 60).

Pairwise comparison of the treatment groups showed that they were significantly dissimilar within and between 10-day intervals (**Supplementary Table 2**). At day 10, the overall microbial composition of the fish gut with diet PD and NPD was different from that with RD. At day 20 there was a difference in microbial composition between NPD and PD, but they did not differ from RD. Over 10-day intervals, there was a temporal change in gut microbial composition with all diets from day 0 to day 10, from day 10 to day 20, from day 20 to day 30, and from day 10 to day 30 (**Supplementary Table 2**). According to SIMPER analysis, the percentage dissimilarity for the pairwise-compared treatment groups ranged from 64.73 to 82.58%.

Effect of Diet and Days of Feeding on Gut Microbial Composition of Rainbow Trout

Two-way ANOSIM revealed that diet and time had a significant influence in shaping the overall gut microbiota composition of trout (**Supplementary Table 3**). The results from the statistical analysis and interaction plot investigating the effect of treatments within and between 10-day intervals of feeding on the abundance of top six OTUs are shown in **Figure 5**. A more detailed description of these data can be found in **Supplementary Tables 4–6**. Day intervals had significant effects on the abundance of *Peptostreptococcus* and *Streptococcus.* Diet and day had significant effects on *Lactococcus* and *Deefgea*. The abundance of *Anaerotruncus* was significantly affected by diet. An interaction effect was observed only for *Deefgea* and *Anaerotruncus*. At day 30, the abundance of *Streptococcus* was significantly different between diets PD and NPD, while the abundance of *Deefgea* was significantly different for diet RD from NPD and PD (**Supplementary Table 5**). Significant increase in abundance from day 0 to day 30 for all diet namely RD, PD and NPD was only observed for *Lactococcus* (**Supplementary Table 5**). At day 0, *Peptostreptococcus* was the dominant taxon, but by day 30 *Lactococcus* was the most abundant taxon for all diets. The PCA results revealed that the occurrence of *Peptostreptococcus* and *Streptococcus* was positively and negatively correlated, respectively, with that

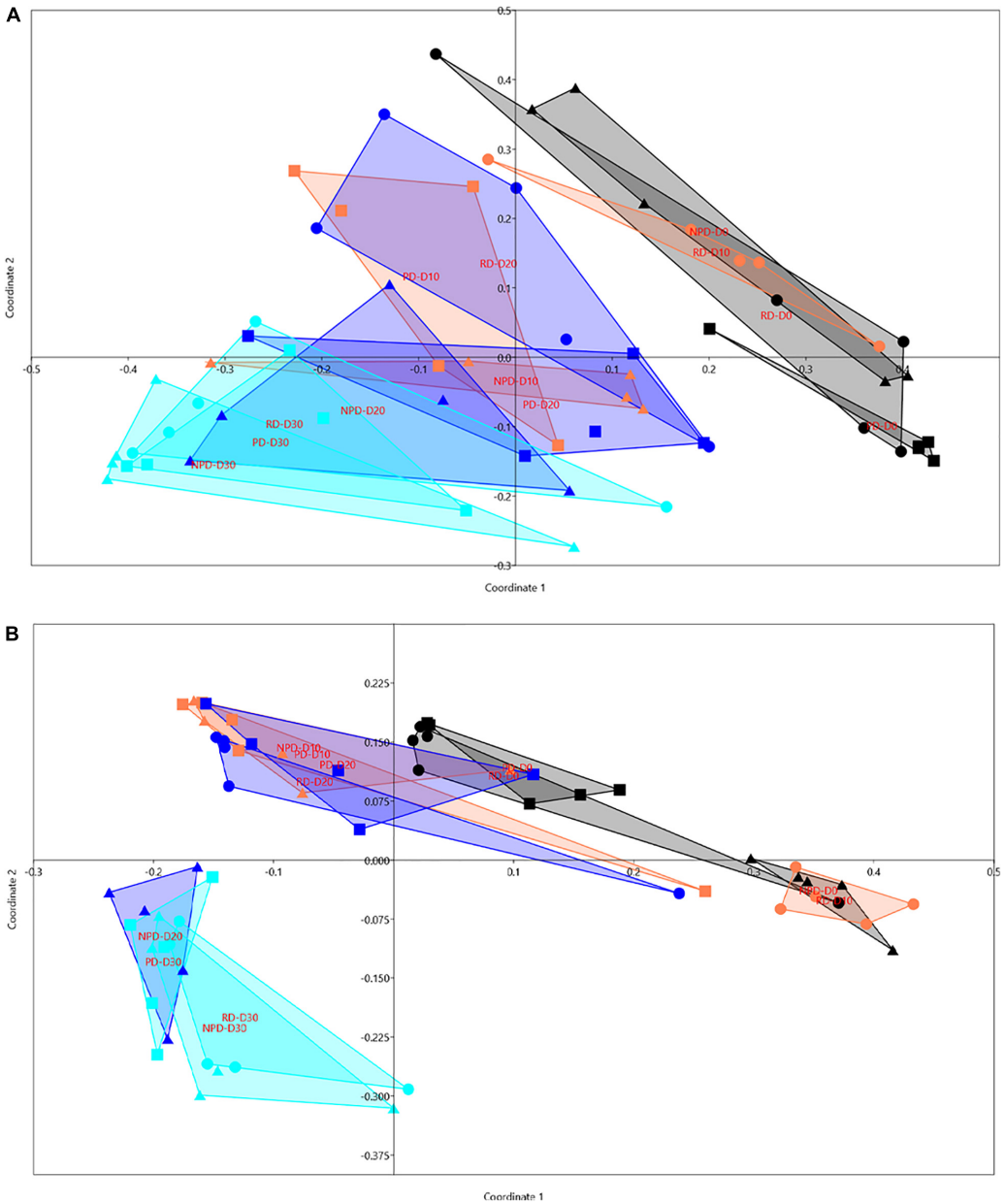

FIGURE 4 | Principal coordinate analysis plot based on **(A)** Bray Curtis index and **(B)** Jaccard dissimilarity showing the shift in gut bacterial community of rainbow trout with diet and time. Reference diet (0), preconditioned diet (□), non-preconditioned diet (Δ). Different colors represent day interval; days 0 (●), 10 (●), 20 (●) and 30 (●). Until day 0, all fish had been fed a commercial diet for 10 days.

of *Lactococcus* (**Figure 6**). These results were confirmed by Spearman correlation analysis (**Supplementary Figure 1**).

Growth Performance

Over the 30-day study period, the fish achieved a mean weight gain of 45.6 ± 3.1%, 44.7 ± 1.5% and 45.1 ± 3.3% for diet RD, NPD, and PD, respectively. These values were not significantly different. All diets were consumed without obvious changes in the intake pattern and zero mortality was recorded during the experimental period.

DISCUSSION

Shift in Overall Gut Microbial Composition With Diet and Time

The PCoA, ANOSIM, and SIMPER results demonstrated that the overall differences seen in gut microbial community were based on type of diet and feeding period. There was a gradual shift in bacterial communities between fish fed the commercial diet (day 0) and those fed the experimental diets (day 10–30) (**Figures 4A,B** and **Supplementary Tables 1**, **2**). Bacterial

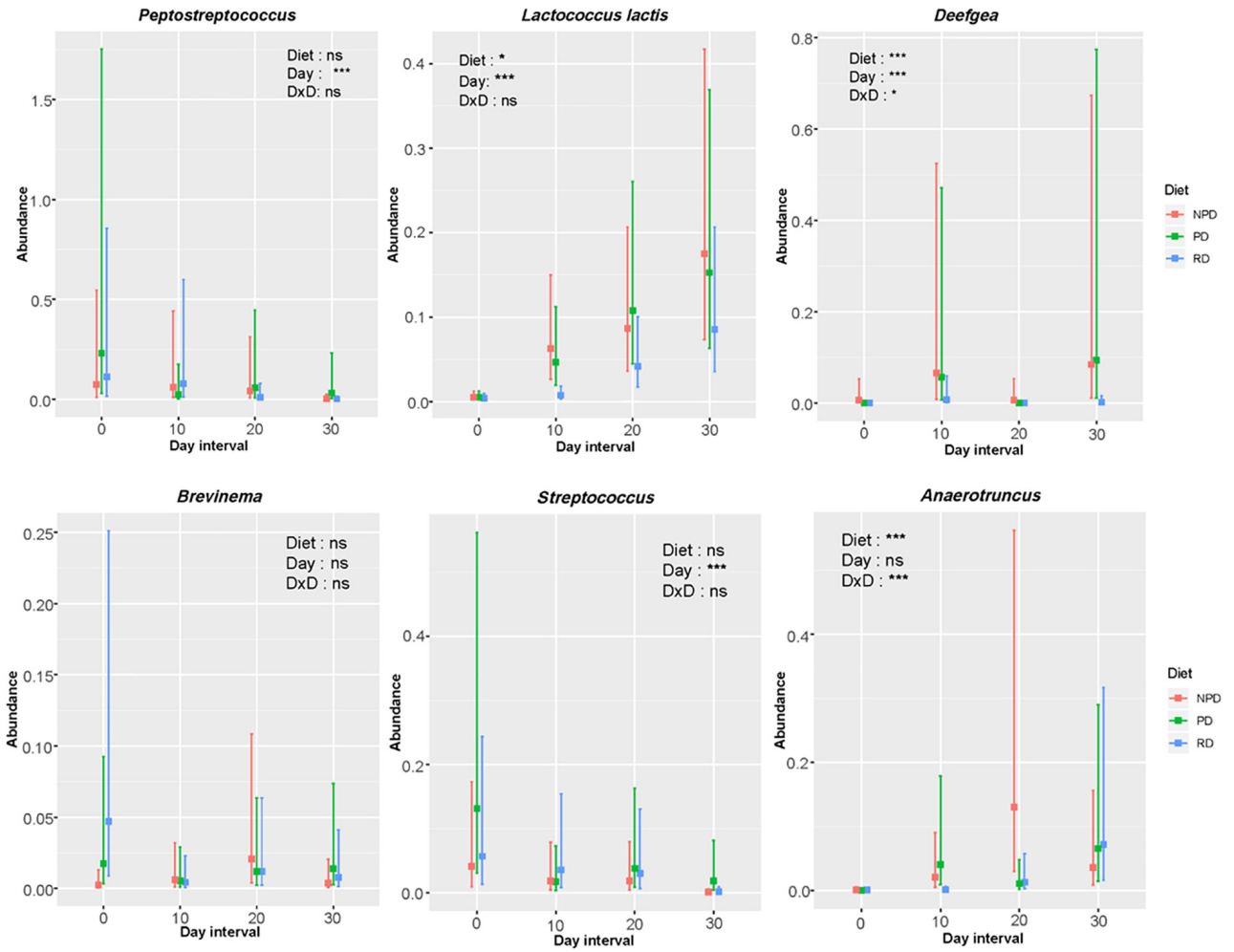

FIGURE 5 | Interaction plot showing relative abundance of the top six bacterial taxa in the gut bacterial community of rainbow trout. RD: reference diet, PD: preconditioned (heat-processed) diet, NPD: non-preconditioned diet. Until day 0, all fish had been fed a commercial diet. The vertical bar in the plot represents confidence interval and overlapping bars means the difference is not significant. Significance: ***$p < 0.001$, ** $p < 0.01$, *$p < 0.05$, and ns $p > 0.05$.

composition was expected to be similar at day 0, since fish in all treatment tanks were fed the same commercial diet during the previous acclimatization period. Within 10 days of experimental diet feeding, microbial composition in the fish gut had changed significantly, indicating that all diets had a significant effect on microbial composition. Previous studies have also reported a change in gut microbiota following a change in diet for salmon, rainbow trout, and brown trout (*Salmo trutta*) after first feeding (Ingerslev et al., 2014b; Michl et al., 2017, 2019). In the studies by Michl and co-workers, trout were fed 0, 50, and 90% plant protein-based diets until 54 days after hatching and then fed a cross-over diet for another 39 days, and a change in microbiome was observed at both 54 and 93 days of feeding showing occurrence of gut microbiota is influenced diet and depend largely on time of sampling. In the present study, diet and 10-day period both had an effect in shaping the gut communities. However, Michl et al. (2017) observed no change in the gut microbiota over time, after a certain point or with longer feeding duration with the same diet, and concluded that microbiota composition depends largely on the actual diet fed at the time of sample collection. In the present study, the gut microbiota differed significantly at day 10 and day 20, but was similar at day 30 irrespective of different treatment diets, suggesting that obtained microbiota at this time point is not influenced by two of the environmental variations in this case heat processing of diet nor *N. intermedia* inclusion. However, temporal change for all diets from day 20–30 was evident. Until day 20, differences in microbial composition can be in order to adapt to the environment due to dietary intervention. Longer periods of study are needed to confirm this.

Little information is available on the effect of thermal processing of feed on the fish gut microbiome. In the presented study, there was no difference in overall microbial composition between the preconditioned (thermal-processed) diet (PD) and the non-preconditioned diet (NPD) at day 30. However, Zhang and Li (2018) observed a decrease in gut microbiota at taxonomic

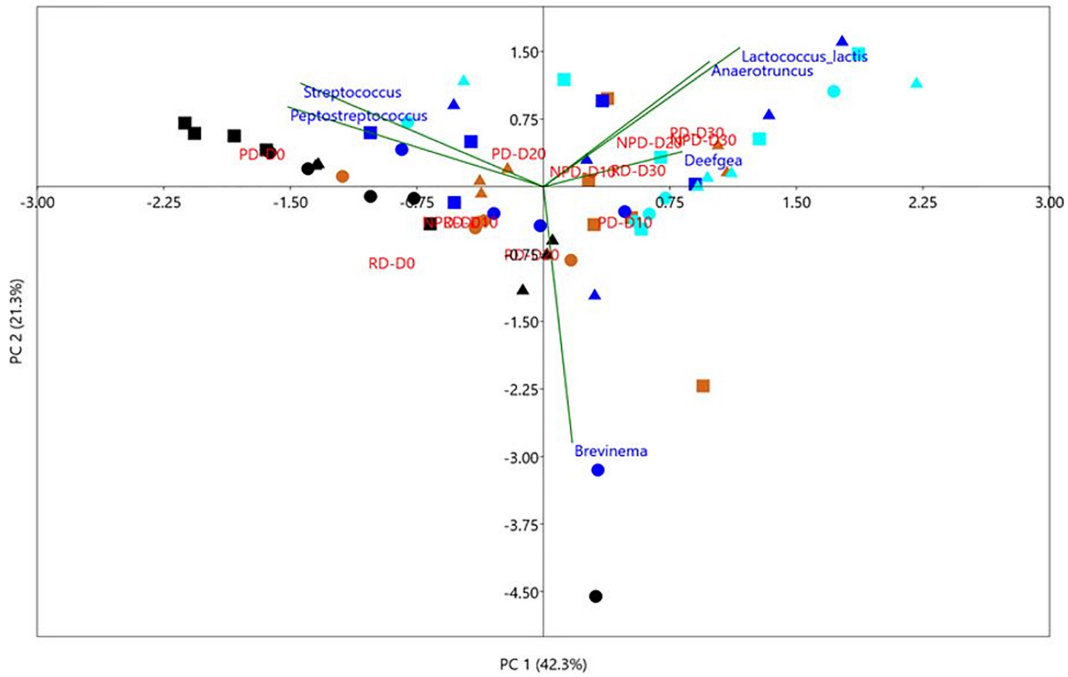

FIGURE 6 | Principal component analysis (PCA) plot showing the correlation of the top six operational taxonomic units (OTUs) in the gut bacterial community of rainbow trout with diet and day interval. Reference diet (0), preconditioned diet (□), non-preconditioned diet (Δ). Different colors represent day interval; days 0 (●), 10 (●), 20 (●), and 30 (●). Until day 0, all fish had been fed a commercial diet for 10 days.

and OTU levels when catfish were fed thermal-processed fish as food (steam, 100°C for 15 min) compared with non-processed food or feed.

Effect of Diet and Duration of Feeding on Core Gut Microbiome

In the present study, high ubiquitous abundance of Tenericutes, dominated by two *Mycoplasma* OTUs, was found which is similar to findings in previous studies on rainbow trout (Lowrey et al., 2015; Lyons et al., 2017a,b; Huyben et al., 2018). According to Holben et al. (2002), *Mycoplasma* can be a natural resident in the gut of both farmed and wild salmon. As the biological function of *Mycoplasma* is not known and very high dominance of the two otus took over the statistical analyses, thus it reduced chance to identify associations among the other microbes possibly associated with the diets.

Data analysis revealed that the next most common phylum in the core gut microbiota after Tenericutes was Firmicutes, folowed by Proteobacteria (**Figure 1B**), as found in other studies on salmonids (Nayak, 2010; Gajardo et al., 2017). One previous study has found that the core gut microbial composition of rainbow trout is resistant to change due to diet type, but that conclusion was reached by comparing data at phylum level (Wong et al., 2013). Another study suggested that there might be differences at lower taxonomic ranks, particularly at species or genus level, rather than at higher taxonomic ranks (Michl et al., 2017). This was the case in the present study, where the abundance of *Lactococcus* (Lactobacillales), *Deefgea* (Neisseriales), and *Anaerotruncus* (Clostridiales) was significantly enriched from day 0 to day 30 and fish on the preconditioned diet (PD) had higher abundance of *Streptococcus* than those on the non-preconditioned diet (NPD). Lower abundance of bacteria of the genera *Deefgea* and *Anaerotruncus* was observed, as also found in the gut microbiota of humans, rainbow trout, and Atlantic salmon isolated through 16s sequencing (Namsolleck et al., 2004; Perez-Fuentes et al., 2018; Ricaud et al., 2018). *Peptostreptococcus* and *Streptococcus* are generally present in high abundance in protein-rich environments, and play an important role in amino acid catabolism and absorption in the gut (Dai et al., 2011; Davila et al., 2013; Neis et al., 2015). The abundance of one taxa can suppress that of another depending on nutrient availability for growth. A shift in microbial composition from *Streptococcus* to *Lactobacillus* has been reported in Atlantic salmon fed fishmeal-free diets or diets with fishmeal replaced with plant protein (Hartviksen et al., 2014). This is comparable to the results in the present study, where *Peptostreptococcus*, the dominant taxon at day 0 (all fish fed the commercial diet) decreased in abundance with time, whereas abundance of *Lactococcus* increased conferring the change due to the substrate exchange.

Diet and Duration of Feeding Promotes Abundance and Dominance of Intestinal *Lactococcus lactis*

Gut bacterial composition in rainbow trout fed plant protein-based diets and Atlantic salmon fed a fishmeal-free diet is

reported to show an increase in abundance of Lactobacillales (Schmidt et al., 2016; Michl et al., 2017). A study using PCR-TTGE-dependent bacterial quantification showed an increase in *Lactobacillus* and *Lactococcus* in Atlantic salmon fed diets in which 30% of the fishmeal was replaced with fermented soy meal (Catalán et al., 2018). Lactic acid bacteria are natural inhabitants of the fish gut and have the ability to adhere and colonize and play a beneficial role in the gut (Seppola et al., 2006; Gatesoupe, 2008). Additionally, growth of *L. lactis* is highly substrate-dependent (Rombouts et al., 2020). It is possible that components in the cell wall of *N. intermedia*, such as beta glucan, chitin, and glycoproteins, act as fermentable substrate for Lactobacillales. Increased Lactobacillales abundance has been observed in Artic charr and rainbow trout fed yeast (Huyben et al., 2017; Nyman et al., 2017). Lactobacillales from fish is known to be slow-growing and the recommended growth period on agar media at low temperatures is up to 4 weeks (Ringø and Gatesoupe, 1998), which is in line with the findings in this study of highest Lactobacillales abundance on day 30. Studies have shown that use of *L. lactis* as a probiotic can enhance weight, immunity, and disease resistance in fish (Sun et al., 2012; Heo et al., 2013; Xia et al., 2018). *Lactobacillus lactis* has also been shown to improve the gut architecture and modulate the intestinal microbial composition in fish (Dawood et al., 2016; Xia et al., 2019; Won et al., 2020).

CONCLUSION

The filamentous fungi *Neurospora intermedia* has a good nutritional profile with high protein content and healthy gut microbiota profile with dominance of lactic acid bacteria. It can be advocated as a protein source to replace fishmeal in the diet of cultured fish, for sustainable feed production and aquaculture. Changes due to environmental interventions, in this case diet and feeding duration were more pronounced for modulating the fish gut microbes at overall and at lower taxonomic levels than feed preprocessing. Preconditioning (steam-processing) of the diet had no effect on shaping the overall microbial gut composition as they were similar on day 30. Diets containing *N. intermedia* promoted abundance of *Lactococcus* compared with the commercial diet. Thus duration of feeding should be taken into account when studying changes in the gut microbial community in rainbow trout following diet manipulation. Based on our findings, a minimum 30-day feeding period is recommended in studies on feed-host interactions. Since, the overall gut microbiota continuously changed until day 30, a future research should investigate the further trends of their occurrence.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: www.ncbi.nlm.nih.gov/bioproject, PRJNA743247.

ETHICS STATEMENT

The animal study was reviewed and approved by Swedish Board of Agriculture (diary number: 5.8.18-16347/2017).

AUTHOR CONTRIBUTIONS

TL and AK conceived the study and experimental design. AS and SK carried out the experimental trial, participated in sampling of the fish material, and wrote the first draft of the manuscript. AS, SK, AV, and ML were performed the feed optimization and feed production. AS and JD were responsible for the DNA analysis and performed the data analysis. TL, AV, and JD participated in editing the final manuscript. SK, JF, and MT were involved in fungus production. All authors contributed to manuscript revision, and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Modulation of Gilthead Sea Bream Gut Microbiota by a Bioactive Egg White Hydrolysate: Interactions Between Bacteria and Host Lipid Metabolism

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This study aimed to highlight the relationship between diet, animal performance and mucosal adherent gut microbiota (anterior intestine) in fish fed plant-based diets supplemented with an egg white hydrolysate (EWH) with antioxidant and anti-obesogenic activity in obese rats. The feeding trial with juveniles of gilthead sea bream (*Sparus aurata*) lasted 8 weeks. Fish were fed near to visual satiety with a fish meal (FM)/fish oil (FO) based diet (CTRL) or a plant-based diet with/without EWH supplementation. Specific growth rate decreased gradually from 2.16% in CTRL fish to 1.88% in EWH fish due to a reduced feed intake, and a slight impairment of feed conversion ratio. Plant-based diets feeding triggered a hyperplasic inflammation of the anterior intestine regardless of EWH supplementation. However, EWH ameliorated the goblet cell depletion, and the hepatic and intestinal lipid accumulation induced by FM/FO replacement. Illumina sequencing of gut mucosal microbiota yielded a mean of 136,252 reads per sample assigned to 2,117 OTUs at 97% identity threshold. The bacterial diversity was similar in all groups, but a significantly lower richness was found in EWH fish. At the phylum level, Proteobacteria reached the highest proportion in CTRL and EWH fish, whereas Firmicutes were decreased and Actinobacteria increased with the FM/FO replacement. The proportion of Actinobacteria was restored by dietary EWH supplementation, which also triggered a highest amount of Bacteroidetes and Spirochaetes. At a closer look, a widespread presence of Lactobacillales among groups was found. Otherwise, polysaccharide hydrolases secretors represented by *Corynebacterium* and *Nocardioides* were increased by the FM/FO replacement, whereas the mucin-degrading *Streptococcus* was only raised in

fish fed the plant-based diet without EWH. In addition, in EWH fish, a higher abundance of *Propionibacterium* was related to an increased concentration of intestinal propionate. The antagonism of gut health-promoting propionate with cholesterol could explain the inferred underrepresentation of primary bile acid biosynthesis and steroid degradation pathways in the EWH fish microbiota. Altogether, these results reinforce the central role of gut microbiota in the regulation of host metabolism and lipid metabolism in particular, suggesting a role of the bioactive EWH peptides as an anti-obesity and/or satiety factor in fish.

Keywords: bioactive peptide, egg white hydrolysate, gut microbiota, bile salts, lipid metabolism, *Sparus aurata*

INTRODUCTION

European aquaculture is still dependent on marine ingredients for feeds, though a high grade of replacement of marine feedstuffs (more than 70%) has been currently accomplished in Norwegian salmon feeds (Ytrestøyl et al., 2015). A high level of fish meal (FM) and fish oil (FO) replacement has also been achieved in a typically carnivorous marine fish, such as European sea bass (*Dicentrarchus labrax*) (Kousoulaki et al., 2015; Torrecillas et al., 2017). Likewise, plant-based diets with less than 10% of marine ingredients have been proven to support maximum growth from early life stages to completion of sexual maturity in gilthead sea bream (*Sparus aurata*) (Benedito-Palos et al., 2016; Simó-Mirabet et al., 2018). However, wide-serum metabolomics profiling revealed nutritionally mediated effects in processes of mucosal tissue repair and DNA stability (Gil-Solsona et al., 2019). Furthermore, feeding gilthead sea bream with plant-based diets induced negative effects at gut level, including changes in the expression of mucins, mucosal immunoglobulins (IgT) and other immune-relevant genes (Pérez-Sánchez et al., 2013; Piazzon et al., 2016). This biomarker profile leads to a pro-inflammatory condition, with drawback effects in gut integrity and epithelial barrier functions (Estensoro et al., 2016; Piazzon et al., 2017). However, most of these effects, including changes in sex reversal from male to female, or in mucosal adherent intestinal microbiota composition, were restored by dietary sodium butyrate supplementation, resulting in an improved disease outcome in fish exposed to bacteria or myxozoan parasites (Piazzon et al., 2017; Simó-Mirabet et al., 2018).

Gut microbiota has the capacity to modify and possibly activate food constituents, providing benefits for health (Brown et al., 2015; Davis, 2016; Aoun et al., 2020). Certainly, the persistent imbalance of the gut's microbial community resulting from exposure to diverse environmental factors, including unhealthy diets, drugs, toxins, and pathogens, has a major impact on health (Chávez-Talavera et al., 2017; Hasan and Yang, 2019; Lee et al., 2020). Among them, diet micro- and macro-nutrients are considered one of the main factors that modulate gut microbiota (Zhang et al., 2018; Leeming et al., 2019). Therefore, understanding the modulation of the gut microbiome by dietary nutrients and vice-versa becomes essential for the development of novel strategies to improve animal and human health. In fact, the targeting of gut microbiota is a promising tool to improve the health and welfare of farmed fish, and gilthead sea bream in particular. Certainly, the intestinal gut microbiota is highly modulated by age and sex reversal in a protandrous hermaphrodite fish such as gilthead sea bream (Piazzon et al., 2019). In addition, the gut microbiota of gilthead sea bream families selected for fast-growth showed a high level of plasticity, which makes them more flexible upon dietary changes, showing at the same time, a better ability to deal with intestinal parasites (Piazzon et al., 2020).

In other studies of this special issue of nutrition and gut microbiota in aquaculture, we also analyzed the effect of probiotics and alternative FM replacers on gilthead sea bream gut microbiota (Moroni et al., 2021; Solé-Jiménez et al., 2021). Meanwhile, bioactive peptides derived from food proteins are considered important modulators of various biological processes, which occur both systemically and locally within the gastrointestinal tract (Mougham et al., 2014). Historically, milk proteins have been considered a rich source of bioactive peptides, but there is now evidence of a wide range of animal and plant protein sources for production of biologically active peptides, comprising meat, bone, eggs, cereals, legumes, yeast, seaweed, and fungi (Brown et al., 2015). In particular, a bioactive egg white hydrolysate (EWH) treated with pepsin showed potent *in vitro* and *in vivo* antioxidant and anti-inflammatory properties (Dávalos et al., 2004; Miguel et al., 2004; Garcés-Rimón et al., 2016a), improving oxidative stress and inflammation biomarkers on genetically and diet-induced obese rats (Garcés-Rimón et al., 2016b; Moreno-Fernández et al., 2018a,b). The antioxidant properties of EWH have also been associated with the prevention of metabolic complications arising from the exposure to heavy metals (Rizzetti et al., 2017; Martínez et al., 2019; Gomes Pinheiro et al., 2020). Although earlier studies targeting intestinal microbiota support that EWH has the potential to revert microbial dysbiosis in a rodent model of genetic obesity (Requena et al., 2017), the modulating effects of food-bioactive peptides upon gut microbiota have been much less studied (Wu et al., 2021). Thus, in an attempt to focus on the link between mucosal adherent microbiota and the bioactive properties of EWH in other animal model, we analyzed the potential benefits of dietary EWH supplementation in farmed gilthead sea bream juveniles fed plant-based diets. Special attention was also paid to the evaluation of growth performance, allocation of body fat depots, hepatic and intestinal histopathological scoring, antioxidant status, and intestinal concentration of lactic acid and short chain fatty acids (SCFAs).

MATERIALS AND METHODS

Ethics Statement

Fish manipulation and tissue collection were carried out according to the Spanish (Royal Decree RD53/2013) and the current EU (2010/63/EU) legislations on the handling of experimental fish. All procedures were approved by the Ethics and Animal Welfare Committee of the Institute of Aquaculture Torre de la Sal (IATS-CSIC, Castellón, Spain), CSIC (permit number 869/2019) and "Generalitat Valenciana" (permit number 2020/VSC/PEA/0010).

Animals

Juveniles of gilthead sea bream (March 2020) were purchased from a Mediterranean hatchery (Piscimar, Burriana, Spain) and adapted for 2 months to the indoor experimental facilities of IATS-CSIC under natural photoperiod and temperature conditions (40°5′N; 0°10′E). Seawater was pumped ashore (open system), oxygen content of water effluents was always above 85% saturation, and unionized ammonia remained below 0.02 mg/L. During the acclimation and experimental period (May–July 2020), water temperature increased from 18°C in May to 25°C in July.

Diets

Extruded isoproteic and isolipidic diets (2, 3 mm pellet size) were formulated by Sparos Lda. (Olhão, Portugal), following current industry practices with plant protein and oil sources as main replacers of fish meal (FM) and fish oil (FO) (**Table 1**). The inclusion level of FM (including fish protein hydrolysates) and FO in the control diet (CTRL) was 35% and 5%, respectively. In the diet named L-FM/FO, the FM and FO inclusion levels were reduced to 12.5% for FM and fish protein hydrolysates, and to 3% for FO, being this diet conveniently supplemented with monocalcium phosphate, L-tryptophan and DL-methionine. The EWH diet was formulated to be a L-FM/FO diet with EWH added at 7.5% instead of plant proteins and fish protein hydrolysates. The EWH was prepared by pepsin hydrolysis of crude egg white as previously described by Garcés-Rimón et al. (2016a). Briefly, commercial pasteurized egg white was hydrolyzed for 8 h with BC Pepsin 1:3000 (E.C. 3.4.23.1; from pork stomach, E:S:2:100 w:w, pH 2.0, 37°C), purchased from Biocatalysts (Cardiff, United Kingdom). Enzyme inactivation was achieved by increasing the pH to 7.0 with 10 N NaOH. The hydrolysate was centrifuged at 2,500 × *g* for 15 min and the supernatants were frozen and lyophilized until use as fish feed ingredient.

TABLE 1 | Ingredients and chemical composition of control and experimental diets.

Ingredients	CTRL (%)	L-FM/FO (%)	EWH (%)
Fishmeal super prime	30	10	10
Fish protein hydrolysate	5	2.5	
Soy protein concentrate	12.5	12.5	10
Pea protein concentrate		4.5	2.5
Wheat gluten	5	12.5	10
Corn gluten meal	5	10	10
Soybean meal 48	5	10	10
Rapeseed meal	5	5	5
Sunflower meal 40	5	10	10
Wheat meal	9.07	3.99	5.49
Whole peas	4	2	2
Vitamin and mineral premix*	1	1	1
Vitamin C35	0.03	0.03	0.03
Betaine HCl	0.2	0.2	0.2
Antioxidant powder	0.2	0.2	0.2
Sodium propionate	0.1	0.1	0.1
Monocalcium phosphate	0.6	2.5	2.5
L-Tryptophan		0.03	0.03
DL-Methionine		0.25	0.25
Fish oil	5	3	3
Soybean oil	5.3	8	8.6
Linseed oil	2	1.7	1.6
EGG hydrolysate			7.5
Chemical composition (proximate analyses)			
Dry matter, % feed	92.9	93.2	92.8
Crude protein, % feed	47.9	47.9	47.9
Crude fat, % feed	16.1	16.1	16.1
EPA + DHA, % feed	2.4	1.2	1.2
Ash, % feed	8.7	8.0	7.6

**Vitamin and mineral premix: INVIVONSA Portugal SA, Portugal: Vitamins (IU or mg/kg diet):* DL*-alpha tocopherol acetate, 100 mg; sodium menadione bisulfate, 25 mg; retinyl acetate, 20,000 IU;* DL*-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium pantothenate, 100 mg; choline chloride, 1000 mg; betaine, 500 mg. Minerals (g or mg/kg diet): copper sulfate, 9 mg; ferric sulfate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulfate, 7.5 mg; sodium chloride, 400 mg; excipient wheat gluten.*

Feeding Trial

In May 2020, fish of 20–24 g body weight were randomly distributed in nine 90 L tanks to establish triplicate groups of 20 fish each (initial rearing density, 4.8–4.9 kg/m^3). All fish were tagged with passive integrated transponders (PIT) (ID-100A 1.25 Nano Transponder, Trovan, Madrid, Spain) into the dorsal skeletal muscle, and were individually weighed and measured at initial, intermediate and final sampling points (every 4 weeks), using a FR-200 Fish Reader W (Trovan) for data capture and pre-processing. The trial lasted 8 weeks, and fish were fed by hand once daily (12 a.m.), 6 days per week, near to visual satiety with CTRL or experimental diets for the entire duration of the trial. Feed intake was registered daily, and normal fish behavior was assessed routinely by camera monitoring. No mortalities were registered through the entire experimental period.

Sample Collection

At the end of the trial and following two fasting days, nine fish per diet (three fish/tank) were anaesthetized with 0.1 g/L of tricaine-methanesulfonate (MS-222, Sigma-Aldrich, St. Louis, MO, United States). Blood was taken from the caudal vessels

with heparinized syringes, centrifuged at 3,000 × *g* for 20 min at 4°C, and plasma samples were stored at −80°C until analyzed. Before tissue collection, fish were sacrificed by cervical section. Liver, intestine (excluding the pyloric caeca) and mesenteric fat were weighed and measured (intestine length) to calculate the hepatosomatic index (HSI), mesenteric fat index (MSI), and intestine weight (IWI) and length (ILI) indexes. Tissue portions (∼0.4 cm) of liver, anterior intestine (AI; immediately after the pyloric caeca) and posterior intestine (PI; immediately before the anal ampoule) were fixed in 10% neutral buffered formalin for subsequent histological analyses. The remaining AI was opened and gently washed with sterile Hanks's balanced salt solution to remove non-adherent bacteria. Intestinal mucus was scrapped off using the blunt edge of a sterile scalpel and collected into sterile 1.5 mL tubes. The anterior intestine portion was selected due to its importance in fish nutrient absorption and metabolism (Sundell and Rønnestad, 2011). The autochthonous bacteria were selected, because these populations are capable of colonizing the mucosal surface, directly impacting the fish physiology (Hao and Lee, 2004). Mucus samples were kept on ice and DNA extraction was performed immediately after the sampling. Additional fish (10 fish per diet) were sampled 8 h after feeding for the analysis of intestinal SCFA. Briefly, animals were anesthetized and sacrificed by cervical section, intestine was cut out, and the intestinal content was collected by stripping. During the two sampling days corresponding to fasting and postprandial sample collection, all samples were obtained in a short-period lasting 2–3 h, alternating among replicates of each dietary group to avoid biases due to sampling time.

Histological Analysis

Formalin fixed pieces of liver, AI and PI were processed for paraffin embedment, 4 μm-sectioned and stained with Giemsa and periodic acid-Schiff (PAS) following standard procedures. Sections were examined with a Leitz Dialux 22 light microscope connected to an Olympus DP70 camera, and representative microphotographs were taken. The histological alterations observed were scored according to semiquantitative scales. In intestinal sections, cell abundance of differentially stained goblet cells (light- or dark-stained with Giemsa), intraepithelial lymphocytes (IELs) and eosinophilic granular cells (EGCs) were scored ranging from 0 (absence) to 3 (very abundant, meaning 25–30 cells/microscope field at 500× magnification). The degree of lipid vacuolization in enterocytes and the degree of hyperplasia in the lamina propria-submucosa were scored from 0 (absence) to 3 (severe). In liver sections, the degree of lipid and glycogen storage in hepatocytes was scored from 0 (absence) to 3 (pervasive) by Giemsa or PAS staining, respectively. In addition, melanomacrophage centers were quantified in the liver.

Antioxidant Capacity

The oxygen radical absorbance capacity (ORAC) assay was used to measure the total plasma antioxidant capacity as previously described (Garcés-Rimón et al., 2016b). ORAC values were quantified by a fluorimeter Polarstar Galaxy plate reader (BMG Labtechnologies GmbH, Germany) with wavelength excitation at 485 nm and wavelength emission measured at 520 nm. Results were expressed as μmol of Trolox (Sigma, United States) equivalent (eq)/mL of plasma.

Lactic Acid and Short Chain Fatty Acid Determinations

Intestine content (200 mg) was homogenized with 0.1% peptone solution with 0.85% NaCl (500 μL) and centrifuged at 10,000 × *g* for 5 min at 4°C. The supernatant was filtered and 0.2 μL were injected on a HPLC system (Jasco, Tokyo, Japan) equipped with a UV-975 detector. Lactic acid and SCFA were separated using a Rezex ROA Organic Acids column (Phenomenex, Macclesfield, United Kingdom) following the method described by Sanz et al. (2005). The mobile phase was a linear gradient of 0.005 M sulfuric acid in HPLC grade water, and flow rate was 0.6 mL/min. The elution profile was monitored at 210 nm, and peak identification was carried out by comparing the retention times of target peaks with those of standards. Calibration curves of formic acid, acetic acid, propionic acid, butyric acid and lactic acid were prepared in the concentration range of 1 to 100 mM.

DNA Extraction From Mucus Samples

Intestinal mucus samples (200 μl) were treated with 250 μg/mL of lysozyme (Sigma) for 15 min at 37°C. Then, DNA was extracted using the High Pure PCR Template Preparation Kit (Roche) following the manufacturer's instructions. DNA concentration, quality and purity were measured using a Nanodrop 2000c (Thermo Scientific), and agarose gel electrophoresis (1% w/v in Tris-EDTA buffer). DNA was stored at −20°C until sequencing.

Illumina MiSeq Sequencing and Bioinformatic Analysis

The V3-V4 region of the 16S rRNA gene (reference nucleotide interval 341–805 nt) was sequenced using the Illumina MiSeq system (2 × 300 paired-end run) at the Genomics Unit from the Madrid Science Park Foundation (FPCM). The details on the PCR and sequencing of amplicons were described elsewhere (Piazzon et al., 2019). Raw sequence data were uploaded to the Sequence Read Archive (SRA) under Bioproject accession number PRJNA705868 (BioSample accession numbers: SAMN18105342-68). Raw forward and reverse reads were quality filtered using FastQC[1] and pre-processed using Prinseq (Schmieder and Edwards, 2011). Terminal N bases were trimmed in both ends and sequences with >5% of total N bases were discarded. Reads that were <150 bp long, with Phred quality score < 28 in both of the sequence ends and with a Phred average quality score < 26 were excluded. Then, forward and reverse reads were merged using fastq-join (Aronesty, 2013).

Bacteria taxonomy assignment was performed using the Ribosomal Database Project (RDP) release 18 as a reference database (Cole et al., 2014). Reads were aligned with a custom-made pipeline using VSEARCH and BLAST (Altschul et al., 1990; Rognes et al., 2016). Alignment was performed establishing high stringency filters (ł90% sequence identity, ł90% query coverage).

[1]http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Taxonomic assignment results were filtered and data were summarized in an operational taxonomic units (OTUs) table. From the annotation obtained, the discussion and interpretation of the results was based at the level of genus, as taxonomic affiliations with 16S rRNA amplicon sequencing might not be accurate enough at the species level (Winand et al., 2020). Sample depths were normalized by total sum scaling and then made proportional to the total sequencing depth, following the recommendations previously described (McKnight et al., 2019).

Inferred Metagenome and Pathway Analysis

Piphillin was used to normalize the amplicon data by 16S rRNA gene copy number and to infer metagenomic contents (Iwai et al., 2016). This analysis was performed with the OTUs significantly driving the separation by diets in the PLS-DA analysis (described in the "Statistics" section). For the analysis, a sequence identity cut-off of 97% was implemented, and the inferred metagenomic functions were assigned using the Kyoto Encyclopedia of Genes and Genomes database (KEGG, October 2018 Release). Raw KEGG pathway output from Piphillin was analyzed with the R Bioconductor package DESeq2 using default parameters, after flooring fractional counts to the nearest integer (Love et al., 2014; Bledsoe et al., 2016; Piazzon et al., 2020).

Statistics

Data on growth were analyzed by one-way ANOVA using SigmaPlot v14 (Systat Software Inc., San Jose, CA, United States). Normality of the data was verified by Shapiro–Wilk test, and Dunn's post-test was used for multiple comparisons among groups. Analysis of semiquantitative and quantitative histological data was carried out with the non-parametric Kruskal–Wallis test, followed by Dunn's post-test for the multiple comparisons. SCFA results were analyzed by one-way ANOVA followed by Holm–Sidak post-test. Rarefaction curves (plotting the number of observed taxonomic assignations against the number of sequences), species richness estimates, and alpha diversity indexes were obtained using the R package phyloseq (McMurdie and Holmes, 2013). Differences in species richness, diversity indexes and phylum abundance were determined by Kruskal–Wallis test using the Dunn's post-test, with a significance threshold of $P < 0.05$. Beta diversity across groups was tested with permutational multivariate analysis of variance (PERMANOVA), using the non-parametric method *adonis* from the R package Vegan with 10,000 random permutations. To study the separation among groups, supervised partial least-squares discriminant analysis (PLS-DA) and hierarchical clustering of samples were sequentially applied, using EZinfo v3.0 (Umetrics, Umeå, Sweden) and the R package ggplot2, respectively. Values of normalized counts of OTUs present in five or more samples were included in the analyses. The contribution of the different genes to the group separation was determined by the minimum Variable Importance in the Projection (VIP) values achieving the complete clustering of the conditions with a VIP value ≥ 1.2. Hotelling's T^2 statistic was calculated by the multivariate software package EZinfo v3.0. All points in the current study were within the 95% confidence limit for T^2, thus no outliers were detected and discarded. The quality of the PLS-DA model was evaluated by the parameters R2Y (cum) and Q2 (cum), which indicate the fit and prediction ability, respectively. To assess whether the supervised model was being over-fitted, a validation test consisting on 500 random permutations was performed using SIMCA-P+ v11.0 (Umetrics). The inferred metagenomic pathways were considered differentially represented using a FDR-corrected significance threshold of 0.05.

RESULTS

Growth Performance and Antioxidant Capacity

Data on growth performance are reported in **Table 2**. Final body weight, feed intake and condition factor were significantly lower ($P \leq 0.004$) in EWH fish than in CTRL fish with intermediate values in fish fed the L-FM/FO diet. Specific growth rates (SGR) also varied significantly from 2.16 in CTRL fish to 1.88 in EWH fish, again with intermediate values (2.03) in fish fed the L-FM/FO diet. The opposite trend (not statistically significant, $P = 0.06$) was found for the feed conversion ratio (FCR) that varied from 1.03 in CTRL fish to 1.10 in EWH fish. HSI, MFI, and IWI were not significantly altered by dietary treatment. However, the intestine length of L-FM/FO fish was larger, and the resulting

TABLE 2 | Effects of dietary treatment on growth performance and antioxidant capacity of gilthead sea bream juveniles fed to visual satiety from May to July (8 weeks) with control (CTRL), low fish meal/fish oil (L-FM/FO) diet, and EWH diets.

	CTRL	L-FM/FO	EWH	P^1
Initial body weight (g)	21.93 ± 0.38	21.98 ± 0.43	21.95 ± 0.40	0.996
Final body weight (g)	71.75 ± 1.08^a	66.89 ± 1.01^b	61.41 ± 0.96^c	**<0.001**
Final condition factor[2]	2.67 ± 0.02^a	2.68 ± 0.03^a	2.58 ± 0.02^b	**0.004**
Feed intake (g DM/fish)	51.09 ± 0.02^a	45.84 ± 0.01^{ab}	43.46 ± 0.16^b	**0.004**
FCR[3]	1.03 ± 0.02	1.02 ± 0.02	1.10 ± 0.02	0.062
SGR (%)[4]	2.16 ± 0.02^a	2.03 ± 0.02^b	1.88 ± 0.02^c	**<0.001**
Liver weight (g)	0.73 ± 0.03	0.73 ± 0.03	0.67 ± 0.03	0.232
Mesenteric fat (g)	0.85 ± 0.13	0.77 ± 0.10	0.85 ± 0.11	0.970
Intestine weight (g)	2.60 ± 0.07^{ab}	2.79 ± 0.14^a	2.32 ± 0.08^b	**0.008**
Intestine length (cm)	10.12 ± 0.53	11.56 ± 0.38	10.31 ± 0.35	**0.042**
HSI (%)[5]	1.06 ± 0.04	1.05 ± 0.03	1.13 ± 0.03	0.292
MFI (%)[6]	1.18 ± 0.14	1.11 ± 0.12	1.37 ± 0.15	0.380
IWI (%)[7]	3.75 ± 0.10	3.99 ± 0.14	3.91 ± 0.08	0.284
ILI (%)[8]	73.21 ± 3.86^b	84.29 ± 2.34^a	75.4 ± 2.68^b	**0.041**
ORAC[9]	6.42 ± 0.6	6.02 ± 0.67	6.41 ± 0.67	0.881

Data on body weight, feed intake and growth indices are the mean ± SEM of triplicate tanks. Data on organosomatic indices are the mean ± SEM of 18 fish. Different superscript letters in each row indicate significant differences among dietary treatments (Holm–Sidak post-test, P < 0.05, stated in bold).

[1] Result values from one-way analysis of variance.

[2] CF = 100 × (body weight/standard length^3).

[3] Feed conversion ratio = dry feed intake/wet weight gain.

[4] Specific growth rate = 100 × (ln final body weight − ln initial body weight)/days.

[5] Hepatosomatic index = 100 × (liver weight/fish weight).

[6] Mesenteric fat index = 100 × (mesenteric fat weight/fish weight).

[7] Intestinal weight index = 100 × (intestine weight/fish weight).

[8] Intestinal length index = 100 × (intestine length/standard length).

[9] Oxygen radical absorbance capacity = μmol eq Trolox/mL plasma.

ILI was significantly higher in this group of fish in comparison to CTRL and EWH groups. Intestine weight of L-FM/FO fish was also larger, although this was not reflected in significant differences in IWI among groups. No statistical differences in plasma antioxidant capacity were observed between different groups, with ORAC values around 6 μmol eq Trolox/mL plasma.

Histological Scoring

The dietary replacement of FM/FO provoked a hyperplasic inflammation in the intestines of both, L-FM/FO and EWH fish, compared to the CTRL fish (**Figures 1**, **2**). Inflammatory cell infiltrates in the epithelium and lamina propria-submucosa consisted mainly of lymphocytes and eosinophilic granular cells. In the AI, a significant submucosal hyperplasia was found in L-FM/FO and EWH fish, though the increase of EGCs and IELs was not significant. In this segment, the abundance of light-stained goblet cells was significantly reduced by the L-FM/FO diet and this effect reverted by the EWH diet. This goblet cell type presented a PAS+ staining pattern indicative of neutral mucins. By contrast, the hyperplasic effect was less severe (not significant) at the PI, where the increase of intraepithelial lymphocytes was the only significant inflammatory sign observed in L-FM/FO and EWH fish. The enterocytes of the CTRL and L-FM/FO fish presented a medium degree of lipid vacuolization, which was significantly reduced by the EWH diet. Remarkably, this lipid depletion in EWH PIs co-occurred with a decrease of lipid depots in the hepatocytes of EWH fish, which were increased with L-FM/FO diets. No differences in glycogen storage were found in the liver of fish fed the different diets, though a significant increase of liver melanomacrophage centers was observed in EWH fish, compared to the other two diets.

Intestinal Content in Lactic Acid and Short Chain Fatty Acid

Butyric acid could not be detected in any of the analyzed samples. No statistically significant differences were found among groups for the intestinal concentration of lactic acid, formic acid, acetic acid, or total SCFA (**Figure 3** and **Supplementary Table 1**). The only difference was found in propionic acid, present in significantly higher concentrations in the intestinal content of fish fed EWH (7.40 μmol/g) when compared to the CTRL group (4.14 μmol/g).

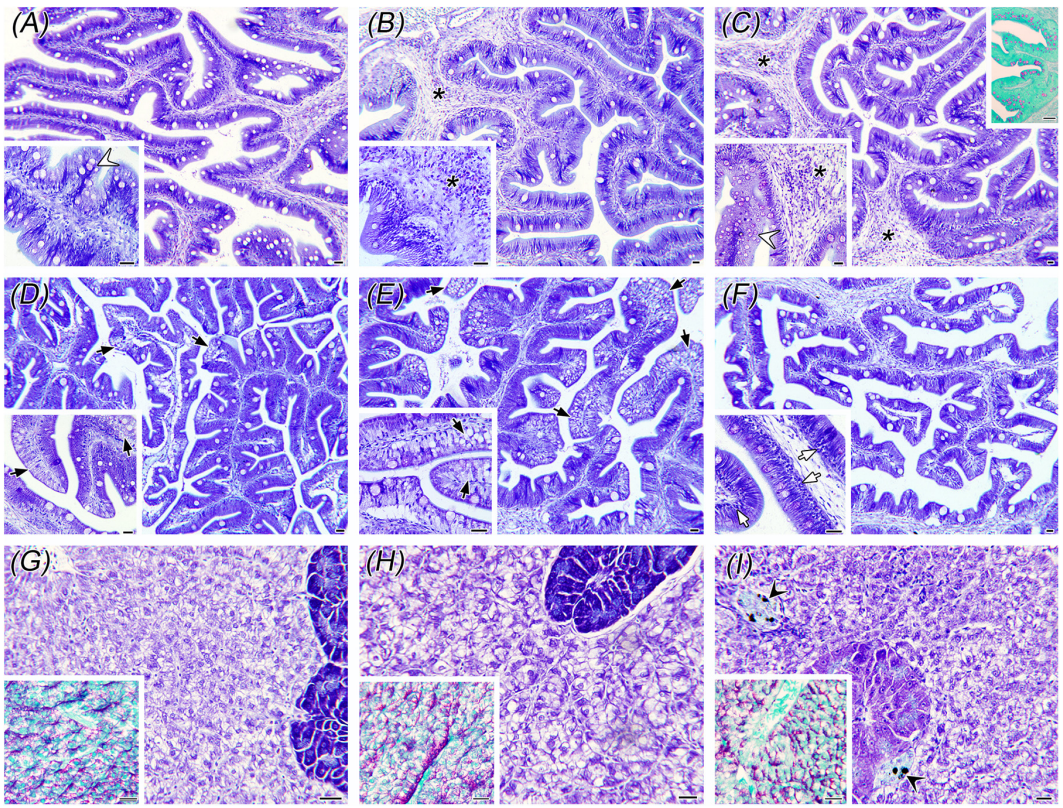

FIGURE 1 | Histological alterations in the AI **(A–C)**, PI **(D–F)** and liver **(G–I)** of gilthead sea bream. Panels **(A**,**D,G)** correspond to CTRL fish; **(B,E,H)** to fish fed the L-FM/FO diet; and **(C,F,I)** to fish fed the EWH diet. In AI, note the high abundance of light-stained goblet cells (white arrowheads) in CTRL **(A)** and EWH **(C)** fish, as well as the submucosal hyperplasia (asterisks) in L-FM/FO **(B)** and EWH **(C)** fish. The upper insert in panel **(C)** shows the PAS-stained goblet cells. In the PI, note the presence of lipid vacuolization in enterocytes (black arrows) in CTRL fish **(D)**, which is intensified in L-FM/FO fish **(E)** and decreased in EWH fish **(F)**. PI of EWH fish presented high abundance of intraepithelial lymphocytes (white arrows). In livers, note the higher lipid storage in L-FM/FO fish **(H)** and the presence of early melanomacrophage centers (black arrowheads) in EWH fish **(I)**. Glycogen storage did not change among groups (PAS-stained inserts). Scale bars = 20 μm.

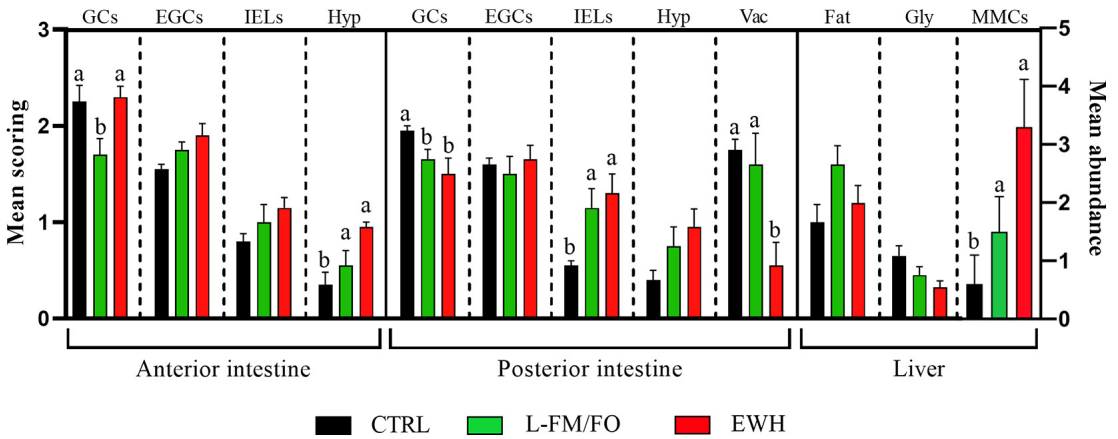

FIGURE 2 | Scoring of histological alterations in fish fed CTRL (black bars), L-FM/FO (green bars) and EWH (red bars) diets. Mean semiquantitative scoring (+SEM) from 0 (absence) to 3 (very abundant) is shown for presence of PAS+ goblet cells (GCs), eosinophilic granular cells (EGCs) and intraepithelial lymphocytes (IELs) in two intestinal segments. Mean semiquantitative scoring (+SEM) from 0 (absence) to 3 (severe) is shown for the degree of hyperplasia in the submucosa (Hyp), vacuolization of enterocytes (Vac) in two intestinal segments, and fat (Fat) and glycogen (Gly) storage in liver. Total melanomacrophage centers (MMCs) were quantified in liver (mean abundance, right *y*-axis, +SEM). Different letters within each alteration indicate statistically significant differences among diets ($P < 0.05$).

Alpha Diversity and Microbial Composition

Illumina sequencing of the 27 analyzed samples yielded 3,678,804 high quality reads, with a mean of 136,252 reads per sample (**Supplementary Table 2**). The reads were assigned to 2,117 OTUs at a 97% identity threshold. Rarefaction analysis showed curves that approximated saturation (horizontal asymptote), thus a good coverage of the bacterial community was achieved and the number of sequences for analysis was considered appropriate (**Supplementary Figure 1**).

In a first attempt to unravel the effects of dietary intervention on gut mucosal microbiota, we analyzed the bacterial diversity of all dietary groups, and no significant differences were found in Shannon and Simpson diversity indexes, but a significantly lower richness (ACE value, $P < 0.05$) was found in EWH fish (**Table 3**). At the phylum level (**Figure 4**), Proteobacteria were the most abundant bacteria, significantly varying from more than 55% in fish fed the L-FM/FO diet to 67.8% in EWH fish. In parallel, a significant decrease in the phylum Firmicutes was found both in L-FM/FO and EWH (16.6–16.8%) groups in comparison to CTRL fish (26.5%). Conversely, the phylum Actinobacteria raised up from ∼6% in CTRL and EWH fish to 18.2% in fish fed the L-FM/FO diet. Finally, in EWH fish, the less abundant Bacteroidetes and Spirochaetes phyla were significantly increased, with values of 2.8% and 2.2%, respectively.

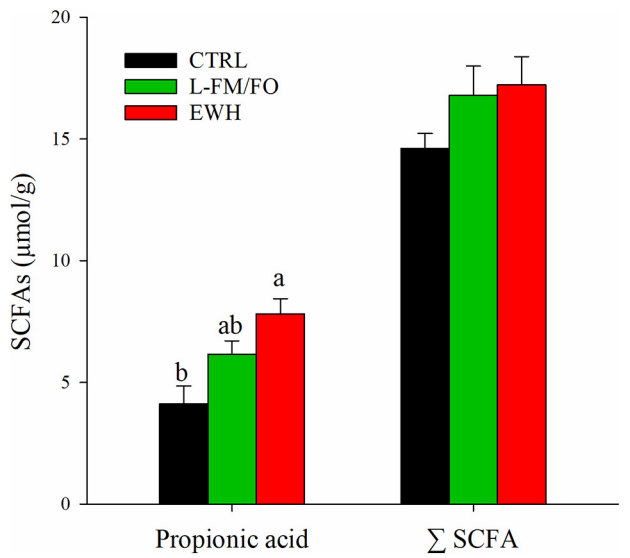

FIGURE 3 | Concentration of intestinal propionic acid and total short chain fatty acids (ΣSCFA) in fish fed CTRL (black bars), L-FM/FO (green bars), and EWH (red bars) diets. Significant differences (one-way ANOVA, Holm–Sidak post-test, $P < 0.05$) are indicated by different letters, which correspond to pairwise comparisons within each dietary group.

Microbiota Discriminant Analysis

Permutational multivariate analysis of variance test highlighted statistically significant differences in bacterial composition when comparing animals fed different diets ($P = 0.048$, $F = 1.115$,

TABLE 3 | Species richness estimators (observed and ACE) and diversity indexes (Shannon and Simpson) of fish fed CTRL, L-FM/FO, and EWH diets.

	CTRL	L-FM/FO	EWH	*P*-value
Observed	278.78 ± 75.29	243.78 ± 55.77	193.56 ± 88.21	0.06
ACE	394.13 ± 81.16[a]	377.09 ± 70.49[a]	266.12 ± 115.75[b]	**0.046***
Shannon	2.78 ± 0.26	2.50 ± 0.32	2.60 ± 0.51	0.23
Simpson	0.89 ± 0.04	0.87 ± 0.09	0.86 ± 0.10	0.67

Values are mean ± SEM of 9 fish. Asterisk (*) and bold font indicates significant differences among groups (Holm–Sidak, P < 0.05) denoted by different superscript letters.

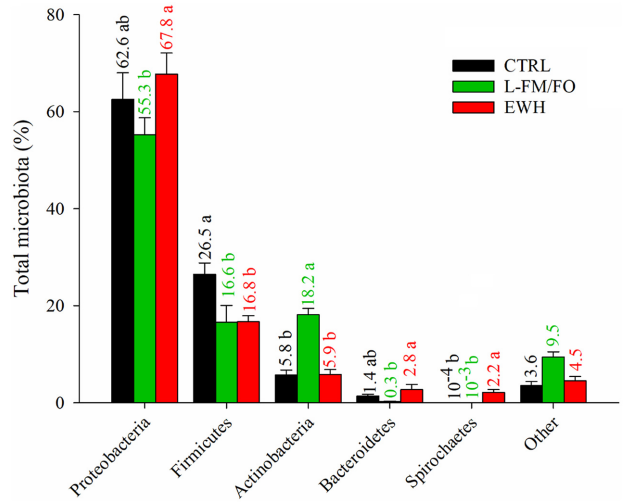

FIGURE 4 | Relative abundance of bacterial phyla in the anterior intestine of fish fed CTRL (black bars), L-FM/FO (green bars), and EWH (red bars) diets. Significant differences (Kruskal–Wallis, Dunn's post-test, $P < 0.05$) are indicated by different letters, which correspond to pairwise comparisons within each phylum among dietary groups. The numbers above each bar represent the mean abundance in percentage for each group.

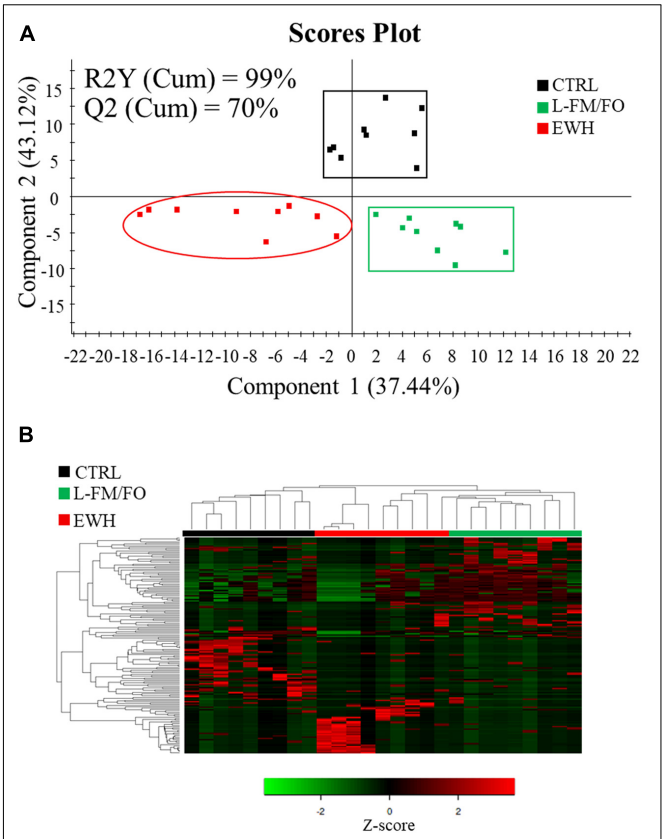

FIGURE 5 | (A) Two-dimensional PLS-DA scores plot constructed using the variable diet. The validation by the permutation test can be found in **Supplementary Figure 2**. **(B)** Heatmap representing the abundance distribution (*Z*-score) of the OTUs identified to be driving the separation by diet among all dietary groups.

R^2 = 0.085). Although R^2 values detected were quite low, they were in line with what was reported in other microbiota studies (He et al., 2018) due to the complexity and variability of microbiota samples. To validate and study these differences in more detail, a PLS-DA model (R2Y = 99%, Q2 = 70%) with three score components was constructed and statistically validated (**Figure 5A** and **Supplementary Figure 2**). The first two components explained more than 80% of total variance, clearly separating CTRL fish from fish fed L-FM/FO diets along *x*-axis (component 1, 37.4%), whereas component 2 (43.2%) separated the L-FM/FO diets with/without EWH along *y*-axis. To determine which groups of bacteria were driving these separations at a high level of confidence, the minimum VIP value driving the correct separation of groups in the model was determined throughout a heatmap representation (**Figure 5B**). Such approach disclosed 165 OTUs (VIP $\geq$ 1.2), which can be accessed in **Supplementary Table 3**.

Figure 6 shows the list of most abundant bacteria (at least 1% in one of the groups; 46 OTUs out of the 165 with VIP $\geq$ 1.2) that exclusively drove the separation by dietary groups. For these abundant bacteria, a first type of response was mediated by 16 OTUs that were increasing with the FM/FO replacement and decreasing again in EWH fish. In this group, the presence of Neisseriaceae family and species of *Ralstonia*, *Lactobacillus*, *Streptococcus*, *Corynebacterium*, and *Nocardioides* genera was remarkable. A second type of response grouped 15 OTUs present in a significant proportion in the CTRL group, but decreasing in fish fed the two L-FM/FO diets. In this case, dietary plant ingredients produced the decrease of the Comamonadaceae family and *Novosphingobium*, *Mesorhizobium, Klebsiella*, *Acinetobacter, Brochotrix, Bacillus*, *Clostridium sensu stricto*, and *Exiguobacterium* genera. The remaining 15 OTUs increased their proportion in fish fed the EWH diet, being in a very low proportion in the other two dietary groups. This response triggered the presence of Bacteroidetes and Spirochaetes phyla, and more specifically of the Flavobacteriaceae family and *Cloacibacterium* genus. The Rhodospirillales order also increased with the addition of EWH, as well as *Granulicatella, Serratia*, *Bradyrhizobium*, *Propionibacterium*, and *Photobacterium* genera.

Inferred Metagenome and Pathway Analysis

With the aim of assessing the biological significance of the diet-induced differences in the microbiota of the different groups, a pathway analysis was conducted with the inferred metagenomes of the 165 OTUs that drove the separation by diet (**Table 4**). The results showed that 15 pathways could be significantly changing in the comparison between fish fed CTRL and the L-FM/FO diet without EWH, whereas the comparison between EWH and CTRL groups rendered 28 pathways. In both comparisons, pathways related to signaling pathways of rat sarcoma (RAS), sphingolipids, GnRH, cAMP, and Fc gamma R-mediated phagocytosis were strongly overrepresented in the two groups of fish fed L-FM/FO diets, whereas Staurosporine biosynthesis, neuroactive ligand–receptor interaction and cholesterol metabolism were

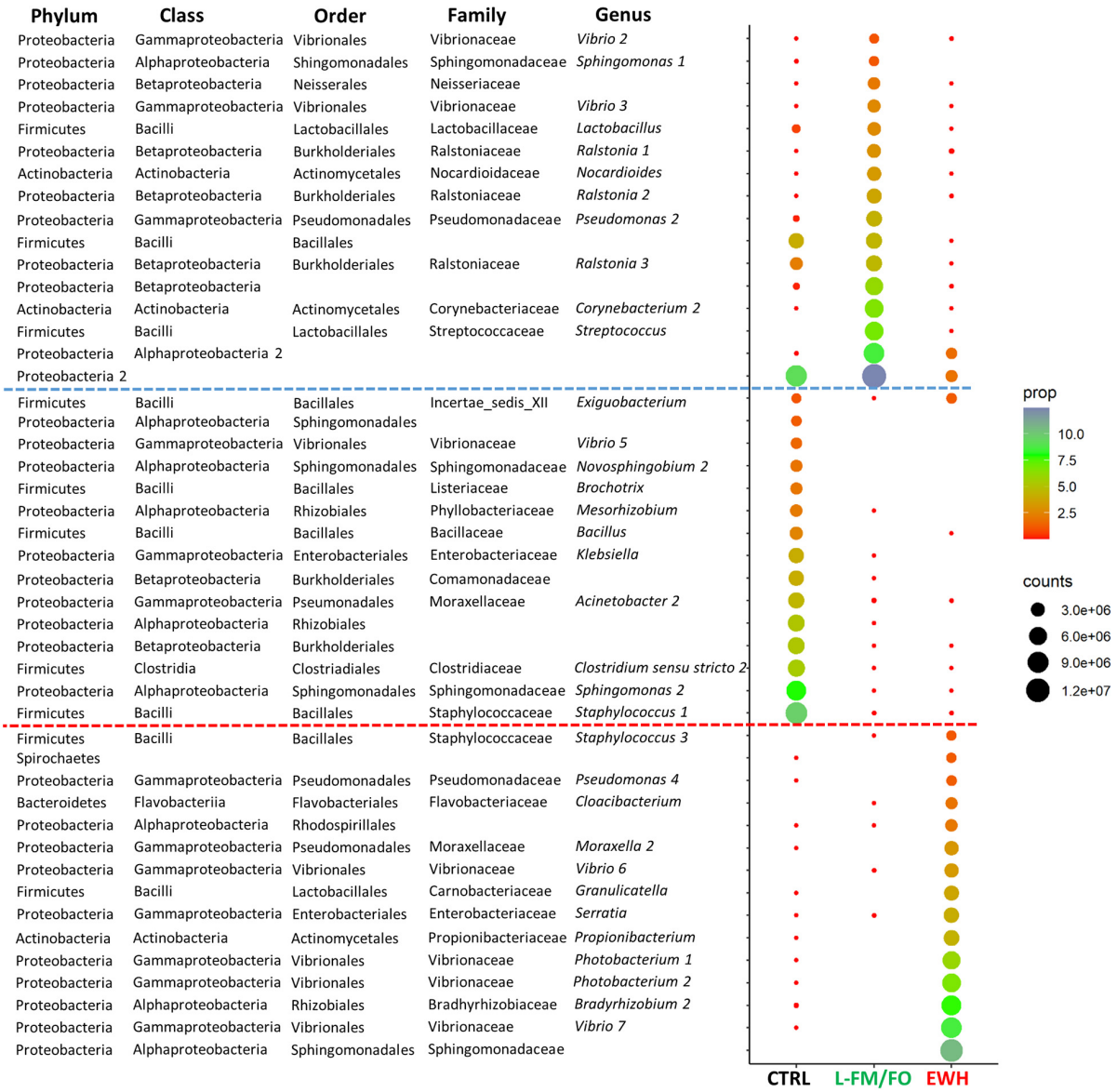

FIGURE 6 | Dotplot map depicting the most abundant genera (more than 1% of the total microbiota in at least one dietary group) from the 165 significant OTUs identified in **Figure 5B**. The size of the dots represents the normalized counts in each dietary group (CTRL, L-FM/FO, and EWH). The color scale represents the mean abundance, in percentage, of each genus within each group. OTUs above the blue dotted line showed an increased abundance in L-FM/FO groups; OTUs between the dotted lines showed an increased abundance in the CTRL group; OTUs below the red dotted line showed an increased abundance in the EWH group. The numbers after the genus names correspond to different OTUs assigned to the same genus that probably belong to different species.

underrepresented. By contrast, only two pathways corresponding to primary bile acid biosynthesis and steroid degradation were consistently underrepresented in the microbiota of EWH fish when comparisons are made with the other two groups. This was coupled to an overrepresentation of the longevity regulating pathway.

DISCUSSION

Enzymatic hydrolysis of animal and plant proteins has been used as a basic method for the conversion of underused protein products into highly digestible peptides (Benjakul et al., 2014; Egerton et al., 2018a). Additionally, protein hydrolysates containing antioxidant peptides possess a high therapeutic potential for the management of chronic diseases, but also as safe additives to halt lipid peroxidation, improving the quality and consumer satisfaction of several food products (Auwal et al., 2017; Cicero et al., 2017). Thus, the antioxidants and anti-inflammatory properties of EWH have shown beneficial effects in different experimental rat models (Requena et al., 2017), and we discussed herein the potential benefits of dietary EWH supplementation in fish fed experimental diets with a high replacement of marine feedstuffs by alternative plant ingredients.

TABLE 4 | Pathway analysis from predicted metagenome.

Generic	Process	1	2	3
Cellular process	Endocytosis		4.63	
	Flagellar assembly		−1.7	
Environmental information processing	Ras signaling pathway	5.47	7.1	
	Sphingolipid signaling pathway	6.97	4.73	
	cAMP signaling pathway	6.72	4.51	
	Neuroactive ligand–receptor interaction	−3.41	−2.69	
Genetic information processing	Proteasome	4.73	4.28	
	Basal transcription factors	4.19	3.13	
	Ribosome biogenesis in eukaryotes		0.8	
	Sulfur relay system		0.75	
Metabolism	Flavonoid biosynthesis		5.24	
	Stilbenoid, diarylheptanoid and gingerol biosynthesis		5.24	
	Secondary bile acid biosynthesis		4.19	
	Vitamin B6 metabolism		0.63	
	Nitrogen metabolism		0.52	
	Aminobenzoate degradation		−1.01	
	Dioxin degradation		−1.56	
	Bisphenol degradation		−2.81	
	Primary bile acid biosynthesis		−3.22	−3.02
	Steroid degradation		−4.16	−3.41
	Steroid biosynthesis		−6.17	
	Staurosporine biosynthesis	−5.35	−6.72	
	Photosynthesis – antenna proteins	8.23		
	Arginine and proline metabolism	−0.61		
Organismal systems	Fc gamma R-mediated phagocytosis	6.16	5.58	
	GnRH signaling pathway	6.14	5.58	
	Cholesterol metabolism	−3.42	−2.7	
	Renin secretion		−4.83	
	Regulation of lipolysis in adipocytes		−5.48	
	Retrograde endocannabinoid signaling	−3.55	−5.85	
	Longevity regulating pathway – multiple species			0.62
	Parathyroid hormone synthesis, secretion and action	4.14		
	Bile secretion	4.24		

Values represent the log_2FC of the comparisons: 1 = L-FM/FO vs. CTRL; 2 = EWH vs. CTRL; 3 = EWH vs. L-FM/FO (FDR < 0.05).

From our results, it is conclusive that dietary EWH supplementation triggered a reduced feed intake and a slight impairment of feed conversion ratio in gilthead sea bream. In rodents, EWH administration reduced body weight gain in obese animals, and this decrease was related to a reduced deposit of fat in different tissues, especially white adipose tissue, but no effects on growth or food intake were observed in obese or control rats fed EWH (Miguel et al., 2006; Moreno-Fernández et al., 2018a). It should be also noted that proteins in their natural state do not contribute to the flavor of food, but hydrolyzed derived peptides can modify the sensory quality of proteins causing food rejection (Iwaniak et al., 2019). Therefore, we cannot exclude a taste effect on the apparent satiety effect of the EWH in our experimental gilthead sea bream model. Moreover, fish protein hydrolysates, absent in EWH diet, are known feed attractants in aquaculture, which could enhance the feed palatability of CTRL and L-FM/FO diets (Kasumyan and Døving, 2003; Aguila et al., 2007). Alternatively, the modulation of the intestinal microbiota, particularly with respect to production of SCFA, might also contribute to explain the observed effects on growth and feed intake. SCFA, such as butyrate, propionate and acetate, are end products of microbial fermentation implicated in a multitude of physiological functions (Morrison and Preston, 2016), but similar to fiber, protein fermentation also produces SCFA (Macfarlane, 1992). However, while propionate production remains relatively stable, the rate of acetate and butyrate production is lowered when they are generated from protein fermentation (Aguirre et al., 2016). In agreement with this, the intestinal concentration of propionate was much higher than for other SCFA in our model of a carnivorous fish fed hyperproteic diets. It is difficult to categorize individual SCFA into purely obesogenic or anti-obesogenic, though acetate seems to be predominantly obesogenic, whereas butyrate and propionate are broadly anti-obesogenic (Chakraborti, 2015). The beneficial effects of butyrate have been reported in a large extent in gilthead sea bream as a highly promising additive to counteract undesired effects of plant-based diets at the local and systemic level (Robles et al., 2013; Benedito-Palos et al., 2016; Piazzon et al., 2017; Simó-Mirabet et al., 2018). However, we found than intestinal butyrate was below the detection limit in all the studied groups, whereas the highest concentration of propionate was achieved in EWH fish, and its relevance is further discussed later on.

Gut microbiota studies are emerging as effective approaches for promoting farmed fish health, contributing to improve the productivity of the aquaculture sector (Brugman et al., 2018; Egerton et al., 2018b; Egan et al., 2020). In this regard, it is noteworthy that measurements of gut microbiota diversity are considered a good indicator of animal health, becoming dietary factors one of the main regulators of intestinal microbial diversity (Moschen et al., 2012). Thus, the ability of replacement diets and/or feed additives to retain a gut microbiome composition close to that of fish fed diets with high contents of FM and FO is envisaged in gilthead sea bream (Fontinha et al., 2021), as well as in other fish species of interest in aquaculture (Egerton et al., 2020; Niu et al., 2020). Though it exists a high variability of response to protein supplements across different animal models (Clarke et al., 2014; Liu et al., 2014; Butteiger et al., 2016; Beaumont et al., 2017), we did not detect changes in gut microbiota diversity of gilthead sea bream fed EWH diet. Regarding gut microbiota richness, a negative correlation with obesity has been largely reported in humans (Turnbaugh et al., 2008; Le Chatelier et al., 2013; Sze and Schloss, 2016; Peters et al., 2018). However, recent evidence suggests that this association cannot be considered as widespread among the population (Stanislawski et al., 2019). Similarly, the lower microbiota richness of our EWH fish with a reduced feed intake was mainly driven by three animals with extreme low richness values (**Supplementary Figure 1**).

Despite all the above findings, changes in the composition of mucosal adherent bacterial communities are already found at the phylum level (**Figure 4**). Proteobacteria, Firmicutes,

Actinobacteria, and Bacteroidetes Phyla dominated the autochthonous microbiota of the intestine of fish, as it has been also observed in previous studies in this species (Kormas et al., 2014; Estruch et al., 2015; Piazzon et al., 2019), with a fifth phylum, Spirochaetes, increasing in EWH fish. Furthermore, discriminant analysis (**Figure 5**) helped to disclose 46 dominant bacteria (VIP $\geq$ 1.2, >1% in abundance) (**Figure 6**) with a significantly higher presence associated to a particular diet, and a decrease in the other groups. This fact revealed a specific organization of the gut microbiota in response to each dietary treatment, which allowed to discover differences at all taxonomic levels. Firstly, Proteobacteria (facultative anaerobic organisms) were presented in all analyzed fish, as it is considered one of the most abundant symbionts in marine fish because of their highly flexible metabolic properties (Tarnecki et al., 2017; Ikeda-Ohtsubo et al., 2018). Within this phylum, high abundance values were found for the Vibrionaceae family in all dietary groups. Species of this family help to digest organic substances due to the production of lipases, amylases and proteases, but some of those species can also produce harmful enzymes like neuraminidases and act as causative agents of fish pathologies (Egerton et al., 2018b).

Firmicutes are also some common intestinal symbionts in fish and mammals (Lozupone et al., 2012; Ghanbari et al., 2015). In our farmed fish, Firmicutes ranged from 26.5% in the CTRL group to ∼17% in the L-FM/FO and EWH groups. Both plant-based diets shared important proportions of Lactobacillales, known to inhibit fish pathogens due to the natural production of bacteriocins (Balcázar et al., 2007; Sugita et al., 2007; Shahid et al., 2017; Ringø et al., 2018). This abundance of Lactobacillales in plant-based diets is caused by their ability to use indigestible fiber and fermentable polysaccharides for their metabolism and growth (Gajardo et al., 2017; Theilmann et al., 2017). In the last decade, Lactobacillales have been extensively studied due to their potential use as probiotics (Gillor et al., 2008; Heo et al., 2012), with particular importance to aquaculture, where the avoidance of the use of antibacterial drugs for facing fish pathogens is one of the main challenges (Sahoo et al., 2016). Bacteria of the family Carnobacteriaceae and of the genera *Lactobacillus* and *Streptococcus*, all of them present in our plant-based dietary groups, are prone to produce these antimicrobial agents (Elayaraja et al., 2014). However, the remarkable proportion of *Streptococcus* in L-FM/FO can also display negative effects as this genus has been described among the reduced group of bacteria capable of producing all the enzymes needed for complete mucin degradation (Derrien et al., 2010). Hence, the goblet cell depletion observed on the L-FM/FO fish could be partly explained by the higher abundance of this genus, which is practically not present in the CTRL and EWH groups. Protective mucus at the intestinal mucosa consists of a gel overlying the epithelium based on the production and secretion of mucins, mostly by goblet cells but also by enterocytes (Pelaseyed et al., 2014). The amount of goblet cells of the AI was altered by FM/FO replacement, but interestingly the profile of CTRL fish was restored by EWH supplementation, suggesting that EWH could be re-stimulating mucus secretion in the AI. In a similar manner, milk-derived peptides have already demonstrated to stimulate rat intestinal mucus secretion and improve intestinal barrier (Giromini et al., 2019). Here we hypothesize that these effects could be, at least in part, induced by the modulation of mucin degrading bacterial populations.

The phylum Bacteroidetes increased in EWH fish, with a predominance of bacteria assigned as *Cloacibacterium*. In gilthead sea bream, fermentation produced by species of this genus is a major process for the metabolism of glucose in SCFA that might be used later in other chemoautotrophic processes (Kormas et al., 2014). The Firmicutes/Bacteroidetes (F/B) ratio is a widely documented factor correlated with obesity in mammals. Changes in these phyla proportions are regarded as dysbiosis (Stojanov et al., 2020), and an increased F/B ratio has been related with obesity in humans (Ley et al., 2006) and rats (Requena et al., 2017). However, the correlation between obesity and F/B ratio in mammals can be controversial (Magne et al., 2020) and has not been demonstrated in fish. In any case, in our fish model, Firmicutes suffered a decrease from 26.5% in CTRL group to ∼17% in the plant-based diets, whereas Bacteroidetes were only increased in the EWH group. Thus EWH fish showed the lowest F/B ratio (6 in EWH fish vs. 18.9 and 55.3 in CTRL and L-FM/FO fish, respectively). Firmicutes and Bacteroidetes represent more than 90% of the total bacterial communities in mammals (Magne et al., 2020), whereas, in fish, Proteobacteria are among the most abundant. Thus, although our results are in agreement with previous studies in mammals, further works are needed to determine the validity of this ratio in fish, and the possible implication of Proteobacteria in these correlations.

A wide range of Spirochaetes is found in aquatic habitats, but this phylum usually comprises a low proportion (<1%) of fish intestinal microbiota (Givens et al., 2015; Le and Wang, 2020). In mammals, the presence or increase of Spirochaetes has been associated to lean individuals in fecal microbiomes of captive cynomolgus monkeys (Koo et al., 2019) and in oral microbiomes of diabetes mellitus type 2 patients (Tam et al., 2018). Concordantly, in this study, the addition of EWH in the diet significantly increased the abundance of this phylum up to a 2.2%, pointing to a potential role of these bacteria in the reduced feed intake and decreased weight gain of this group of fish.

The phylum Actinobacteria significantly increased in the L-FM/FO group, mainly due to the increase in *Corynebacterium* and *Nocardioides*, which represented ∼10% of the overall bacterial population in this dietary group. These bacteria have been described to produce polysaccharide hydrolases (Anandan et al., 2016), which is compatible with the higher fiber content of our plant-based diets. Lastly, 73% of the total Actinobacteria found in the EWH group belongs to the genus *Propionibacterium*, present in significantly lower proportions in the other two groups. Since *Propionibacterium* is the best natural producer of propionate (Zárate, 2012; González-Garcia et al., 2017), this observation supported the higher concentrations of intestinal propionic acid in fish fed the EWH diet. Microbial production of propionate has been related to a healthier gut state (Hosseini et al., 2011; Louis and Flint, 2017), lowering lipogenesis (Weitkunat et al., 2016) and triggering the secretion of satiety peptides, such as glucagon-like peptide-1 and peptide YY (Chambers et al., 2015). Moreover, propionate acts as an inhibitory factor of food intake *via* its antagonism with the cholesterol synthesis (Harris et al., 2012; Chakraborti, 2015).

In agreement with this, we found herein that primary bile acid biosynthesis and steroid degradation were consistently underrepresented in the inferred metagenome of EWH fish in comparison to the other two groups (**Table 4**). To clarify, these results do not imply that bacteria are expressing primary bile acid biosynthesis genes, but that some bacteria within the detected populations might be expressing molecules that could affect such pathway. Indeed, bile acids represent a significant host factor that modulates the microbiome of obese mice and the digestion and absorption of dietary lipids (Zheng et al., 2017). Likewise, in the present study, the down-regulation of bile acids biosynthesis, together with the decreased F/B ratio and the increased Spirochaetes phylum in the EWH group could be describing the link between the bioactive egg white hydrolysate and an anti-obesogenic response. This assumption is supported by the reduced lipid vacuolization in intestines, and by the restoration of normal liver fat deposition in association with an increase in the number of hepatic melanomacrophage centers, as already found during feed restriction in lesser guitarfish (*Zapteryx brevirostris*) (Neyrão et al., 2019). Studies addressing the gut metatranscriptome in close association to host changes of metabolism and intestinal transcriptome should be conducted to validate this hypothesis and unravel the molecular interactions behind the effects.

In summary, altogether, these results reinforce the central role of gut microbiota in the regulation of host metabolism and lipid metabolism in particular, which might suggest a role of the EWH derived bioactive peptides as an anti-obesity and/or satiety factor in fish, although the ultimate mechanisms of action still remains to be established. From a practical point of view, the potential use of this functional food ingredient in finishing diets, and the role of gut microbiota in tuning filet fatty acid composition of marketable fish merits further research.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, SAMN18105342-68 and PRJNA705868.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics and Animal Welfare Committee of the Institute of Aquaculture Torre de la Sal (IATS-CSIC, Castellón, Spain), CSIC (Permit number 869/2019) and "Generalitat Valenciana" (permit number 2020/VSC/PEA/0010).

AUTHOR CONTRIBUTIONS

FN-C, MCP, IE, MM, and JP-S contributed to the formal analysis. FN-C, MM, JP-S, and MCP contributed to the writing-original manuscript. IE, AS-B, GW, ML-M, JC-G, MCP, and TR contributed to the writing-review and editing. GW, MM, AS-B, and JP-S contributed to the conceptualization. All authors read and approved the final manuscript and contributed to the experimental investigation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.698484/full#supplementary-material

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