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Dietary Replacement of Fish Oil with Plant Oils and *Schizochytrium limacinum* Biomass Modulates Gut Microbiota Composition and Functional Potential in European Sea Bass (*Dicentrarchus labrax*)

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Abstract

Aquaculture sustainability requires a reduction in the reliance on marine-derived raw materials such as fish oil in aquafeeds while maintaining fish health and product quality. This study investigated the effects of replacing fish oil with plant oils supplemented with DHA-rich *Schizochytrium limacinum* biomass on the gut microbiota of European sea bass (*Dicentrarchus labrax*). *S. limacinum* SR21—an oleaginous microalga naturally rich in omega-3 fatty acids—was produced through heterotrophic fermentation using crude glycerol, a waste stream from biodiesel production, within a circular economy framework. A 21-week feeding trial was conducted in an indoor recirculating aquaculture system using 280 fish distributed across eight tanks. Four experimental diets were tested: fish oil-based (FO), plant oil-based without microalga (VO + 0), and plant oil-based supplemented with 5% (VO + 5) or 10% (VO + 10) microalgal biomass. Gut microbiota was analyzed in 22 fish per group using 16S rRNA gene sequencing. While alpha and beta diversity analyses of gut microbiota revealed modest structural shifts at phylum and class ranks, genus-rank differences were evident, with *Lactobacillus* and *Clostridium sensu stricto* associated with FO and VO + 0 diets, and *Pseudomonas* and *Staphylococcus* enriched in microalga-supplemented groups. Functional inference highlighted enhanced bile acid biosynthesis and carbohydrate metabolism in VO + 0, whereas antioxidant-related pathways, including ubiquinone and carotenoid biosynthesis, were stimulated in VO + 5 and VO + 10 groups. These results demonstrate that *S. limacinum* biomass modulates microbiota functional capacity, potentially contributing to oxidative stress mitigation and host resilience. The findings support microbiota-informed feed formulation strategies to advance sustainable aquaculture.

Keywords: gut microbiota; sustainable aquafeeds; DHA; single-cell oils; circular economy

Key Contribution: The manuscript reports diet-dependent modulation of European sea bass gut microbiota composition and inferred functions following fish oil replacement with plant oils and *Schizochytrium limacinum* biomass.

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1. Introduction

The aquaculture sector has long represented a cornerstone of aquatic food production, with a steady rise over the last few decades and the achievement in the year 2022 of a higher total biomass production compared to capture fisheries, which amount to 94 and 91 million tons, respectively [1,2]. However, although aquaculture is commonly considered to have much less impact than other animal production sectors, the path towards better environmental sustainability is still ongoing. Feed composition remains one of the most critical challenges, as it traditionally relies on ocean-derived fishmeal (FM) and fish oil (FO), key sources of high-quality protein and long-chain polyunsaturated fatty acids (LC-PUFAs) such as eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), essential for fish health and metabolism [3]. Despite their great importance, in fact, the use and global production of FM and FO has declined, citing a rise in the prices due to high demand, and forcing feed manufacturers to reduce their inclusion rates, especially in grower diets, and to focus their use on specific stages of production such as the early life stages or for broodstock nutrition [4–6].

The principal alternative and sustainable dietary sources successfully introduced over time and widely used today in response to the reduction in marine-derived feed ingredients include vegetable feedstuff, considering both lipid and protein fractions [7,8]. Regarding vegetable oils (VOs), soybean represents the principal source, but not the only one. There is a large spectrum of valuable alternatives such as rapeseed, palm, linseed, and canola oils among others, usually included as a blend to ensure the correct supply of lipid species to the diet. Although lacking in LC-PUFAs, especially EPA, DHA, and arachidonic acids (ARAs), the use of VOs is of great interest due to their availability, lower cost, and higher resistance to lipid oxidation [9]. From fish performance and health perspectives, partial or total FO replacement using vegetable sources has also been supported over time by the lack of negative effects on animals, as far as the minimum essential fatty acid (EFA) requirements, which mostly come from FM, were concerned [5]. Indeed, several studies have demonstrated positive outcomes or performance comparable to control diets in different fish species, including salmonids, greater amberjack (*Seriola dumerili*), and sturgeons [10–13].

Over the past few decades, the use of single-cell ingredients (SCIs) has become increasingly widespread. SCIs represent a broad class of biomaterials primarily produced by bacteria, yeasts, and microalgae, which can be used individually or in combination [7,14]. The use of SCIs offers several advantages, including efficient growth under controlled conditions independent of seasonal or climatic fluctuations, as well as tolerance to a wide range of cultivation environments, resulting in stable, reproducible, and consistently standardized products [15,16]. In contrast to probiotics, these ingredients do not involve live microorganisms but consist of their derivatives, such as extracts and dried biomass, providing proteins, amino acids, bioactive compounds (e.g., astaxanthin and peptidoglycans), and lipids, especially n-3 LC-PUFAs [17–20]. The inclusion of single-cell oils (SCOs) in aquafeed formulations represents a valuable strategy to enhance the content of polyunsaturated fatty acids (PUFAs), enabling the development of fish oil (FO)-free diets. Among the various microorganisms investigated, species of the genus *Schizochytrium*, particularly *S. limacinum*, have gained increasing attention as alternative and sustainable producers of n-3 fatty acids. This marine microorganism synthesizes docosahexaenoic acid (DHA) via an oxygen-independent polyketide synthase pathway and is characterized by high lipid accumulation capacity, with intracellular oil content exceeding 35% of total fatty acids and more than 50% of dry biomass [21]. These characteristics, together with the relative ease of cultivation, genetic manipulation, and optimization of fermentation processes, have contributed to the widespread adoption of this microorganism in the aquaculture

sector [22,23]. Previous studies in various fish species have documented the benefits of including *Schizochytrium* spp. in aquafeeds, not only in terms of zootechnical performance, but also with respect to immune response and fillet quality [24–28].

Beyond their effects on growth performance and feed conversion efficiency, the evaluation of alternative ingredients intended to replace marine-derived components should also consider their impact on the intestinal microbiota. The gut microbial community plays a crucial role in fish physiology, contributing to nutrient digestion and absorption, the regulation of energy metabolism, and maintenance of overall health [29–31]. In particular, bacterial communities can enhance nutrient availability both directly and indirectly by producing vitamins and digestive enzymes capable of hydrolyzing complex compounds such as chitin, cellulose, and other plant polysaccharides. This enzymatic activity generates simpler metabolites and promotes the production of short-chain fatty acids (SCFAs), which can be absorbed and utilized by the host at both local and systemic levels [32,33]. Although several studies have reported positive effects of microalgae and other single-cell organisms as partial or total replacers of marine-derived ingredients [7,34], relatively few have investigated their combined use with VOs. In this context, the present study evaluated the effects of graded inclusion levels of whole-cell *S. limacinum* biomass, rich in DHA, combined with a mixture of VOs, as a sustainable alternative to FM and FO, on the intestinal microbiota of European sea bass (*Dicentrarchus labrax*). By analyzing gut bacterial community composition and predicted metabolic functions, this approach explores a circular economy-based strategy for aquafeed formulation, valorizing a low-value industrial by-product and reducing the reliance on marine-derived ingredients, thereby potentially lowering the environmental footprint of aquaculture.

We hypothesized that the partial or total replacement of FO with VOs supplemented with DHA-rich *S. limacinum* biomass would modulate gut microbiota composition and functional pathways without causing major disruption to overall microbial community stability.

2. Materials and Methods

2.1. Ethics Statement

All animal experiments were performed according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes. The Italian Ministry of Health approved the animal experiments [REF: 483/2017-PR (response of Prot. Nr. 344C6.6 of 13 March 2017)] in accordance with Art.31 of D.lgs. 26/2014.

2.2. Experimental Diets and Feeding Trial

The feeding trial and the four experimental diets used in the present study were already reported by [27]. Briefly, after a one-week acclimation period in the indoor recirculating saltwater facility at the University of Insubria (Varese, Italy), a total of 280 European sea bass with an initial mean weight of 22.38 ± 0.3 g were randomly distributed into eight tanks at an initial stocking density of 1.56 kg/m^3. Fish were fed the experimental diets for 21 weeks, with each diet administered to duplicate groups (2 tanks per diet). The feeding rate was maintained at 1.5% of the total biomass. Throughout the trial, fish were reared under controlled conditions, including a 12 h light: 12 h light dark photoperiod, a constant temperature of 19 ± 1.5 °C, pH 8.3 ± 0.4 and dissolved oxygen levels maintained above 85% saturation. Total ammonia nitrogen and salinity were periodically monitored. The four experimental groups were defined based on dietary administration. In particular, a non-oiled commercial feed, manufactured by VRM S.r.l. (Naturalleva) feed company (Verona, Italy), was used as base for all the isonitrogenous, isolipidic, and isoenergetic diets. Then, the oil fraction was added as follows: FO group included fish oil, while

VO + 0, VO + 5, and VO + 10 included vegetable oil as FO replacer. Furthermore VO + 5 and VO + 10 also included *S. limacinum* biomass at 5 and 10%, respectively. *Schizochytrium* sp. biomass was produced through fermentation process using crude glycerol waste, generated from a palm oil biodiesel plant, as substrate. Detailed information of the process and diets was reported by [27]. At the end of the trial (final fish weight: 78.11 ± 2.34), intestinal microbiota samples were collected from 20 overnight-fasted fish per diet, except for the FO group, from which 22 fish were sampled. Prior to sampling, all fish were euthanized with an overdose (320 mg/L) of tricaine-methanesulfonate (MS-222; Sigma-Aldrich, Milano, Italy). Fish were fasted before the final sampling to minimize the presence of transient digesta-associated microbiota. The entire intestine was aseptically removed and opened, and the autochthonous gut microbial fraction was collected by gently scraping the intestinal mucosa, excluding the pyloric caeca, using individually wrapped sterile cotton swabs. Each swab head was immediately placed into a sterile 1.5 mL Eppendorf tube containing 200 µL of Xpedition Lysis/Stabilization Solution and stored at room temperature for up to 24 h prior to bacterial DNA extraction.

2.3. DNA Extraction

DNA was extracted using DNeasyPowerSoil®® Kit (Qiagen, Milano, Italy) following the manufacturer's instructions, except for a few modifications at the lysis step, as previously indicated by Rimoldi et al. [35]. In brief, the lysis of 200 µL of gut bacteria suspension was performed in Power Bead Tubes by means of a TissueLyser II (Qiagen, Italy) for 2 min at 25 Hz. A negative control sample with only lysis buffer was processed in parallel. The concentration of extracted DNA was then measured using NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Rodano, Italy) and stored at −20 °C until the sequencing.

2.4. Illumina Sequencing and Bioinformatic Analysis

The V3–V4 hypervariable regions of the 16S ribosomal RNA (rRNA) gene were amplified with 5′—GTGCCAGCMGCCGCGGTAA—3′ and 5′—GGACTACHVGGGTWTCTAAT—3′ primer pairs with overhanging adapters, according to the 16S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, CA, USA).

All libraries were sequenced on a MiSeq platform (Illumina) in a single 2 × 300 bp paired-end run. The sequencing process was conducted at Biodiversa srl (Treviso, Italy).

FASTQ files were uploaded to the Sequence Read Archive (SRA) under the Bioproject accession number Project PRJEB103764 (Submission ERA35154890).

Raw microbiota reads were processed using QIIME2 (Quantitative Insights Into Microbial Ecology 2) (https://qiime2.org/, accessed on 25 September 2025) [36]. Sequences were demultiplexed with native plugin and DADA2 (Divisive Amplicon Denoising Algorithm 2) [37] was applied to obtain exact sequence variants (ESVs) [38], trimming primers and performing a quality filter with an expected error of 2.0. Chimeric sequences were removed using the consensus method. The taxonomic assignment of representative sequences was carried out using the feature-classifier (https://github.com/qiime2/q2-feature-classifier, accessed on 25 September 2025) plugin implemented in QIIME2, using classify-consensus-vsearch method against the SILVA SSU non-redundant database (138 release), adopting a consensus confidence threshold of 0.8. Sample depth was normalized by total sum scaling and then made proportional to the total sequencing depth.

2.5. Statistical Analysis

For all the data, normality was verified by Shapiro–Wilk test. Statistical evaluations of the differences between experimental groups were tested by one-way ANOVA followed by Tukey's test or by Kruskal–Wallis test followed by Dunn's post hoc test, depending

on the normality of the data. For all tests, the analysis significance threshold was set at $p < 0.05$. For the microbiota analysis, the R package phyloseq (v1.48.0) was used to obtain rarefaction curves, species richness estimates, and alpha diversity indices (ACE, Chao 1, Faith-PD, Pielou, Shannon and Simpson) [39]. To visualize similarities and dissimilarities between beta diversity data, principal coordinate analyses (PCoAs) based on Bray–Curtis and weighted UniFrac distance metric were conducted. Beta diversity was assessed using Bray–Curtis dissimilarity calculated on normalized count data. Differences in microbial community composition were tested using permutational multivariate analysis of variance (PERMANOVA) implemented in the adonis2 function of the vegan R package (v2.6-6), with 1000 random permutations. The model included Diet and the Diet:Tank interaction term to account for the nested tank structure within dietary treatments. Homogeneity of multivariate dispersion was evaluated using the betadisper function followed by permutation testing (permutest, 1000 permutations) to verify that significant PERMANOVA results reflected differences in group centroids rather than unequal within-group dispersion. To further study microbiota differences among groups, partial least squares discriminant analysis (PLS-DA) was performed using the Bioconductor R package ropls (v1.38.0). The outlier's identification was performed using Hotelling's T2 statistic, setting a 95% confidence limit, while the quality of the model was evaluated by the parametersR2Y (cum) and Q2 (cum) together with a validation test consisting of 500 random permutations. Functional microbiota profile was obtained through the inferred metagenome analysis using PICRUSt2 protocol and the Kyoto Encyclopedia of Genes and Genomes as reference database (KEGG) [40].

3. Results

3.1. Microbial Richness and Diversity

Considering the 82 gut samples and after removal of unassigned, chloroplast and mitochondria sequences, a total of 581,462 reads were produced. The mean read count was 5063.96, with a median of 4086 reads per sample. The first and third quartiles were 1774.25 and 6125.5, respectively. Reads were taxonomically assigned to 2279 ESVs reaching a high percentage of reads classified up to the genus level (79.7%). As indicated in Supplementary Figure S1, rarefaction curves approached the horizontal asymptote which indicates the saturation plateau, suggesting that the sequencing depth was adequate to capture the diversity of the bacterial community for all the samples sequenced. Alpha diversity analysis (Figure 1) revealed significant differences in richness indices (ACE, Chao1) and Faith's Phylogenetic Diversity (Faith_PD), registering the lowest values for the VO + 0 and VO + 10 groups. In contrast, no significant differences were detected for Pielou's evenness, Shannon, or Simpson indices, suggesting a relatively stable distribution and dominance of taxa across groups.

Regarding beta diversity analysis, the permutational analysis of variance (PERMANOVA), including tank as a nested factor, indicated that microbial community structure differed significantly among tank/dietary administration ($F = 5.51$, $R^2 = 021$, $p = 0.001$). Homogeneity of multivariate dispersion did not highlight significant differences in dispersion among dietary groups ($F = 1.58$, $p = 0.21$), indicating that the observed PERMANOVA significance was not driven by unequal within-group variability. However, dispersion differed significantly among individual tank units ($F = 14.06$, $p = 0.001$), highlighting a slight heterogeneity at the tank level. Despite these significant results, both PCoA approaches using Bray–Curtis (Figure 2a) and weighted UniFrac metrics (Figure 2b) did not highlight spatial differences among the microbial profiles, as was also demonstrated by the variance explained by both models, which stands at around 35.78 and 50.18% for the first two components respectively for the first and second methods used (Figure 2).

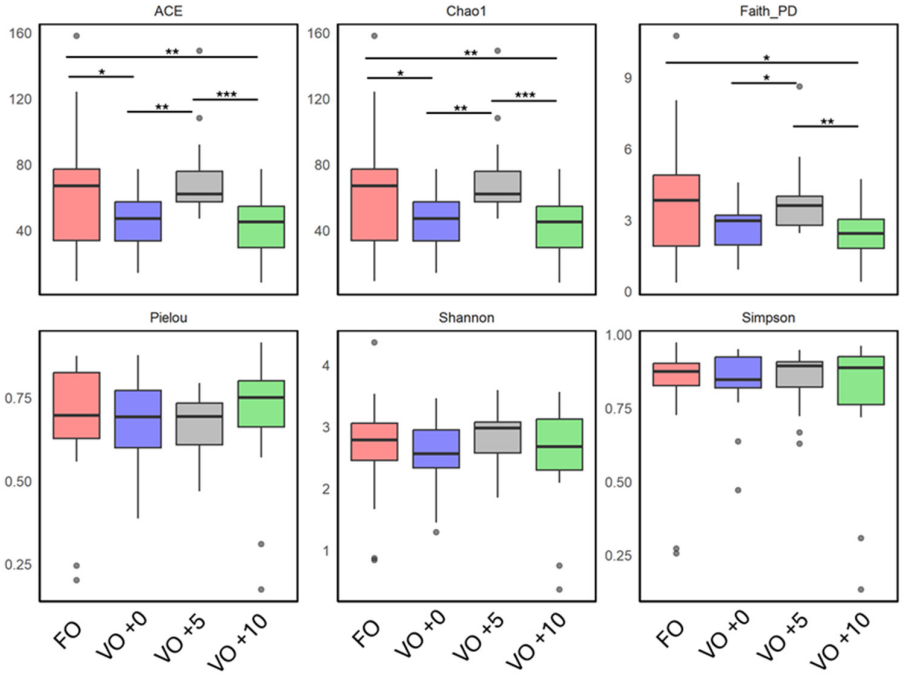

Figure 1. Boxplots representing alpha diversity indices related to richness (ACE and Chao1) and diversity (Faith_PD, Pielou, Shannon and Simpson). Significant values are indicated as follows: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

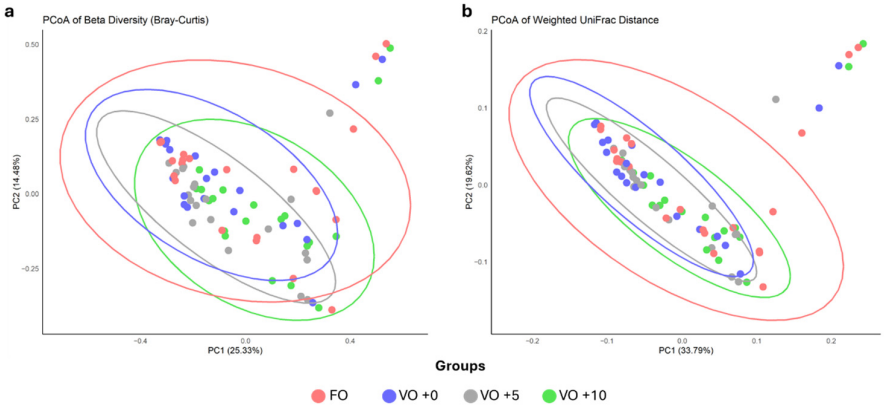

Figure 2. Graphical representation of sample distribution between the first two components of principal coordinate analysis (PCoA) using Bray–Curtis (**a**) and weighted UniFrac (**b**) metrics, driving the separation of the four experimental groups.

3.2. Multivariate Analysis and Microbiota Composition

A more-in-depth analysis through the validated PLS-DA model of the sea bass's intestinal microbiota in the four diets disclosed a clearer separation between the dietary groups (variance explained (R2Y) and predicted (Q2), $p = 0.002$) (Supplementary Figure S2). The model highlighted how the fish from the two groups FO and VO + 0, although fed with diets containing different fat sources, clustered together along the first component of the model, while the two groups containing the *S. limacinum* biomass appeared more separated (Figure 3).

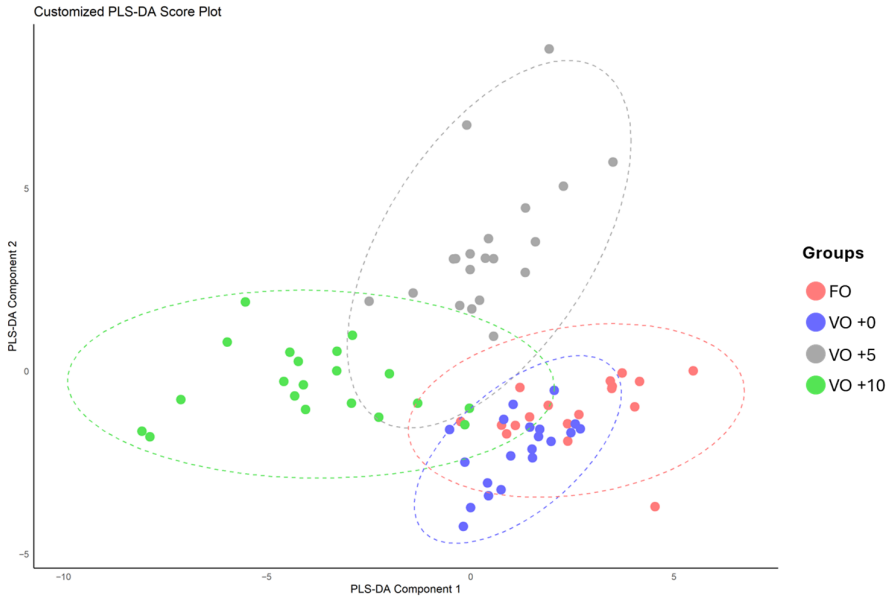

Figure 3. Sample distribution between the first two components of partial least squares discriminant analysis (PLS-DA) model driving the separation of the four experimental groups.

However, even if the analysis revealed a total of 64 bacterial ESVs statistically different between groups (Supplementary Table S1), these differences are not clear in the composition of the microbiota profile at the phylum level. At the higher taxonomy level, in fact, the statistical differences only reveal changes at less abundant taxa, such as Fusobacteriota, Acidobacteriota, Chloroflexi, Deinococcota and Verrucomicrobiota, which account for 2.33% of the total population, on average (Figure S3a). The main phyla which instead dominate the population in all four experimental groups are Firmicutes, Proteobacteria, Spirochaetota and Actinobacteriota, which constitute almost 95% of each experimental group. Conversely, considering the taxonomic rank of family (Figure S3b), different results in abundant taxa such as Lactobacillaceae, which registered the highest value in VO + 0, and Pseudomonadaceae and Staphylococcaceae, which instead highlighted a significantly higher abundance in groups VO + 5 and VO + 10. Genus-level differences mirrored those observed at the family level, displaying significant differences for *Lactobacillus*, *Clostridium sensu strictu* 1 and *Weissella* which showed higher values for FO and VO + 0, while *Pseudomonas*, *Staphylococcus*, *Leuconostoc* and *Enterobacter* were found to be more abundant in the VO + 5 and VO + 10 groups (Figure S3c).

3.3. Functional Characterization of the Gut Microbiome

The functional characterization of the 64 ESVs (Supplementary Table S1) whose abundance was significantly different between the four experimental groups was performed using the PICRUSt2 protocol. The inferred metagenomic results highlighted a total of 173 metabolic pathways identified (Supplementary Table S2) with 45 of them exhibiting a significant relative abundance among the dietary groups (Figure 4).

Among the most represented pathways, secondary bile acid biosynthesis, phosphotransferase system (PTS), fructose and mannose metabolism, and amino sugar and nucleotide sugar metabolism exhibited significantly higher values associated with the VO + 0 group, while showing the same decreasing pattern in the other groups, with VO + 10 expressing the lowest levels. The influence of the two percentages of *S. limacinum* were instead expressed mainly in the ubiquinone and other terpenoid–quinone biosynthesis, non-homologous end-joining, lipoic acid metabolism, geraniol degradation, carotenoid

biosynthesis, caprolactam degradation and the biosynthesis of siderophore-group nonribosomal peptides.

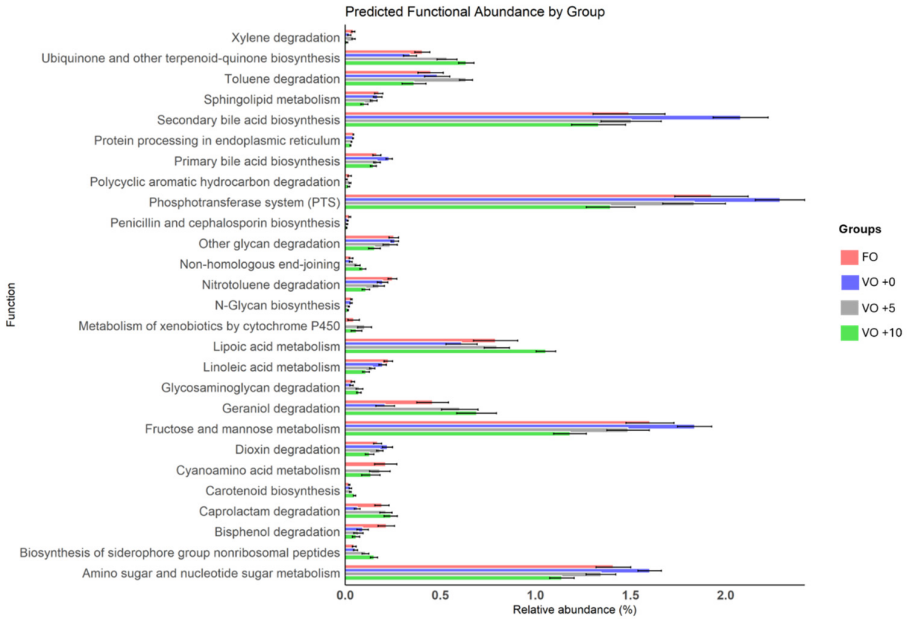

Figure 4. Grouped barplots of the significantly different metabolic pathways of KEGG annotations (Level 3) with an average abundance > 0.01%. Values are reported as relative abundance ± SE. Significance values ($p < 0.05$) are reported in the Supplementary Table S2.

4. Discussion

The application of circular economy principles, which focus on recycling and reusing raw materials, waste and by-products, aligns well with the goal of achieving a lower environmental impact and more sustainable growth in animal production systems, particularly in aquaculture. To this end, introducing alternative protein and lipid sources as substitutes for marine ingredients in aquafeeds presents one of the key challenges facing modern aquaculture [3,6,41]. Accordingly, as already reported by [27], the use of a mixture of VOs and whole-cell biomass of *S. limacinum* as a replacer for the lipid, and partially for the protein fraction, in the sea bass's diets determined an efficient growth performance and feed conversion together with a high level of accumulation of PUFAs in the fillets. This latter point, moreover, represents a crucial aspect as it is well known that the widespread use of plant feedstuff over recent decades has led to a progressive yet significant reduction in the EPA and DHA amount in fish fillets, ultimately decreasing final product quality [42,43]. For this reason, in the present study, the use of the *S. limacinum* SR21 strand was selected due to its ability to use low-cost carbon sources, such as crude glycerol derived from industrial biodiesel, as substrate for heterotrophic fermentation. Through this process, this marine microorganism can transform an initially low-value by-product into a material of high biological value with multiple applications, while accumulating significant amounts of intracellular lipids (more than 60% of the dried weight), of which approximately 35% is represented by DHA [44,45]. However, to better understand how experimental diets containing *S. limacinum* influenced fish physiology, an in-depth analysis of the intestinal microbiota was essential, given its key role in host metabolism, including its contribution in the synthesis and degradation of bioactive compounds, digestion, and assimilation [46,47].

If the benefits of dietary plant and algal oils on growth and immune response are widely reported in fish, the effects of these ingredients on the intestinal microbiota are still not consistent. Several studies have in fact reported different results in terms of alpha diversity, suggesting that the diet may not be the only driving variable of microbiota

modification [48]. Other conditions, such as fish species, farming settings and environment, may also play a key role in combination with diet. An example of this concept is discussed by Trevi and colleagues [49], who investigated different lipid sources as FO replacers in Nile Tilapia (*Oreochromis niloticus*). They reported a decrease in microbial diversity and a shift in the microbial community profile due to aging, regardless of the administered diet [49]. Variable results regarding microbial richness and diversity were also described in sea bream with the dietary inclusion of *Schizochytrium* sp., other algae or even VOs [50–53]. Accordingly, the present results showed a reduction in the richness estimators ACE, Chao1, and Faith's Phylogenetic Diversity (PD) in the VO + 0 and VO + 10 groups, but with statistically higher values registered in the VO + 5 group. As previously defined, these findings do not indicate a clear effect of FO replacement but do suggest a variable tendency to modulate rare or phylogenetically diverse taxa in response to the alternative dietary treatment compared to the control group. The minor effects were further confirmed by the lack of significant differences between groups in richness and evenness indices, such as Shannon, Simpson, and Pielou's evenness, as well as by the absence of a clear clustering of experimental groups in the PCoA plots, based on both Bray–Curtis and Weighted UniFrac metrics. The lack of distinct clustering, consistent with observations by other researchers studying European sea bass [54,55], suggests that these lipid sources have a limited impact on the overall composition of gut microbiota. This finding indicates a consistent distribution of taxa abundances and a community structure characterized by low dominance.

In accordance with the previous findings, PERMANOVA analyses also revealed low R^2 values. Despite being minor, these findings are noteworthy, suggesting that even a small portion of the overall community variance is influenced by dietary intervention, resulting in a slight difference between the experimental groups studied. Interestingly, PLS-DA discriminated the VO + 5 and +10 groups from VO + 0 and FO. This model enabled the detection of minor variations that might have been masked by within-group variability in the PCoA. Taken together, these results revealed that while overall community shifts are modest, specific microbial taxa are driving the separation between groups. The microbiota profile indeed confirmed this tendency. At the phylum level, as already defined by previous studies on sea bass [56–58], the general composition highlighted the dominance of Firmicutes and Proteobacteria, followed to a lesser extent by Spirochaetota and Actinobacteriota, regardless of the dietary treatment. Interestingly, all these majority taxa did not mark differences between experimental groups. Significant variations were instead only observed in a small fraction of the microbial population, which included the phyla Fusobacteriota, Verrucomicrobiota, and Chloroflexi, collectively representing less than 10% of the total community. These findings emphasize the homogeneity and stability of microbial composition at higher taxonomic levels, also suggesting that the subtle differences previously noted between groups are likely driven by shifts at a lower resolution scale. Accordingly, the results showed significant changes in Lactobacillaceae and Pseudomonadaceae families, among the most abundant genera like *Lactobacillus*, *Pseudomonas*, *Clostridium sensu strictu*, *Weissella*, *Leuconostoc* and *Enterobacter*. These taxa are commonly associated with a healthy intestinal microbiota profile in sea bass and different studies have also identified them as highly responsive to various dietary stimuli [56,59–61]. In particular, a previous study published by Lyons and colleagues [62] reported a modulation of this bacterial group due to the administration of a diet containing a 5% whole-cell microalgae ingredient as a partial replacer of FO in rainbow trout (*Oncorhynchus mykiss*) [62]. Apart from *Leuconostoc* and *Enterobacter* genera, which were specifically associated with the VO + 5 and +10 groups and exhibited the same tendency indicated in the previous study, in the present work all the other bacteria showed a different dynamic between the two trials. Although this discrepancy may be attributed to species-specific factors, which can modulate the host–microbe response, it is

worth mentioning that almost all of these microorganisms belong to the lactic acid bacteria (LAB). This heterogeneous group includes among others the genus *Lactobacillus*, which is usually considered as a positive marker of healthy gut microbiota [52,63] and are widely recognized as beneficial taxa to be used as probiotics [64]. In fact, even with relatively low abundance, they play a crucial role in enhancing feed conversion efficiency and maintaining the healthy status of intestinal epithelium through the competitive exclusion of pathogens and the production of antimicrobial compounds [65–67]. Hence, the association between these taxa and the experimental diets of the present study demonstrated the potential of these novel formulations to be valid alternatives to marine-derived ingredients.

To further investigate the host–microbiome interactions within the present study, a functional metagenomic analysis was also performed using the ESVs that were mostly affected by the different dietary sources and strongly marked the differences between the experimental groups. Among the numerous enriched functions identified, the results revealed a clear trend, highlighting the role of VOs used either as the sole substitute for fish oil or in combination with two inclusion levels of *S. limacinum* biomass. As described by Terova et al. [27] in a previous publication on the same feeding trial, fish belonging to the VO + 0 group accumulated a great amount of PUFAs, but a large majority of them belonged to the n-6 [27]. This aspect could influence the modulation of the microbiota composition directly or indirectly, determining a shift in the metabolic adaptation to dietary sources typically associated with vegetable ingredients. Inferred metagenomic analysis, in fact, emphasized a change in the bile acid biosynthesis. Different studies reported a variable regulation of these pathways because of the partial or total replacement of marine ingredients in many fish species, including salmonids, grass carp (*Ctenopharyngodon idellus*), gilthead sea bream and spotted seabass (*Lateolabrax maculatus*) [68–72]. According to the present results, which describe a higher level of Firmicutes and the *Lactobacillus* genus associated with the VO + 0 group, these authors clearly indicated that bile salt hydrolases (BSHs) are widely present across most bacterial phyla, but especially in members of lactobacilli, Clostridium and Bacteroides, corroborating the idea that these taxa played an active role in these processes. Furthermore, the plant-based ingredients, compared to FM and FO, also determined broader changes which mostly include a higher carbohydrate intake, usually not easily digested by carnivorous fish, but also a lower level of phosphorus, which being bound to phytate is only partially available for fish assimilation [73]. These assumptions find strong confirmation in the microbiota's inferred functions. Fructose, mannose, amino and nucleotide sugar metabolism, together with PTS, indeed exhibited significantly higher values associated with the VO + 0 group. A similar trend has been reported in previous studies, which not only observed shifts in the microbiota profile associated with carbohydrate-rich diets [57], but also highlighted its potential role in mitigating the negative effects of thermal stress through alternative substrates metabolized by gut microbiota. Additionally, these studies emphasized that such variations can serve as distinctive features of intestinal bacteria among fish species occupying different ecological niches, as differences in dietary availability across niches strongly influence the baseline for comparing gut microbiota between species [74,75]. In light of this evidence, it is undeniable that the microbiota represents an excellent, sensitive indicator of the physiological and biochemical response of animals. Interestingly, associated with this, a high relative abundance of *Lactobacillus* described in the present results was also described by Guo and colleagues [76], who supported the hypothesis that this genus could essentially contribute to improved phosphorus bioavailability in the host–microbiota intestinal system. *Lactobacillus* is known to facilitate the absorption of small molecules and, in this context, it plays a pivotal role in the increased expression of PTS, triggered by the lower amount of biologically usable phosphorus [76,77].

Different functional patterns were instead obtained in association with the *S. limacinum* administration, despite the vegetable dietary fraction shared with the VO + 0 group. Several metabolic routes were particularly susceptible to the higher level of DHA present in the VO + 5 and 10 groups. Due to their high degree of unsaturation, n-3 FAs are highly prone to peroxidation, which can have strong negative repercussions on the organism's homeostasis due to oxidative stress and ROS production [78]. For this reason, the balancing of large quantities of DHA with antioxidant molecules and detoxification processes represents a unique strategy to maintain a dynamic but stable equilibrium and ensure optimal performance and health. In parallel to the potential host response, the inferred functional profiles suggested that microbial communities may also be involved in these processes. In particular, the significantly higher values of ubiquinone and other Terpenoid biosynthesis, together with geraniol degradation, emphasized the potential shift in the microbiota to enhanced antioxidant capacity in diets containing *S. limacinum*. Numerous studies corroborated these results, confirming the positive effects of this oleaginous marine protist on providing protection against oxidative damage using both enzymatic and non-enzymatic systems [79–81]. Among the latter, terpenoids represent an important source of sterols and steroid precursors, but they also include carotenoids, such as β-carotene, lycopene, and astaxanthin [82]. The presence of these molecules is not surprising, given that they are generally associated with algal inclusion and for this reason they are widely used both in aquaculture and in the food industry for their qualities [21,83]. In the present study, however, bacterial carotenoid biosynthesis may have played a relevant role due to its ability to buffer the potential oxidative stress caused by the high amount of DHA administered. The management and distribution of these n-3 molecules, in fact, has been shown to generate an increase in the activity of the mitochondrial respiratory chain, as confirmed by the increased activity reported for the NHEJ and lipoic acid metabolism pathways [84–86].

However, these functional inferences rely solely on 16S rRNA gene-based predictions and do not provide direct evidence of metabolic activity or host physiological responses. Likewise, the association between *Lactobacillus* abundance and pathways related to energy and nutrient metabolism may represent microbial adaptation to diet composition rather than direct effects on host oxidative status or resilience. Without direct measurements of biomarkers, antioxidant enzyme activity, or inflammatory parameters, the functional significance of these predicted pathways remains speculative.

Overall, these findings indicate that dietary inclusion of *S. limacinum* and vegetable oils can modulate gut microbiota composition and its predicted functional capacity. The observed shifts align with a potential role of microbial communities in nutrient metabolism and redox-related processes. Future studies integrating metagenomics, metabolomics, and direct assessments of host physiology are needed to validate these predictions and elucidate the mechanistic links between diet, microbiota, and host health.

5. Conclusions

The development of sustainable aquaculture requires the identification of innovative nutritional strategies that can limit the use and exploitation of marine-based ingredients, ensuring excellent growth performance and high-quality products. In this context, the results of the present study demonstrated how the combination of VOs and *S. limacinum* biomass, rich in DHA, represents an excellent supplement to a plant-based diet, which enhances the PUFA accumulation in the fillet and positively modulates the intestinal microbiota of sea bass. The analysis identified key bacterial genera belonging to LAB, especially the *Lactobacillus* genus, which have been shown to be abundant and metabolically decisive in mitigating the negative effects of oxidative stress and aligning the host physiological response to novel

dietary ingredients. However, since these functional insights are based on a 16S rRNA gene-based inference, their potential involvement in oxidative stress regulation and host physiological responses should be considered as preliminary and hypothesis-generating rather than conclusive. In light of these findings, future research should therefore integrate multi-omics approaches and direct measurements of host physiological and biochemical parameters to clarify the mechanistic links between diet, microbiota, and host health. Such studies will support the optimization of novel feed formulations that exploit the functional potential of gut microbiota to develop next-generation diets tailored to the digestive and metabolic traits of specific fish species. Lastly, this research contributes to the advancement of strategies that improve sustainability, enhance nutrient utilization efficiency, and minimize environmental impact across diverse aquaculture systems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes11030152/s1, Figure S1: Rarefaction curve of the microbiota samples of the four experimental groups (FO; VO + 0; VO + 5; VO + 10); Figure S2: Goodness of fit and validation plot of PLSDA shown in Figure 3; Figure S3: Stacked barplots representing the relative abundance (%) of the most important bacteria in the microbiota profiles of the four experimental groups at phylum (a), family (b), and genus (c) taxonomic level. Bacteria with lower abundance were pooled and indicated as "Others". Asterisks indicate significant differences ($p < 0.05$) between groups; Table S1: Results of Kruskal–Wallis and post hoc Dunn's test of the whole microbiota population (ASVs). The significative taxa were then used to perform the functional enrichment analysis using Picrust2; Table S2: The inferred metagenomic results highlighted a total of 173 metabolic pathways identified.

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