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Effect of Temperature on Microbial Diversity and Predictive Function in the Intestine of Olive Flounder (*Paralichthys olivaceus*)

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Abstract

While the impact of the environment on the fish microbiome remains under discussion, studies on the intestinal bacterial diversity of olive flounder are still limited. This study investigated the effects of three temperature conditions, 15 °C (Group L), 20 °C (Group M), and 25 °C (Group H), on the structure and diversity of the bacterial community in the gut of olive flounder. During the 35-day experiment, changes were observed in the composition and diversity of the gut microbiota, with alpha diversity estimates gradually decreasing as the temperature increased. At the species level, there were significant differences in the relative abundance of *Sphingomonas echinoides* and *Photobacterium damselae* between Groups L and H. According to LEfSe and correlation analyses, the relative abundances of *Gammaproteobacteria*, *Alphaproteobacteria*, *Sphingomonas*, and *Photobacterium* varied significantly depending on temperature levels. Through functional prediction, the predicted functional pathways based on bacterial abundances showed that those involved in aromatic compound degradation and amino acid metabolism were abundant in Group L, whereas pathogenic factors were abundant in Group H, suggesting a potential impact on host digestive and immune functions. These results suggest that temperature can significantly affect both the diversity and potential functional profiles of the intestinal microbiota in olive flounder.

Keywords: *Paralichthys olivaceus*; bacterial diversity; temperature; laboratory scale; circular consensus sequencing

Key Contribution: Temperature levels alter bacterial diversity and predictive functions in the intestine of olive flounder.

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1. Introduction

Paralichthys olivaceus, also known as olive flounder or Japanese flounder, has been extensively farmed commercially in East Asia since the 1990s [1]. In South Korea, olive flounder production accounts for approximately 30% of total farmed fish production. Therefore, the olive flounder is a species of significant economic value, and aquaculture strategies to improve its health and growth performance have been widely studied in South Korea [2].

Thermal stress is an environmental factor that has a strong effect on olive flounder [3]. It plays a crucial role in regulating fish physiology, behavior, immune response, and reproductive processes. While olive flounder exhibits resistance to wide temperature changes, acute temperature fluctuations can induce osmotic regulation and metabolic stress in flounder, which can result in adverse health effects [4,5]. In particular, elevated temperature stimulation weakens the immune system of flounder, increasing susceptibility to bacterial diseases and causing significant economic losses [6]. Global climate change is causing persistent increases in average water temperature and prolonged thermal fluctuations in aquatic environments. Dramatic environmental changes pose substantial challenges to aquaculture, which is highly impacted by aquatic environmental changes and disease outbreaks [7]. The annual mass mortality rate of flounder has also been steadily increasing each year, presumably due to the environmental changes [8]; therefore, temperature-driven mortality has become a recurring issue in the aquaculture industry [9].

Recently, research has increasingly focused on the gut microbiome as a pivotal factor influencing fish health, growth, and disease resistance [10]. The intestine serves as a major digestive and immune organ in fish, harboring numerous microorganisms, predominantly bacteria [11]. The intestinal bacterial community has a variable composition that is influenced by various environmental factors. The intestine is particularly sensitive to temperature stress [12], and water temperature can indirectly affect the composition of the intestinal bacterial community in fish. Intestinal symbiotic bacteria have co-evolved with their host [13] and carry more genes than the host's genome [14], contributing significantly to host functions. They help the host absorb nutrients by secreting various metabolites and biological enzymes that the host cannot produce [13]. The microbiome regulates energy metabolism to promote the absorption of more efficient energy from dietary nutrients [13], thereby influencing the host body weight. It also maintains the physical barrier functions of the intestine to prevent external infections and modulates host immune responses [15], as well as gut homeostasis, by secreting compounds with anti-inflammatory effects [16].

The impact of water temperature on host physiology and microbial communities has been well established [17,18]. While the diversity of intestinal bacterial composition in aquaculture fish species is also being actively discussed [19,20], studies focusing on microbiomes in olive flounder are still limited. Previous fish microbial community studies have been limited by applying culture-dependent techniques and culture-independent approaches [21–23]. Research has expanded to investigate microbial communities in flounder according to growth stages [24] and organ tissues [25]. Furthermore, the impact of dietary components including probiotics and supplements [26], as well as varying nutritional compositions [27], on the gut microbial structure has been extensively documented. More recently, studies have also focused on the shifts in the intestinal microbiota of olive flounder infected with pathogenic bacteria [20]. Despite these extensive studies regarding the microbiota in olive flounder, research has not been reported on the effects of temperature change on the bacterial composition and diversity. Studies on the changes in genetic richness of microbial communities in flounder in response to temperature stress and their presumed functional implications are also underexplored. Therefore, this study applied a CCS approach to improve bacterial species resolution in order to compare diversity and analyze the functional potential of the intestinal microbiota in olive flounder across different temperature conditions within a controlled laboratory aquaculture environment.

2. Materials and Methods

2.1. Aquaculture Conditions

Nine rearing tanks (70 L, 0.16 m^2) were used to stock the juvenile flounder (average weight of 14.8 ± 0.6 g). The juvenile fish used in this experiment were obtained

from a commercial flounder hatchery (34°43.3172′ N, 128°35.5448′ E, Jeogu Aquaculture, Geoje-si, Republic of Korea). Before the temperature experiment, the juveniles were treated with oxytetracycline (50 ppm) for 1 h to prevent potential bacterial infections and to adapt to laboratory conditions. Previous studies have demonstrated that following 1 h of exposure to 50 ppm OTC, blood OTC levels dropped to the residue limit of 0.2 ppm by 48 h and were no longer detectable by 240 h [28]. After 48 h of acclimation, 30 juveniles with an average weight of approximately 14.8 g were randomly divided into each tank. Thirty juvenile flounder were then stocked in each rearing tank and acclimated at 20 °C for a week. The fish in each tank were fed a commercial feed at 2% of their total body weight per day, divided into two equal feedings. To ensure consistent intake, feed was provided slowly over a 60 min period for each feeding. Any uneaten feed and feces were removed after the 30 min post-feeding window to maintain optimal water quality. It has been reported that physiological changes in olive flounder can be observed even with a temperature difference of at least 3 °C [29]. To observe more distinct changes based on the optimal temperature of 20 °C, this study was conducted using three temperature groups with a 5 °C interval. Three temperature conditions, 15 °C (low), 20 °C (medium, the optimum growth temperature for flounder), and 25 °C (high) were applied to three rearing tanks for each temperature group. The temperature was maintained at 20 °C for the medium-temperature group (hereafter referred to as Group M), or adjusted to 15 °C for the low-temperature group (Group L) and 25 °C for the high-temperature group (Group H). The water temperatures were adjusted by decreasing or increasing them by 1 °C per day until the target was reached. Water temperatures were maintained at 15, 20, and 25 ± 1 °C using heat pumps (DH-3010, Daeil, Busan, Republic of Korea) until the end of the experiment. To minimize thermal stress during the cooling process from 20 °C to 15 °C, the water temperature was gradually decreased using an automated heat pump system. The temperature was lowered by 1 °C per day, followed by a 24-hour observation period for the fish before the next 1 °C reduction was initiated. The seawater quality and commercial feed were kept identical across all experimental tanks. The fish were reared for 35 days after reaching the target temperature. After the temperature levels were set for each group, the fish were reared for 35 days, and seawater quality was maintained by pre-filtration and UV sterilization, with regular monitoring performed twice daily. The seawater quality parameters were maintained as follows: pH 8.2 to 8.4, dissolved oxygen 5.5 to 6.3 mg/L, salinity 29.5 to 31.5‰ (g/kg), and water flow at 1.2 L/min.

2.2. Sample Collection and DNA Extraction

After 35 days, ten individuals were randomly collected from each replicate tank. The fish were anesthetized with 2-phenoxyethanol and gut samples were aseptically collected using sterile scissors and forceps. The isolated intestines were squeezed and the gut contents were collected in sterile microtubes. We performed a one-way ANOVA to compare the growth performance among the experimental groups.

Gut content samples were individually processed to extract total genomic DNA using the EZNA® Soil DNA Extraction Kit (Omega, Norcross, GA, USA). Bead tubes containing 0.1 g of each sample were homogenized at 1500 rpm for 10 min using a Tissuelyser II (Qiagen, Hilden, Germany), followed by extraction according to the manufacturer's instructions. The quality and quantity of DNA were assessed using a Qubit Flex fluorometer (ThermoScientific, Waltham, MA, USA).

2.3. Library Preparation and Amplicon Sequencing

A 16S rRNA gene was amplified using a set of primers, 27F (AGRGTTYGATYMTGGCTCAG) and 1492R (RGYTACCTTGTTACGACTT) primers with a unique SequelII bar-

code for each sample. Amplification was performed using AccuPower Bioneer PCR premix (Bioneer, Daejeon, Republic of Korea) in a 20 μL reaction volume. A polymerase chain reaction (PCR) was conducted at initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 3 min. A total of 90 fish (30 per group) were initially sampled, and of these, 28 successfully amplified samples (n = 10 for Group L, n = 8 for Group M, and n = 10 for Group H) were used for NGS analysis. The successfully amplified PCR products were purified using the Qiagen PCR Product Purification Kit (Qiagen, Hilden, Germany), and pooled after normalization to an equal molar concentration. The final pooled amplicon library sample was then sequenced using the Pacific Biosciences (PacBio, Menlo, CA, USA) Sequel II CCS platform.

2.4. Diversity Analysis

Sequences containing barcode and adapter indices were removed using QIIME2 version 2026.1 [30]. Raw reads underwent initial quality assessment using the SeqKit (v2.10.0) program (https://bioinf.shenwei.me/seqkit, accessed on 18 May 2025). Sequence reads with a length less than 1200 bp or more than 1600 bp, or of low quality (<Q30), were filtered out using the DADA2 (v2026.1.0) pipeline. In addition, the chimera sequences were also removed using the DADA2 pipeline and contiguous sequences were aligned to obtain unique amplicon sequence variants (ASVs). Taxonomic classification and analysis were conducted using the Naïve-Bayes classifier based on the selected 16S SILVA database (v138.2). Mitochondria and chloroplast sequences, and ASVs with a sequence frequency lower than 10, were excluded from further analysis. Bacterial taxa with a relative abundance lower than the average 1% composition were indicated as "Others". A composition chart was visualized using QIIME2 view (https://view.qiime2.org, accessed on 3 September 2025). Alpha diversity, including observed features, ACE, Chao1, PD whole tree, and Shannon, were evaluated using QIIME2 and visualized using Statistical Analysis of Taxonomic and Functional Profiles (STAMP) software (v2.1.3, [31]). Beta diversity analysis was performed to identify differences in bacterial composition between groups using principal coordinate analysis (PCoA) plots based on the weighted and unweighted UniFrac metrics.

2.5. Sequence Annotation and Stastistical Analysis

Comparative analysis of statistical and functional differences between two different sample groups was conducted using bioinformatics tools, including STAMP and R Foundation for Statistical Computing (R, Vienna, Austria; v4.5.2). LEfSe analysis was conducted to identify taxa that significantly contributed to differences in relative abundance between groups. To compare the differential taxonomic abundance of ASVs, linear discriminant analysis (LDA) score-based LDA Effect Size (LEfSe) (v1.1.2) analysis was performed. The LDA log score cutoff for class discrimination was set to 3.0, and ANOVA and Wilcoxon tests were performed with an alpha value of 0.05. Pearson correlation analysis was performed using the corrplot (v0.95, [32]) and Hmisc (v5.2.5, [33]) packages in R software (v4.5.2). Statistical results with p-values less than 0.05 were considered significant. The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2; [34]) analysis was conducted using the picrust2 module (v2.6.3) to predict microbial function. To validate the accuracy of the functional predictions, the Nearest Sequenced Taxon Index (NSTI) was calculated for each sample. The mean NSTI value (indicating high predictive accuracy) across all samples was 0.07 ± 0.06. Predicted pathways were mapped to functional hierarchies (Levels 1, 2, and 3) by referencing the KEGG BRITE database using the KEGGREST package (v1.50.0) in R. Predictive functional profiles were compared using the extended error bar plot in STAMP software. Statistical significance between the groups was

determined using a two-sided Welch's t-test, and 95% confidence intervals were calculated using Welch's inverted method.

3. Results

3.1. Changes in Bacterial Diversity in Intestinal Region

During the 35-day temperature experiment, the flounder were reared collectively until they reached an average body weight of approximately 28.1 g. At the end of the experiment, the average body length and weight of Group L were 12.7 ± 0.6 cm and 21.8 ± 3 g, those of Group M were 14.5 ± 0.7 cm and 30.0 ± 4.4 g, and those of Group H were 14.2 ± 0.8 cm and 32.4 ± 6.1 g. Although the growth performance was lowest in Group L, no significant differences were observed between the other temperature groups ($p > 0.05$).

In total, 28 gut content samples were used for analysis, with 10, 8, and 10 samples from Groups L, M, and H, respectively. After quality filtering and denoising of reads, a total of 107 species were observed across all intestinal samples, with a mean sequence length of 1461 bp. Of these, 4 species were shared among all three groups, 22 species were shared between two groups, and 81 species were observed exclusively in individual groups. The highest number of taxa was observed in Group L, whereas the number of taxa was lowest in Group H.

The alpha diversity of bacterial communities in the intestine of flounder under different temperature conditions is shown in Figure 1. The alpha diversity metrics, including observed features (Figure 1a), Chao1 (Figure 1b), Shannon (Figure 1c), Faith's PD (Figure 1d), and evenness, showed different patterns depending on water temperature. In particular, significant differences were observed for all indices between Groups L and H. The observed features (22.6 ± 10.4); Chao1 index (26.6 ± 14.7), representing species richness; and Faith's PD index (4.4 ± 1.7) were highest in Group L, whereas the Shannon index (1.8 ± 0.9), representing species diversity, and evenness index (0.4 ± 0.2) were the lowest in this group. This result indicated that while Group L possessed a high number of bacterial taxa, the community was dominated by a few specific species, leading to lower overall evenness. Intriguingly, although the species richness (as indicated by observed features and Chao1) and phylogenetic diversity (Faith's PD index) were the lowest among all groups, Shannon and evenness (0.8) indices were the highest in Group H. This result demonstrated that while Group H had a lower number of bacterial taxa, these taxa were distributed with greater evenness compared to the other groups. In Group H, where the lowest indices were observed, these three values decreased by more than twofold compared to Group L. Alpha diversity, which is closely related to microbial homeostasis, showed a gradual decline toward Group H, suggesting that this trend is closely associated with a change in community stability.

The PCoA plots revealed that the bacterial communities were clustered according to the three temperature groups, with a clear separation observed between Groups L and H (Figure 2). Specifically, Axis 1 explained 77.7% of the total variation in the weighted PCoA plot (Figure 2b), suggesting that water temperature is a major factor in bacterial community shifts. Samples from Groups L and H formed distinctly independent clusters, while Group M showed a broader distribution. In both weighted and unweighted UniFrac analyses, samples within Group H exhibited tight clustering, indicating a high degree of bacterial community similarity within the group. Taken together, these results suggest that temperature leads to a more convergent and simplified microbial structure in the flounder intestine. To further investigate the differences in community structure, PERMANOVA analysis based on unweighted and weighted UniFrac distances revealed significant differences in the overall intestinal bacterial community structure among the three temperature groups ($p = 0.001$).

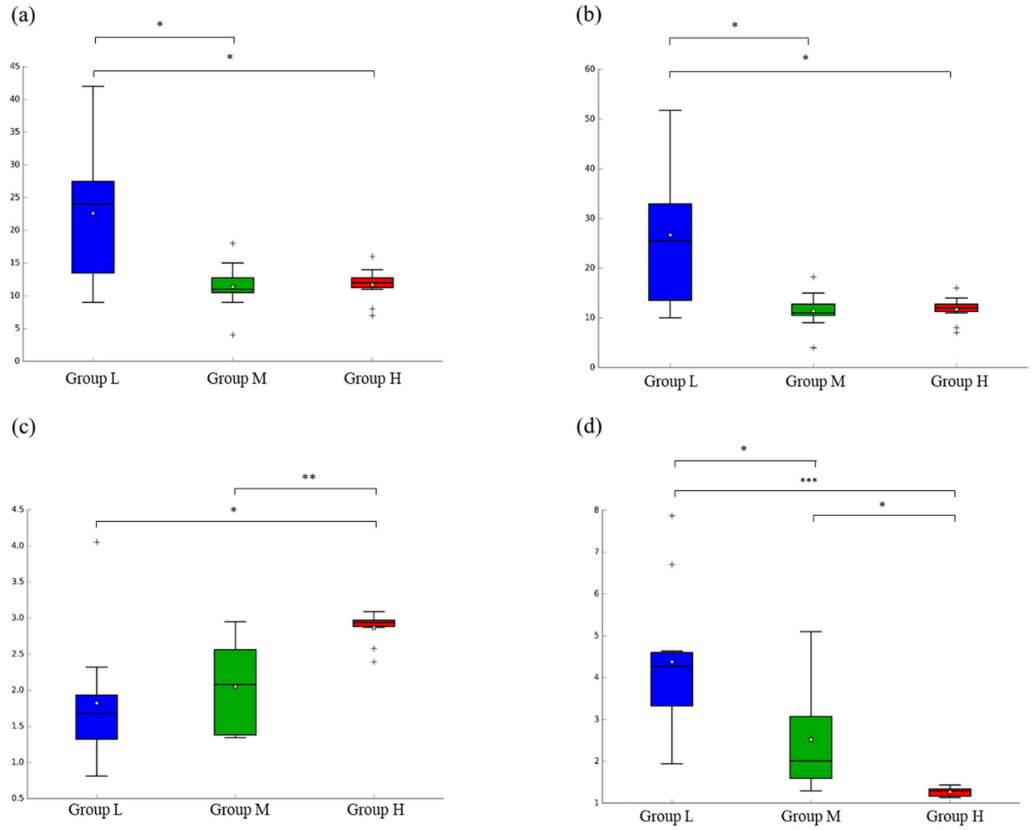

Figure 1. Alpha diversity index plots of the flounder intestinal bacterial community across the three temperature groups. (**a**) Observed features, (**b**) Chao1, (**c**) Shannon, and (**d**) Faith's PD estimates represented as box plots. Group L indicates blue, Group M indicates green, and Group H indicates red. The observed features and Chao1 indices represent species richness, the Shannon index represents diversity, and Faith's PD indicates phylogenetic diversity. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ represent significant differences between groups. + mark indicates an outlier.

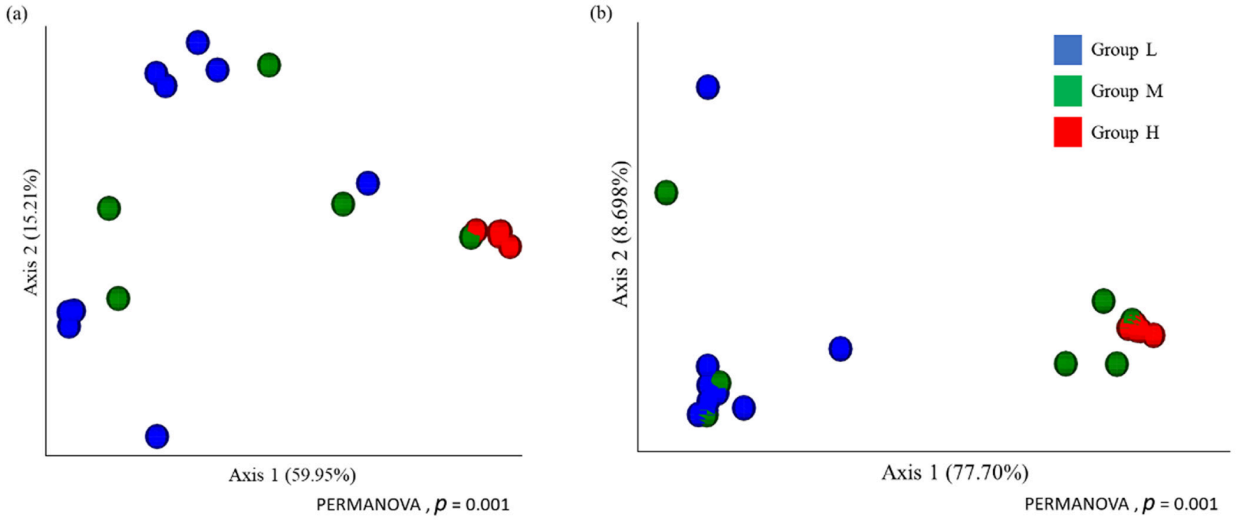

Figure 2. Beta diversity showing the changes in the composition of the intestinal bacterial community of flounder among the three temperature groups based on (**a**) unweighted UniFrac distance and (**b**) weighted Principal Coordinate Analysis (PCoA) plots. Each point in the graph represents a sample, with temperature groups distinguished by color. Group L indicates blue, Group M indicates green, and Group H indicates red. The distance matrix ratio along each axis is indicated as a percentage in parentheses. PERMANOVA, $p = 0.001$.

3.2. Taxonomic Composition of the Intestinal Microbiota

In this study, the bacterial community varied among all temperature groups. Taxonomic analysis revealed that the ASVs in flounder were classified into a total of 10 phyla across all temperature groups. The overall bacterial profile, including the top three phyla of samples from each group, is presented in Figure 3. At the phylum level, *Pseudomonadota* dominated all groups with a relative abundance of over 93.5%. *Bacillota*, the second most abundant phylum, had a relative abundance exceeding about 5.1% in Groups L and M, except for Group H. The ratio of *Pseudomonadota* to *Bacillota* was consistently below 93.0:6.0%, highlighting the distinct predominance of *Pseudomonadota*. Notably, the relative abundance of *Pseudomonadota* increased, whereas that of *Bacillota* decreased toward Group H. At the class level, all groups were dominated by *Gammaproteobacteria* except Group L. Contrary to other temperature groups, *Alphaproteobacteria* exhibited the highest relative abundance at 73.1% in Group L, which decreased markedly in the order of Groups M and H. In Group H, the proportion of *Gammaproteobacteria* was the highest at approximately 99.5%, while the combined abundance of *Bacilli* and *Alphaproteobacteria* was less than 0.4%, indicating a low distribution in Group H. *Bacilli* were observed at a relative abundance over 5.0% only, except in Group H. Toward Group H, the richness of *Gammaproteobacteria* increased, whereas that of *Alphaproteobacteria* decreased. At the species level, *Sphingomonas echinoides* was dominant in Group L, and *Photobacterium damselae* predominated in Group H, with an average relative abundance of 69.0% and 88.8% for each group, respectively. Additionally, the proportion of *Stenotrophomonas* sp. also decreased toward Group H. In Group M, the ASVs of *Aliivibrio finisterrensis*, *Staphylococcus* sp., *Stenotrophomonas* sp., and *Vibrio scophthalmi* each comprised approximately 5.0% of the bacterial community. These results demonstrated that Group M has a higher evenness compared to Group H, where a single species, *P. damselae*, was highly dominant.

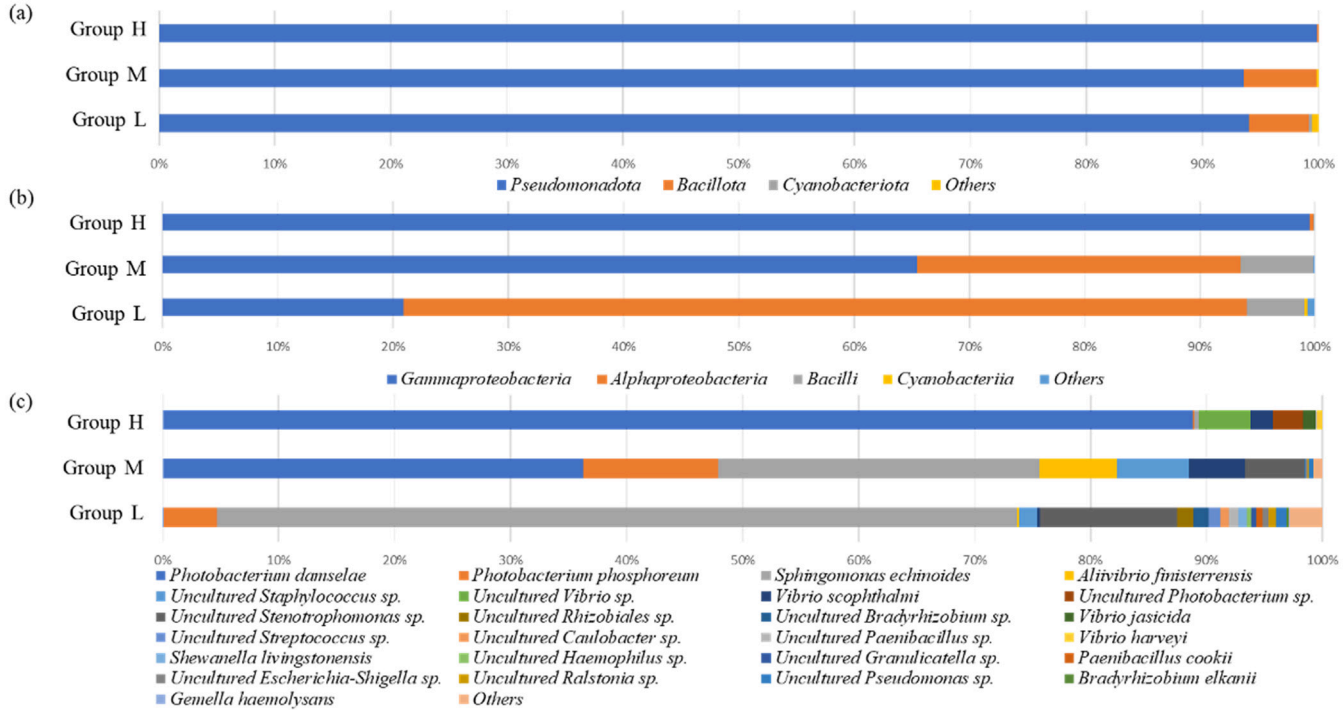

Figure 3. Bar plot showing the average taxonomic abundance of the flounder intestinal bacterial community in Groups H (n = 10), M (n = 8), and L (n = 10). The relative abundance (%) of the bacterial community according to temperatures is shown at the (**a**) phylum level, (**b**) class level, and (**c**) genus level. Each bar represents a group sample, with different colors indicating taxonomic units. Only bacterial taxa with a relative abundance of 1% or more are shown.

3.3. Differential Abundance and Correlation Analysis of Intestinal Microbiota Across Temperatures

According to the LEfSe analysis based on temperature (Figure 4), a total of 42 bacterial branches were identified, including 3 phyla, 4 classes, 11 orders, 13 families, and 11 genera. In Groups L and H, 31 and 5 phylotypes, respectively, were found to have an LDA threshold greater than 3, indicating significant differences ($p < 0.05$). At the class level, *Alphaproteobacteria* and *Actinobacteria* were notably abundant in Group L, while *Gammaproteobacteria* were in Group H. At the order level, *Sphingomonadales*, *Paenibacillales*, *Pseudomonadales*, and *Caulobacterales* were dominant in Group L, whereas *Enterobacterales* were in Group H. At the family level, *Sphingomonadaceae* were more abundant in Group L, while *Vibrionaceae* were in Group H. At the genus level, *Sphingomonas*, *Paenibacillus*, *Pseudomonas*, and *Ralstonia* were abundant in Group L, whereas *Photobacterium* was predominant in Group H. In addition, *Bacillota*, *Bacilli*, and *Bacillales* were abundant in Group M at the phylum, class, and order levels, respectively.

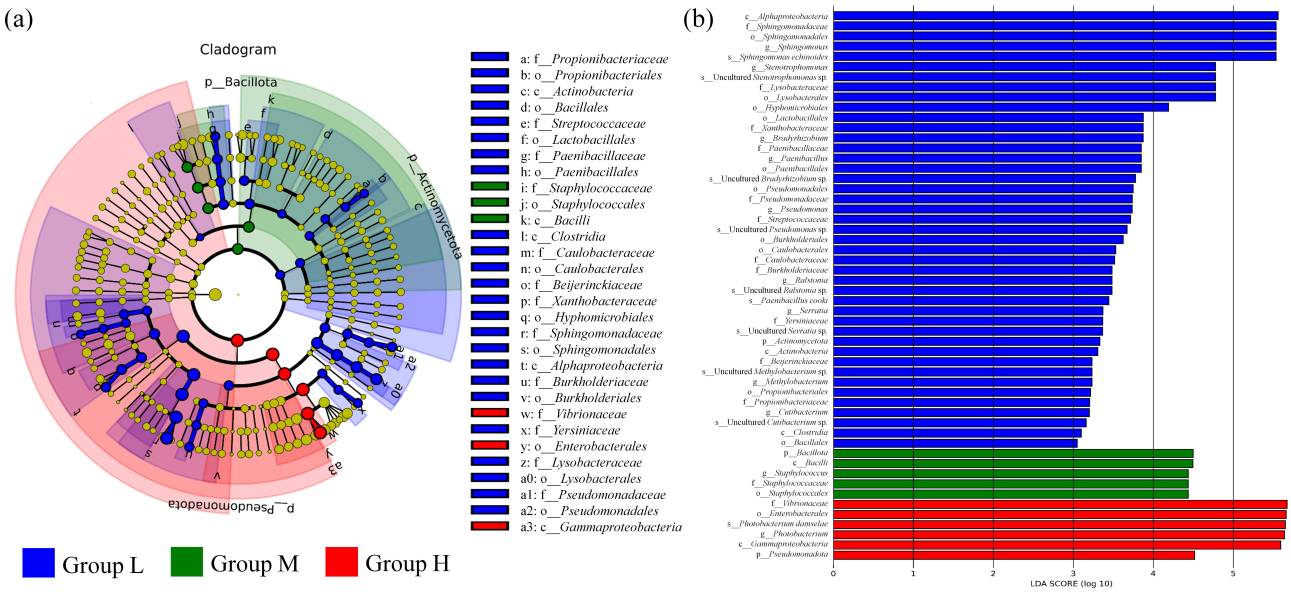

Figure 4. (**a**) Cladogram based on LEfSe analysis (LDA > 3.0, $p < 0.05$) and (**b**) bar graphs of the linear discriminant analysis (LDA) score (log10) of the flounder intestinal bacterial community in the three temperature groups. Taxa abundant according to temperature groups are shown for Group L (blue), Group M (green) and H (red). The color of the bar indicates the group with higher relative abundance. The relative proportion of phylotypes is indicated by the diameter of the circles. LEfSe analysis was performed across all three groups (L, M, and H) using the Kruskal-Wallis (KW) sum-rank test and the Wilcoxon rank-sum test.

Moreover, Pearson correlation analysis (Figure 5) clearly showed the correlations between temperature variables and bacterial taxa (>1% relative abundance). The analysis revealed that water temperature had a significant effect on the composition of intestinal microbiota. The thermal elevation in water showed a strong negative correlation with several taxa, including *Alphaproteobacteria*, *Stenotrophomonas* sp., *Ralstonia* sp., *Pseudomonas* sp., and *S. echinoides*. Conversely, *Gammaproteobacteria*, *P. damselae*, and *V. harveyi* had significant positive correlations with temperature. Lower temperature groups exhibited a strong positive correlation with common environmental or beneficial bacteria. However, these taxa, which significantly decreased toward Group H, were replaced by the proliferation of opportunistic pathogens. In addition, negative correlations were observed between *Pseudomonadota* and *Bacillota*, as well as between *Gammaproteobacteria* and *Alphaproteobacteria*. In contrast, positive correlations were observed between *Gammaproteobacteria* and *P. damselae*, as well as between *Alphaproteobacteria* and *S. echinoides*.

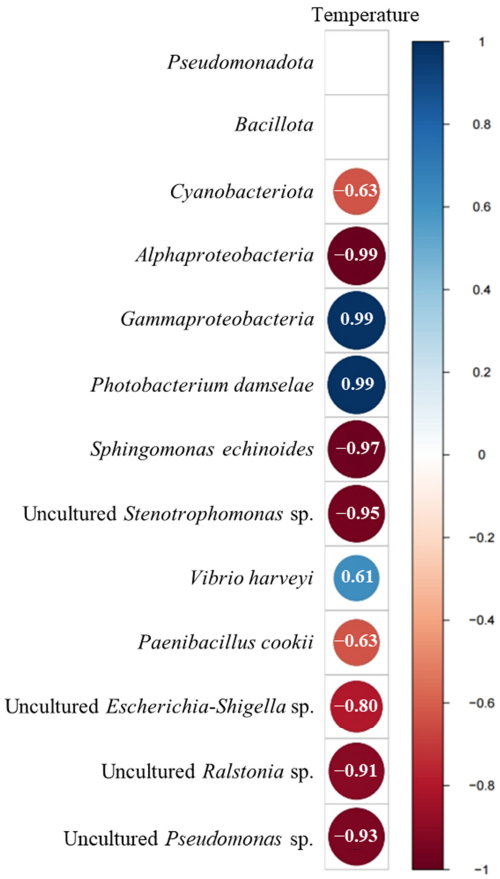

Figure 5. Corrplot of Pearson's *r* correlation matrix between bacterial abundance of dominant microbial taxa and temperature affected by water temperature. Positive correlations are shown in blue, while negative correlations are shown in red. Only statistically significant correlations ($p < 0.05$) are displayed. The correlation coefficients are represented by color density, circle size, and numbers within the dots.

3.4. Functional Prediction of Metabolic Pathways Across Temperatures

Through PICRUSt2 analysis, a total of 421 metabolic pathways, 2067 enzymes and 6110 genes were predicted. The metabolic pathways included 172 (40.9%) related to biosynthesis and 143 (34.0%) involved in biodegradation. The 37 and 25 KEGG pathways ($p < 0.05$) that were dominant in each group differed significantly between Groups L and H, respectively (Figure 6). At KEGG pathway level 3, the predicted functional profiles based on bacterial gene abundance indicated potential differences between the groups. Group L showed a higher abundance of predicted pathways associated with xenobiotics and organic pollutant degradation, degradation of carbohydrates, amino acids, and fatty acids, and the biosynthesis of secondary metabolites. In particular, putative functional genes involved in the degradation of xenobiotics, including chlorocyclohexane, chlorobenzene, fluorobenzoate, and bisphenol, were highly abundant. Furthermore, predicted pathways involved in carbohydrate metabolism, including starch, sucrose, and glyoxylate and dicarboxylate metabolism, and in amino acid metabolism, including valine, leucine, methionine, tryptophan, and phenylalanine, were found to be abundant. In addition, predictive microbial functions related to flavonoid and carotenoid biosynthesis were also abundant. Additionally, Group L has higher proportions of pathways related to beta-lactam antibiotic resistance, and the biosynthesis of penicillin, cephalosporin, butirosin, and neomycin. In contrast, Group H was predicted to exhibit higher abundance of pathways related to pathogenic factors and bacterial survival response. Potential functional genes involved in lipopolysaccharide (LPS) biosynthesis proteins were highly abundant. Specifically, the

predicted functions related to environmental sensing and intracellular transport, including the two-component system, and ABC transporters appeared to abundant. Additionally, potential functions associated with genetic information processing and cell growth such as DNA replication, ribosomal proteins, amino acid-related enzymes, and chaperones and folding catalysts were also abundant in Group H. In addition, putative functions related to the biosynthesis of fatty acids, including unsaturated fatty acids, and related to the metabolism of vitamins and cofactors, including terpenoid backbone biosynthesis, were also frequently observed.

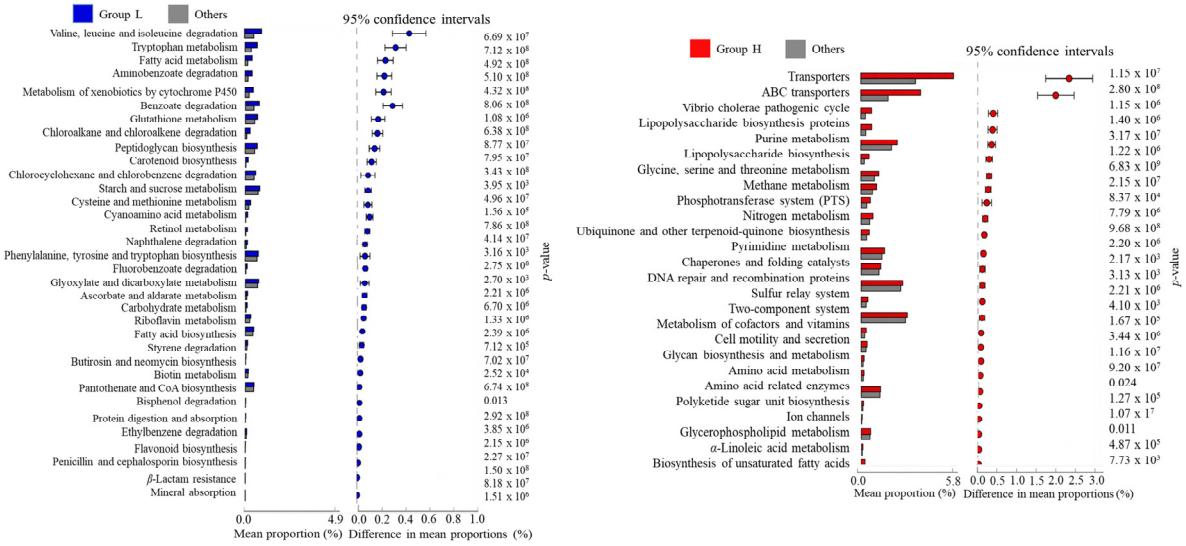

Figure 6. Extended error bar plot for the abundance of predicted metabolic pathways between the Groups L (blue) and H (red). Data are presented as mean proportions with 95% confidence intervals and sorted by effect size in descending order. Bars shown on the left side represent the mean proportion in each pathway. Error bars represent the standard deviations in each group. Corrected *p*-values are shown on the right and were calculated using STAMP software.

4. Discussion

Paralichthys olivaceus is a commercially important species in marine aquaculture. However, comprehensive studies on its bacterial community remain limited, and the impact of water temperature on its microbiota has not been fully explored. Understanding the functional characteristics of the intestinal microbiota is essential to elucidate its potential roles in host physiology, metabolism, and immune responses. In this study, PacBio CCS was utilized to profile the intestinal microbiota of flounder under different temperature conditions in a laboratory aquaculture environment, providing high-resolution analysis through full-length 16S rRNA sequencing. Furthermore, we identified the predicted metabolic impacts of the observed community shifts on the host, providing insights into the functional consequences of temperature-induced microbial changes.

Our results showed that temperature affected the composition and diversity of the intestinal microbiota, with distinct shifts in community diversity observed across groups (Figures 1 and 2). Alpha and beta diversity indices revealed that Group L was characterized by significantly higher species richness. Furthermore, the PCoA plot and Faith's PD metrics showed that Group L comprised a broader range of distantly related bacterial taxa, suggesting a more complex and stable community structure. This high diversity in Group L was further supported by a greater number of dominant bacterial species, indicating the formation of a robust and diverse fundamental microbiota. Overall, alpha diversity indices tended to decrease toward Group H. Specifically, Group H exhibited relatively low community richness but higher evenness, characterized by a more balanced

distribution of taxa with minimal compositional variance. Additionally, a distinct community comprising phylogenetically closely related taxa was observed in this group. This supports previous findings that a small number of bacteria colonize specific niches in the intestine and establish themselves as resident species [35]. Moreover, high-temperature stress reduces bacterial community diversity, including alpha diversity, in fish [36]. Generally, higher microbiome diversity [37] and greater alpha diversity [38] are considered beneficial to host health. In contrast, a lack of diversity can lead to microbial dysbiosis, and such disturbances can profoundly impact the host's physiological state [39,40]. Consequently, the observed shifts in diversity indicate significant quantitative differences in microbial composition between Groups L and H, suggesting that the taxonomic structure of the community is replaced depending on the water temperature.

In addition, distinct differences in the taxonomic composition of the intestinal microbiota were observed among the three temperature groups (Figure 3). The major phyla commonly found in the intestine of flounder were *Pseudomonadota* and *Bacillota*. These phyla are known to be predominant in common aquaculture fish [22,41]. These two major phyla are particularly associated with digestion and energy metabolism in fish. Members of *Pseudomonadota* are closely related to gut health, including fermenting complex carbohydrates [41,42]. *Bacillota* mainly encompass numerous probiotic strains that primarily support growth and immunity, contributing to disease resistance and health maintenance [43,44]. Additionally, our results support previous findings that as temperature increases, the relative abundance of *Pseudomonadota* increases, while that of *Bacillota* decreases significantly [45,46]. An increase in *Pseudomonadota* suggests microbial dysbiosis [47], and has been frequently observed in the gut of fish with intestinal inflammation [48]. At the genus level, *Sphingomonas* had a higher proportion in Group L, whereas *Photobacterium* was predominant in Group H. Both species are ubiquitous in marine environments and belong to opportunistic Gram-negative bacteria genera capable of rapidly establishing dominance within the gut [49]. The genus *Sphingomonas* is recognized as one of the core community members of fish microbiota [50] and is involved in the synthesis of immune-regulating molecules and nutrient processing [51–53]. Similarly, *Stenotrophomonas* sp., which was also abundant in Group L, possesses the ability to secrete enzymes that degrade complex compounds [54]. On the other hand, *Photobacterium* species exhibit strong adhesion to the gut and mucus of carnivorous fish, including olive flounder [55,56]. They can also act as part of both commensals and opportunistic fish pathogens [57]. At the species level, *S. echinoides* in Group L and *P. damselae* in Group H were particularly prominent, representing an average of 69.0% and 88.8% of the total abundance in each respective group. Specifically, *P. damselae*, dominant in Group H, is mainly found in tropical and subtropical environments and exhibits the ability to secrete digestion and pathogenic enzymes [58,59]. Prolonged temperature stress increased the abundance of potential opportunistic pathogens [60], including the genus *Vibrio* [61] and *Photobacterium* [62], which belong to the class *Gammaproteobacteria*. The direct stimulation of opportunistic pathogen proliferation by rising water temperatures indicates that thermal stress destabilizes microbiota, including commensal and beneficial bacteria. A reduced abundance of commensal bacteria, including *Pseudomonas*, toward Group H suggests that thermal conditions significantly impact the intestinal colonization of these beneficial taxa. This suggests that the fish gut is highly sensitive to changes in seawater temperature [12], and is presumed to be an environment where it is difficult for predominantly psychrophilic bacteria to establish dominance in thermal stress. The consequent dysbiosis likely weakens the physical barrier role of the gut microbiota in preventing pathogens and modulating immunity, allowing fast-growing *Vibrio* species to dominate the gut environment.

The most differentially abundant taxa between Groups L and H were observed at the class and genus levels in LEfSe analysis (Figure 4), and their correlation with temperature was confirmed (Figure 5). *Alphaproteobacteria*, *Sphingomonas*, *Paenibacillus*, *Pseudomonas*, and *Ralstonia* were identified as biomarkers in Group L, whereas *Gammaproteobacteria* and *Photobacterium* were abundant in Group H. As shown in the Group L results, an increase in *Alphaproteobacteria* is associated with a decrease in potential pathogens [52]. *Alphaproteobacteria*, which influences host immunity, may defend against pathogens by first occupying niche positions on the mucosa [63]. Specifically, high-temperature stress appears to have reduced the abundance of beneficial bacteria like *Bacillota* [36], which appeared as significant biomarkers in Group M, while promoting the expansion of *Pseudomonadota*, including potential pathogens. Such a reduction in *Bacillota* has been associated with intestinal functional impairment [45]. Among the dominant taxa observed in this study, the *Photobacterium* genus possesses physiological traits, such as a relatively high optimal growth temperature [64] and strong adhesion to the intestinal mucosa [54,55], that provide it with a competitive advantage over beneficial microbes that thrive at lower or moderate temperatures. Interestingly, despite the dominance of these potentially pathogenic species, including *P. damselae* and *V. harveyi*, in Group H, no disease-related mortality was observed during the experimental period. Although we observed potential signs of infection, such as intestinal hemorrhaging, in fish from Group H, the absence of mortality suggests that these fish may have been in a state of subclinical infection. A high abundance of potential pathogens has also been observed in other healthy fish [52]. Therefore, the increased abundance of *P. damselae* and *V. harveyi* in Group H could be interpreted as a potential response to thermal stress rather than definitive disease progression. To determine the actual effects of the excessive intestinal proliferation of opportunistic pathogens such as *P. damselae* due to thermal stress on the immune and antioxidant systems of olive flounder, further physiological and biochemical studies, including Lysozyme and SOD (superoxide dismutase) activity assays and related gene expression analyses, are necessary. However, further research is needed to fully confirm how the proliferation of these bacteria affects the health of the fish in long-term practice.

Based on the 16S rRNA bacterial taxonomic composition, PICRUSt2 was used to infer the potential metabolic capacity of the flounder intestinal microbiota indirectly (Figure 6). In Group L, predicted functional pathways involved in the degradation of aromatic compounds, organic pollutants and PAHs, as well as carbohydrate, amino acid, and fatty acid metabolism, were significantly abundant. The increased biological functions of the microbiota of these pollutant degradation systems provide the microbiota with additional carbon and energy sources under restricted environmental conditions [65]. The abundance of these pathways may be closely related to the predominance of *Pseudomonadota* and *Sphingomonas* [65–67], which possess the ability to degrade pollutants. These taxa act as biological filters to degrade residual pollutants from seawater, and contribute to bioremediation [67]. In addition, the increase in functions related to carbohydrate and amino acid metabolism, protein and mineral absorption, and fatty acid decomposition promotes gut health by providing essential energy sources, maintaining homeostatic stability, and reinforcing the intestinal barrier [68,69]. The intestinal microbiota can modulate the host's amino acid pool to facilitate digestion, absorption, and protein synthesis [70]. These functions that affect the content of fatty acids in the gut are likely related to the fatty acid storage capabilities of several *Bacillota* species [71]. Our results support that temperature itself, or changes in host metabolism, may continuously shape the intestinal populations of both *Bacillota* and *Pseudomonadota*. In contrast, Group H was characterized by an abundance of predicted functional pathways with pathogenic factors, including the *Vibrio cholerae* pathogenic cycle and lipopolysaccharide (LPS) biosynthesis.

As a major component of the Gram-negative outer membrane, LPS plays a crucial role in the bacterial adhesion process [72]. LPS is widely recognized for its endotoxicity and involvement in oxidative stress response mechanisms, which can trigger inflammation and mitochondrial damage in fish [13]. Furthermore, Group H exhibited higher proportions of predicted pathways involved in ABC transport associated with essential osmotic homeostasis, nutrient uptake, and peptide and cell signaling, which suggests an enhanced capacity for resistance to and export of endotoxicity in bacterial cells [73]. It should be noted that taxonomic assignment at the species level, particularly for the genera *Photobacterium* and *Vibrio*, remains challenging even with full-length 16S rRNA gene sequencing. While our analysis using PacBio CCS and the SILVA database identified these sequences as *P. damselae* and *Vibrio* sp., these assignments should be considered putative. Future studies using whole-genome sequencing or metagenomics may be required for definitive strain-level confirmation.

Functional differences in Group H, related to ABC transporters and the PTS system, were consistent with previous studies [74,75]. While Group L possessed health-promoting metabolic functions and robust immune defenses, thermal stress disrupted these beneficial pathways. Consequently, these changes in the predicted functions of the gut bacterial community due to temperature fluctuations may imply a fundamental shift in the intestinal community structure.

5. Conclusions

Our findings support that water temperature is crucial in determining the composition and functional capacity of the microbial community in the fish intestine, and these predicted functional shifts inherently reflect fundamental changes in the community structure. However, this is a predicted functional analysis inferred from the bacterial community at the experimental level, and the exact relationship between these metabolic functions and the actual gut microbiota of flounder has not yet been clearly elucidated. Additional analyses are required to understand the long-term effects of temperature variations on the physiology and metabolism of flounder and to clarify the exact roles and mechanisms of these metabolic processes in the microbiome.

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