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Thyroid-Active Agents Triiodothyronine, Thyroxine and Propylthiouracil Differentially Affect Growth, Intestinal Short Chain Fatty Acids and Microbiota in Little Yellow Croaker *Larimichthys polyactis*

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Abstract: Thyroid dysfunction may affect the intestinal microbiota through short-chain fatty acids (SCFAs) in marine fish. This study investigated the effects of triiodothyronine (T3, 20 ng/g) and thyroxine (T4, 20 ng/g), and propylthiouracil (PTU, 5000 ng/g) on growth performance, intestinal SCFA profiles, and microbiota composition in little yellow croakers *Larimichthys polyactis*. The results showed that dietary thyroid-active agent supplementation significantly decreased weight gain, and specific growth ratio. Moreover, dietary T3, T4, and PTU induced the states of hyperthyroidism, hyperthyroidism, and hypothyroidism, respectively, leading to differential alterations in intestinal SCFA profiles. Specifically, only dietary T4 supplementation significantly increased the diversity of intestinal microbiota. Our findings suggest that the genera *Vibrio* and *Sediminibacterium* play key roles in multiple metabolic pathways within the host intestine. Correlation analyses further indicated that intestinal acetic acid and isobutyric acid were characteristic metabolites involved in the alteration of the genus *Vibrio* abundance. These results provide a foundation for further investigation into the effects of thyroid-disrupting activities on growth, intestinal SCFA profiles, and microbiota composition in marine fish.

Keywords: thyroid; short-chain fatty acids; intestine; microbiota; little yellow croaker

Key Contribution: This study was conducted to investigate the effects of various dietary thyroid-active agents on growth performance, intestinal short chain fatty acids, and microbiota in little yellow croaker and to explore the possible correlations.

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1. Introduction

Thyroid hormones (THs) play a crucial role in regulating the growth, development, osmoregulation, and metabolic pathways of multiple organs in fish [1]. Previous studies have demonstrated that the thyroid status in fish can be modulated through the exogenous administration of drugs such as T3, T4, and PTU, which are used to investigate the effects of different thyroid functions on physiological processes. However, the underlying regulatory mechanisms of these effects in fish remain unclear [2,3]. Exogenous administration of T3 and PTU can induce hyperthyroidism and hypothyroidism in GH-transgenic coho salmon (*Oncorhynchus kitsutch*), respectively, leading to inverse effects on growth and skeletal abnormalities [4]. Both T3 and T4 have been shown to enhance the activity of

a-GPD and increase mitochondrial protein content in the liver and muscle of adult toads (*Bufo melanostictus*) [5]. Furthermore, T3 and PTU exert differential effects on thyroidal status in vivo, which in turn modulates various immune responses in the head kidney, spleen, and peripheral blood of rainbow trout (*Oncorhynchus mykiss*) [2,6]. Recent studies also indicate that THs regulate gastrointestinal physiological processes, such as intestinal development, nutrient absorption, and metabolism in mammals [7,8]. However, the role of THs in regulating gastrointestinal physiology in fish remains underexplored, and there is limited information regarding the potential immunomodulatory effects of THs in fish. Physiological processes in the gut are known to depend on the interactions between the gut microbiota and host metabolism [9]. The gut microbiota has been shown to perform functional organ-like roles, primarily residing on the mucosal surfaces of the host [10]. Studies have revealed that the composition of gut microbiota can affect energy absorption and the onset of diseases in the host through their metabolites [11–13]. The vagus nerve plays an essential role in the microbiota–gut–brain axis; specific bacteria can synthesize and release neuroactive substances to regulate the host behavior [14,15]. Recent research emphasizes that the microbiota–gut–brain axis functions as a bidirectional regulatory pathway [16–18]. These processes are mediated by immune and endocrine systems, including humoral and cellular regulation, hypothalamic–pituitary–adrenal (HPA) and hypothalamic–pituitary–thyroid (HPT) axes [19,20]. Many autoimmune disorders, like hypothyroidism and hyperthyroidism, have been linked to excessive microbial growth or alterations in microbiota composition [21,22], indicating that thyroid function may also interact with gut microbiota. Given the critical role of the thyroid in animal physiology, understanding the stability of thyroid function and its impact on gut microbiota is an important area of research. However, to date, studies on the effects of thyroid homeostasis on gut microbiota, and their potential crosstalk, particularly in fish, remain limited.

Microbial metabolites, particularly short-chain fatty acids (SCFAs), such as propionate, butyrate, and acetate, serve as important anions in the colonic lumen and can affect both the morphology and physiological functions of the gut [23]. SCFAs can enhance T3 metabolism and stimulate the secretion of prolactin [24]. Together, SCFAs and T3 work synergistically to maintain the development and homeostasis of the intestinal epithelium [25]. Furthermore, SCFAs may indirectly adjust thyroid function by affecting gut microbiota and immune function [26]. Recent studies have highlighted that SCFAs are involved in the crosstalk between thyroid function and gut microbial metabolism, contributing to the regulation of disease resistance in aquatic species [27–29]. However, few studies were focused on the effects of thyroid functions on SCFA metabolism and gut microbiota [29].

Little yellow croaker (*Larimichthys polyactis*), a widely cultured species in the coastal areas of southeastern China, is an economically significant marine fish in China, Korea, and Japan. Environmental factors in aquaculture can easily induce metabolic disorders in the digestive system of little yellow croaker through the endocrine system [30]. Investigating the interactions within the thyroid–gut microbiota axis may provide valuable insights into the regulation of gastrointestinal physiological homeostasis in fish. In the present study, we used thyroid-active agents, including T3, T4, and PTU, to induce varying states of thyroid dysfunction in little yellow croaker. The aim of this study was to explore the effects of thyroid dysfunction on the composition of the intestinal microbiota and SCFA metabolism. The results of this study will lay the foundation for a better understanding of the interplay between thyroid function and the intestinal microbiota in fish.

2. Materials and Methods

2.1. Animal Ethics

The fish experiments were conducted following the regulations of "Guidelines for Experimental Animals" of the Ministry of Science and Technology (Beijing, China), which was approved by the Committee of Laboratory Animal Experimentation at Zhejiang Academy of Agricultural Sciences (Permit Number: 2023ZAASLA29). The scientists in charge of the experiments received training and personal authorization.

2.2. Diets

The experimental diets were labeled as Control (Con), T3, T4, and PTU. The commercial diets (45% crude protein, 12% crude lipid) were purchased from the Fuzhou Haima Feed Co., Ltd., (Fuzhou, China), and commercial diets were supplemented with these thyroid-active agents to prepare four experimental diets following standard protocols. Based on previous studies [2,3], the concentrations of T3, T4, and PTU were selected, and selected concentrations of T3, T4, and PTU were 20 ng/g, 20 ng/g, and 5 mg/g, respectively. Referring to the methods in a previous study [4], T3, T4, and PTU were prepared as a 99% alkaline ethanol solution (20 mL 99% ethanol + 0.4 mL 0.1 N NaOH) and uniformly applied to feed pellets via spray-coating, with subsequent oven-drying at 60 °C for solvent removal. The control group underwent identical processing steps with ethanol application but lacked the pharmacological additives.

2.3. Fish Feeding Experiment

Little yellow croakers were obtained from the Xiangshan harbor aquatic seedling Co., Ltd. (Xiangshan County, Ningbo, China). Prior to the feeding trial, these fish were acclimated to the experimental diet and conditions by being stocked in indoor cage tanks for 14 days. The healthy fish (initial weight 23.14 ± 1.08 g) were randomly assigned to 12 laboratory tanks (200 L), with 10 individuals in each tank. Each diet was assigned to triplicate tanks. Fish were hand-fed twice daily to apparent satiation (05:00 and 17:00), and the amount of diet consumption in each net cage was recorded daily. The feeding trial lasted for 28 days, during which the water temperature ranged from 28.0 to 30.5 °C, salinity ranged from 24.2‰ to 25.7‰, and dissolved oxygen was not less than 8.0 mg/L.

2.4. Sample Collection and Zootechnical Parameters

At the end of the feeding experiment, all fish in each tank were anesthetized with MS-222 (Sigma Diagnostics INS, St. Louis, MO, USA) at a concentration of 100 mg/L and weighed. A total of six fish from each group (two fish per tank) were randomly selected for serum collection. Blood was collected from these fish, and serum was separated via centrifugation (1500× *g*, 4 °C, 30 min), with the individual serum samples pooled into a single tube. The pooled serum was stored at −80 °C until further analysis. Subsequently, the posterior intestinal content from the 6 fish per group were ground in liquid N_2 and pooled into a tube. These samples were stored at −80 °C for further determination of SCFA profiles and intestinal microbiota.

Zootechnical parameters such as weight gain (WG), and specific growth rate (SGR) were calculated using the formulae described below.

Weight gain (WG, %) = 100 × (final body weight (g) − initial body weight (g))/initial body weight (g).

Specific growth ratio (SGR, % day^{-1}) = 100 × (Ln (final body weight) − Ln (initial body weight))/t (culture period).

2.5. Serum Biochemical Analysis

Serum T3 and T4 levels were measured via radioimmunoassay (RIA) using commercial kits (Beijing North Institute of Biotechnology, China). The detailed method for measuring was described in our previous study [31]. T3/T4 ratios were calculated for each individual.

2.6. Intestinal SCFA Quantitative Analysis

The intestinal SCFA content was determined by GC-MS (Gas Chromatography–Mass Spectrometer), and the protocol was described in a previous study [32].

2.7. Analysis of Intestinal Microbiota

The E.Z.N.A.® Soil DNA kit (Omega Biotek, Norcross, GA, USA) was used to extract microbial DNA. The extracted bacterial DNA was sent for sequencing of the V1–V9 regions of the 16S rRNA gene on dedicated PacBio Sequel cells at Shanghai Biozeron Biotechnology Co., Ltd. (Shanghai, China). After sequencing, raw reads were processed through the SMRT Portal, and OTU clustering and species annotation were performed using UPARSE (version 7.1 http://drive5.com/uparse/, accessed on 15 July 2020). Then, analyses of alpha and beta diversity, and Tac4Fun and BugBase analyses were conducted. Related manufacturers' protocols for the above were described in a previous study [33].

2.8. Statistical Analyses

Statistical analysis was performed with SPSS 24.0 (IBM, New York, NY, USA) using one-way ANOVA. For biochemical analyses in serum, intestinal SCFA content, and microbial profiles, statistical comparisons on the measured variables among the dietary treatments were investigated by Tukey's post hoc test. The level of $p < 0.05$ was considered significant and all values expressed as mean ± standard error (SD). The principal coordinates analysis (PCoA), redundancy analysis (RDA), and correlations among the variables assessed with Spearman's correlation and other graphs used the OmicShare tools, a free online platform for data analysis and charting (http://www.omicshare.com/tools, accessed on 30 April 2024).

3. Results

3.1. Growth Performance

The growth performance data are presented in Table 1. Compared with the control (Con) group, the indexes of final weight, weight gain (WG), and specific growth rate (SGR) were significantly lower in all treatment groups ($p < 0.05$). Additionally, the lowest final weight, WG, and SGR were observed in the T4 group ($p < 0.05$). The feed consumption in the Con and T3 groups was significantly higher than those in the T4 and PTU groups ($p < 0.05$).

Table 1. Growth performance, feed utilization, and morphologic index of little yellow croakers fed thyroid-active agents.

Index	Groups			
	Con	T3	T4	PTU
Initial weight	23.14 ± 1.08	23.14 ± 1.08	23.14 ± 1.08	23.14 ± 1.08
Final weight	34.21 ± 0.82 ^{a}	30.32 ± 0.19 ^{b}	28.02 ± 0.20 ^{c}	30.55 ± 0.14 ^{b}
WG (%)	47.84 ± 3.53 ^{a}	31.04 ± 0.82 ^{b}	21.10 ± 0.85 ^{c}	32.01 ± 0.62 ^{b}
SGR (% day^{-1})	1.40 ± 0.09 ^{a}	0.97 ± 0.02 ^{b}	0.68 ± 0.03 ^{c}	0.99 ± 0.02 ^{b}
Feed consumption	270.62 ± 4.74 ^{a}	274.92 ± 2.96 ^{a}	241.88 ± 3.53 ^{b}	249.77 ± 9.97 ^{b}

Note: Values with different superscripts in the same line are significantly different ($p < 0.05$).

3.2. Serum Thyroid Hormone Content

The levels of serum TH are presented in Figure 1. Compared with the Con group, both T3 content and the T3/T4 ratio were significantly higher in the T3 group, while T3 content and the T3/T4 ratio were significantly lower in the PTU group, followed by the T4 group ($p < 0.05$). Moreover, the levels of T4 in the Con group were significantly higher than those in the T3 group, but significantly lower than those in the T4 and PTU groups ($p < 0.05$).

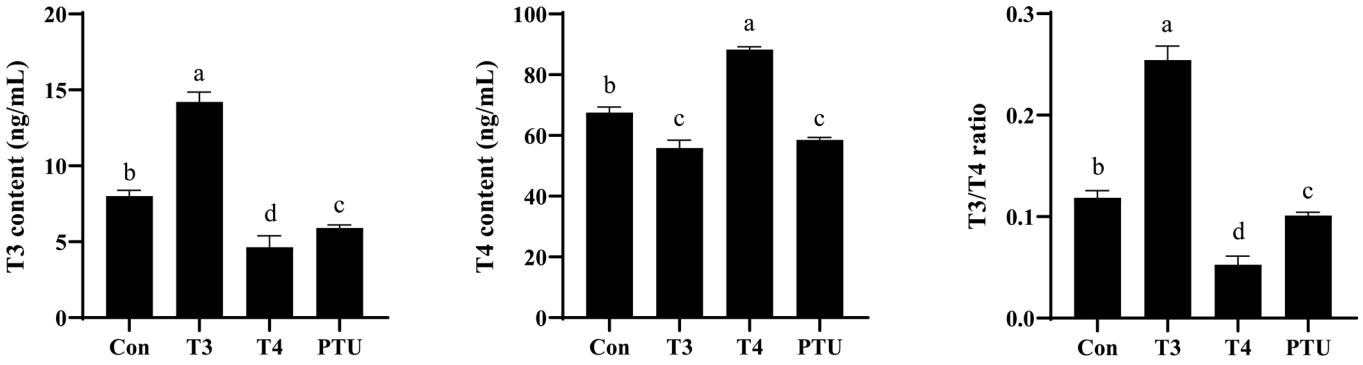

Figure 1. Serum concentrations (ng/mL) of circulating total (T3, A), total T4 (T4, B), and the ratio of total T3 to T4 (T3/T4, C) of little yellow croakers fed various thyroid-active agents. The values are expressed as mean ± SD (N = 6). Different little letters indicate significant differences among all the groups ($p < 0.05$).

3.3. Intestinal SCFA Profiles

The UHPLC-MS/MS data revealed the detection of seven SCFAs in all treatment groups (Figure 2A–G). Acetic acid (AA) was the predominant compound among BAs found in the posterior intestine of the little yellow croaker. Compared to the Con group, significantly higher levels of AA were observed in the T3 and T4 groups ($p < 0.05$), and there were no significant differences in AA content between the Con group and the PTU group ($p > 0.05$) (Figure 2A). The highest levels of butyric acid (BA) were shown in the Con group, followed by the T3 group, while the T4 and PTU groups exhibited the lowest BA levels ($p < 0.05$) (Figure 2B). The caproic acid (CA) content in the Con group was significantly lower than that in the T3 group ($p < 0.05$), but there were no significant differences between the T4/PTU group and the Con group ($p > 0.05$) (Figure 2C). There were no significant differences in the isobutyric acid (IBA) between the T3/PTU group and the Con group ($p > 0.05$), but the IBA content in the T4 group was significantly down-regulated ($p < 0.05$) (Figure 2D). A similar trend of changes in valeric acid (VA) content is observed in Figure 2E. Furthermore, isovaleric acid (IVA) content was significantly higher in both the Con and T3 groups compared to the T4 and PTU groups ($p < 0.05$) (Figure 2F). Finally, different thyroid-active agents did not affect the propionic acid (PA) content ($p > 0.05$) (Figure 2G).

3.4. Composition and Diversity of Intestinal Microbiota

Community richness and diversity were assessed using the Chao1, ACE, Shannon, and Simpson indices across different treatments (Figure 3A). The Shannon and Simpson indices in the T4 group were significantly higher in the PBO groups ($p < 0.05$), whereas no significant differences were observed in the ACE and Chao1 indices between the groups ($p < 0.05$). The up-regulated Shannon and Simpson indices in the PBO groups indicated that dietary T4 supplementation improved the diversity of the microbiota. PCoA analysis based on the Bray–Curtis distances revealed a distinct separation in microbiota composition between the Con and T4 groups, while the Con, T3, and PTU groups exhibited similar microbial profiles (Figure 3B).

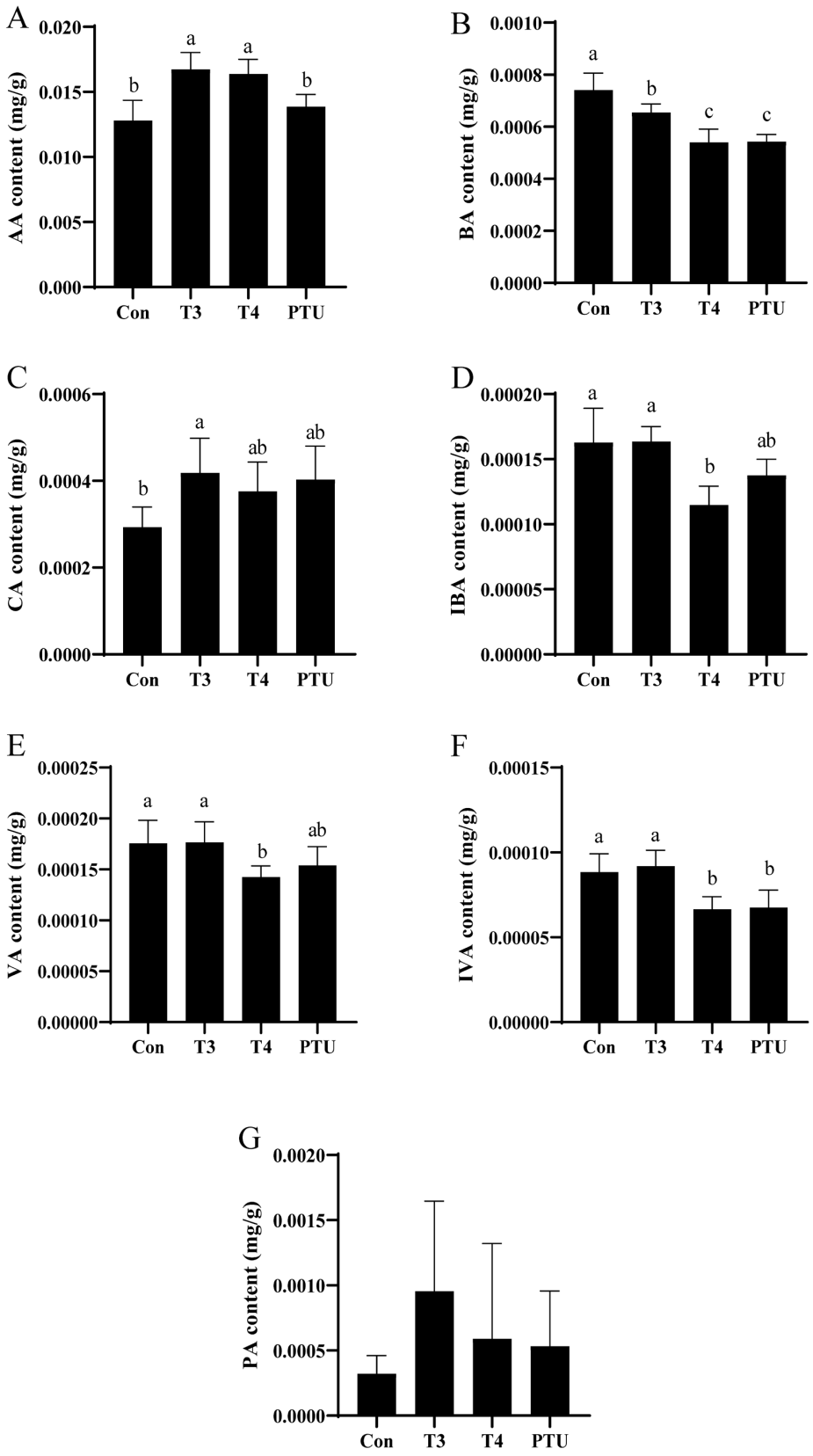

Figure 2. Significantly different content of various SCFAs (ng/g) in the intestine of little yellow croakers fed various thyroid-active agents. (**A**) acetic acid content; (**B**) butyric acid content; (**C**) caproic acid content; (**D**) isobutyric acid content; (**E**) valeric acid content; (**F**) isovaleric acid content; (**G**) propionic acid content. The values are expressed as mean ± SD (N = 6). Different letters indicate significant differences among all the groups ($p < 0.05$).

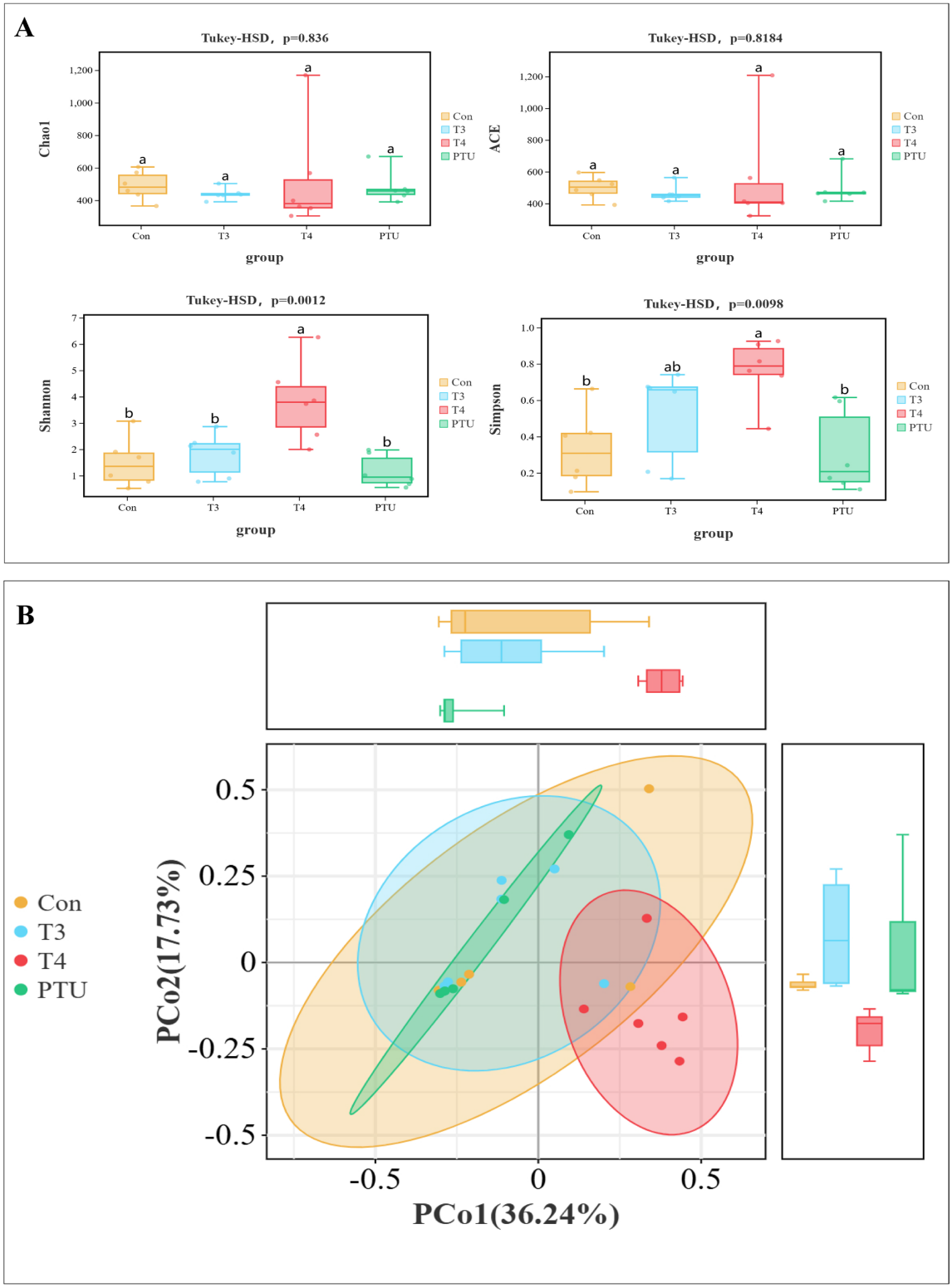

Figure 3. The α diversity index (**A**) and β diversity index (**B**) of gut microbiota. (**A**) The α diversity index contains Chao1, ACE, Shannon, and Simpson indices. (**B**) The β diversity index is revealed by principal coordinates analysis (PCoA) of gut microbiota. Different letters indicate significant differences among all the groups ($p < 0.05$).

The Tukey HSD test was applied to analyze the relative abundance at the phylum and genus levels. At the phylum level (Figure 4A), Proteobacteria and Firmicutes were identified as the dominant phyla in the gut of little yellow croakers. At the genus level (Figure 4B), *Vibrio* and *Acinetobacter* were the predominant genera. Further Lefse analysis

identified *Vibrio* and *Sediminibacterium* as biomarkers for distinguishing the T4 group from the other treatments (LDA = 3.5) (Figure 4C).

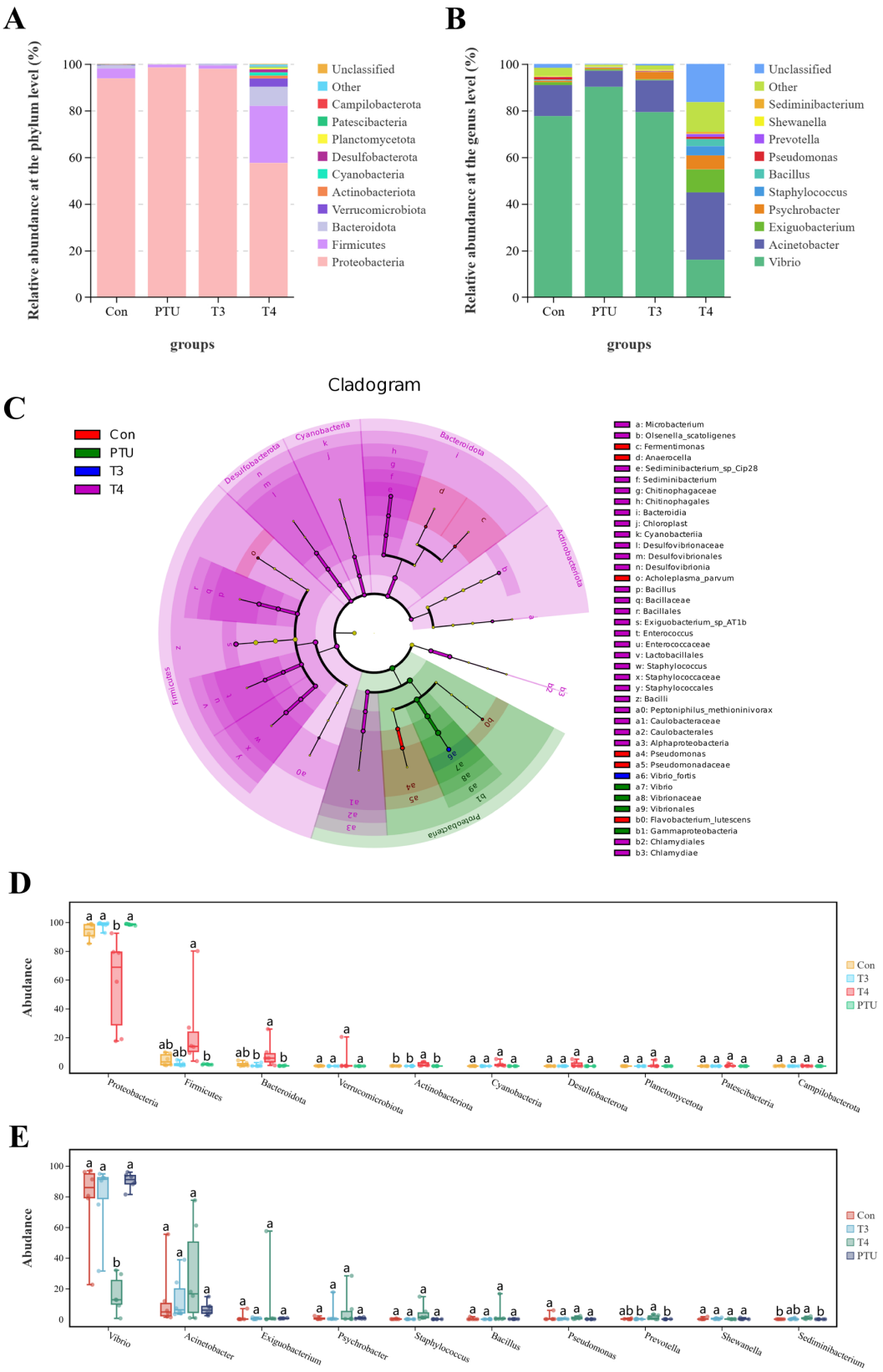

Figure 4. The relative abundance of the intestinal microbial communities in little yellow croakers fed various thyroid-active agents. (**A**) Column chart of the top 10 species in relative abundance at the phylum level. (**B**) Column chart of the top 10 species in relative abundance at the genus level. (**C**) Analysis of

bacterial taxa was identified via LEfSe, and the species with an LDA SCORE > 3.5 were defined as statistically different biomarkers. Intergroup variation in the relative abundance of the intestinal microbial communities at the phylum (**D**) and species (**E**) levels, and different letters indicate significant differences among all the groups ($p < 0.05$).

The top 10 relative abundance of bacterial phyla in little yellow croakers from the four experimental groups are shown in Figure 4D. Compared with the Con group, only the dietary T4 supplement significantly reduced the abundance of Proteobacteria, while it notably increased the abundance of Actinobacteriota ($p < 0.05$). The abundance of Firmicutes in the T4 group was significantly higher than that in the PTU group ($p < 0.05$), and the abundance of Bacteroidota in the T4 group was significantly higher than that in the T3 and PTU groups ($p < 0.05$). The top 10 relative abundance of bacterial genus in little yellow croakers from the four groups are shown in Figure 4E. In comparison to the Con group, only the dietary T4 supplement significantly decreased the abundance of *Vibrio*, while significantly increasing the abundance of *Sediminibacterium* ($p < 0.05$). Furthermore, the abundance of *Prevotella* in the T4 group was significantly higher than that in the T3 and PTU groups ($p < 0.05$).

3.5. Redundancy Analysis and Correlation Coefficients Among the Parameters

To elucidate the relationship between various parameters, the RDA was conducted using THs as explanatory variables and intestinal microbiota abundance at the genus level as response variables (Figure 5A). The first two axes cumulatively explained 100% of the variation in the microbial community structure. Combined with the arrow lines of T3 and T4, these results clearly showed that the T4 was positively correlated with the microbial profile in the T4 group, whereas T3 was positively correlated with the microbial profile in the other groups. Furthermore, the Spearman correlation analyses were used to further investigate the significant correlation between TH profiles and intestinal microbiota abundance (Figure 5B). The genera of *Staphylococcus*, *Bacillus*, *Sediminibacterium*, and *Enterococcus* were clustered together, with their abundance showing a significantly negative correlation with T3 content and a significantly positive correlation with T4 content ($p < 0.05$). In contrast, the abundances of *Vibrio* and *Cobetia* were significantly positively correlated with T3 content, but the abundance of the *Vibrio* genus was significantly negatively correlated with T4 content ($p < 0.05$).

Another RDA was used to further investigate the relationship between intestinal SCFA content and intestinal microbiota abundance at the genus level (Figure 5C). The first two axes cumulatively explained 94.72% of the variation in the microbial community structure. Combined with the length of the arrow lines, the AA was positively correlated with the microbial profile in the T4 group, while other SCFAs were positively correlated with the microbial profile in the other groups. Moreover, correlation analyses were conducted to explore the significant correlation between SCFA profiles and intestinal microbiota abundance (Figure 5D). It was investigated that the abundance of the *Vibrio* genus was significantly negatively correlated with AA content, but significantly positively correlated with IBA content ($p < 0.05$). The abundances of *Staphylococcus*, *Bacillus*, *Sediminibacterium*, and *Enterococcus* were negatively correlated with BA, IVA, VA, and IBA content. The abundance of *Sediminibacterium* was significantly negatively correlated with IBA content ($p < 0.05$).

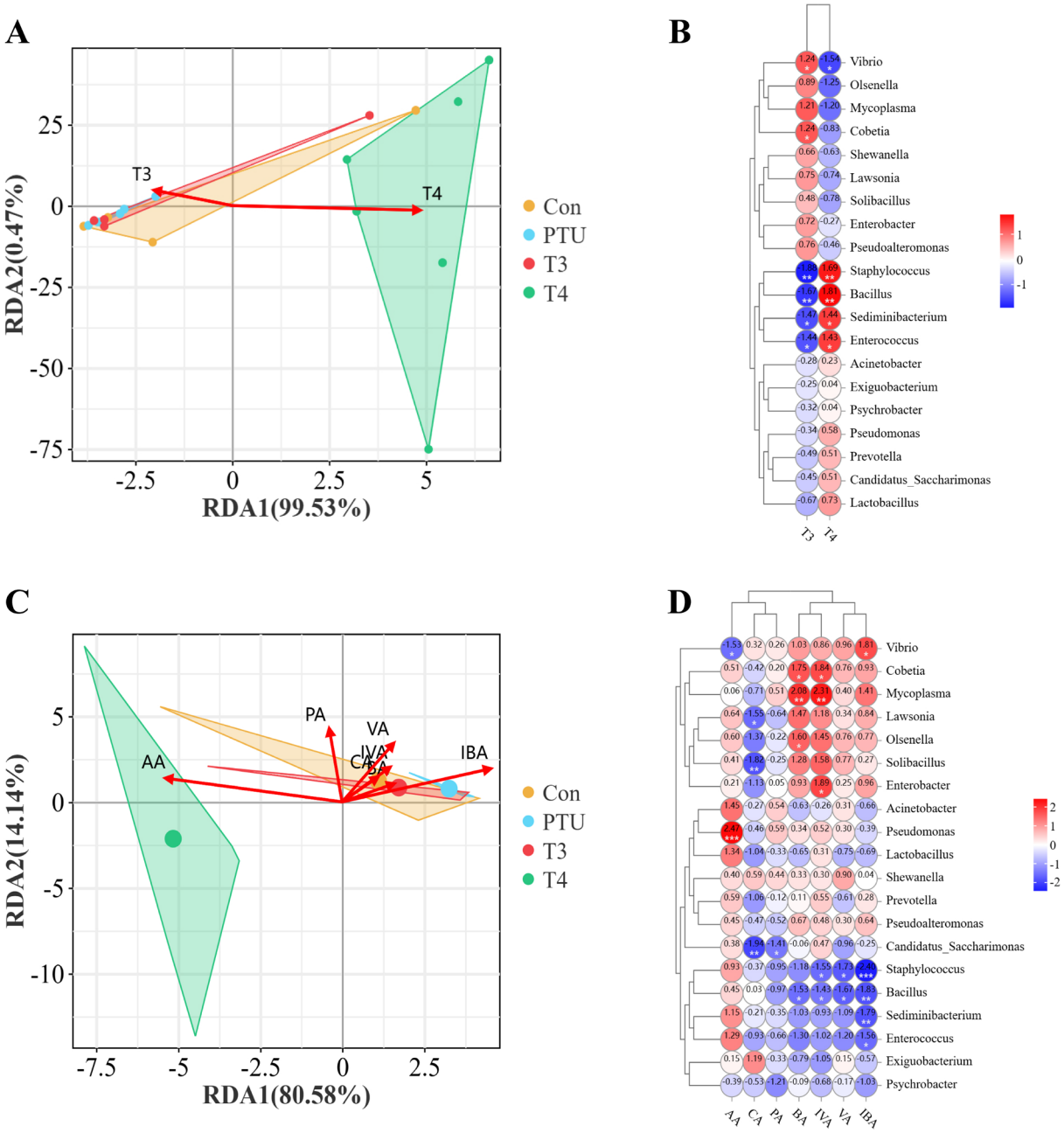

Figure 5. RDA plots showing factors that significantly explain the variance in the microbiota composition. (**A**) RDA of TH levels and relative abundance of gut microbiota at the genus level. (**B**) RDA of SCFA levels and relative abundance of gut microbiota at the genus level. (**C**) Heatmap representation of the Spearman correlation coefficient between TH levels and relative abundance of the top 20 predominant genera. (**D**) Heatmap representation of the Spearman correlation coefficient between SCFA levels and relative abundance of the top 20 predominant genera. Red represents a positive correlation, and blue represents a negative correlation.

3.6. Functional Prediction

The microbial function pathways predicted with the Tax4Fun tool in the T4 group were significantly different from those observed in the other groups (Figure 6A). Compared with the Con group, the T4 group exhibited lower abundances of bacteria related to membrane transport and signal transduction, but higher abundances of bacteria associated with amino acid metabolism, energy metabolism, replication and repair, translation, and lipid metabolism ($p < 0.05$) (Figure 6C).

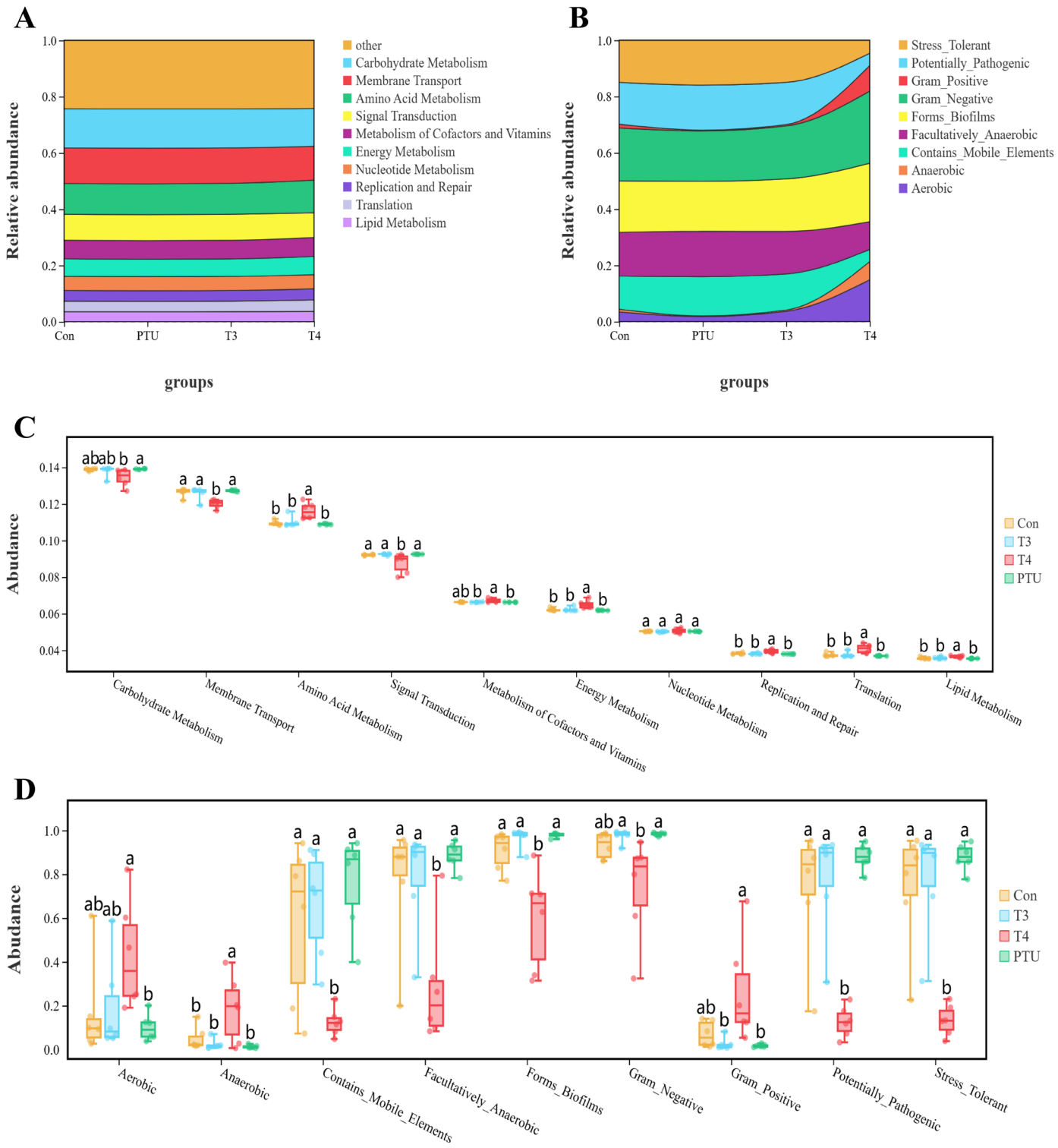

Figure 6. (**A**,**C**) Tax4Fun analysis results of predicted functional pathways in gut microbiota of little yellow croakers fed various thyroid-active agents. (**B**,**D**) BugBase analysis results of predicted bacterial phenotypic classifications of gut microbiota in little yellow croakers fed various thyroid-active agents. Different letters indicate significant differences among all the groups ($p < 0.05$).

The bacterial phenotypic classifications predicted with the BugBase tool in the T4 group also differed markedly from those in the other groups (Figure 6B). Compared with the Con group, the T4 group showed higher abundances of anaerobic bacteria, and lower abundances of bacteria related to the values of contains_mobile_elemants, facul-

tatively_anaerobic, forms_biofilms, potentially_pathogenic, and stress tolerant ($p < 0.05$) (Figure 6D).

4. Discussion

Administration of THs or other thyroactive preparations to fish can alter body growth [34]. In coho salmon (*O. kisutch*), dietary T3 (12 μg/g) was found to increase the growth index, whereas dietary PTU (6 mg/g) resulted in a decrease [34]. Moreover, exposure to T4 (10 nM) for 30 days did not affect WG, SGR, and FER in red tilapia (*Oreochromis mossambicus* × *O. urolepis hornorum*), but a 60-day exposure led to significant increases in WG, SGR, and FBW [35]. Our study revealed that administration of T3, T4, and PTU significantly decreased WGand SGR in the little yellow croakers. Despite these findings, relatively few studies have focused on the effects of thyroid-active agents on growth performance in fish.

Based on previous studies in humans, studies have employed THs or anti-thyroid drugs to induce the status of hyper- or hypothyroidism through disruption of the thyroid axis [36,37]. PTU, a common anti-thyroid drug, is employed to induce hypothyroidism [3,38]. In tilapia (*O. mossambicus*), plasma T3 and T4 levels were significantly down-regulated following PTU feeding (20 μg/g) for 15 days [38]. Similarly, a higher dose of PTU (1.5 or 6 mg/g) administered to coho salmon (*O. kisutch*) for 15 days also led to significant reductions in plasma T3 and T4 concentrations [34]. In line with these findings, our study demonstrated that feeding PTU (5 mg/g) to little yellow croakers for 28 days significantly decreased serum T3 and T4 levels, which could indicate hypothyroidism. On the other hand, both plasma T3 and T4 levels in Nile tilapia (*Oreochromis niloticus*) showed no significant changes after feeding T3 (48 μg/g) or T4 (48 μg/g) for 28 days [3]. In contrast to previous studies, feeding a dose of T3 or T4 (20 μg/g) for 28 days resulted in a significant increase in serum T3 or T4 levels in little yellow croakers, which could serve as indicators of different types of hyperthyroidism. Based on the existing data [3,38], we propose that effective regulation of hyperthyroid or hypothyroid states in fish is relevant to several factors, including drug dosage, treatment duration, and the species' sensitivity to the medication. Consequently, further fundamental research is needed to explore these variables and their interactions.

SCFAs, products of anaerobic fermentation by the intestinal microbiota, play an essential role in primarily anti-inflammatory mechanisms as well as the indirect modulation of energy metabolism [39]. The primary SCFAs in the human intestine are AA, PA, and BA, which are typically present in a ratio of 60:25:15, respectively [26]. Similarly, our results also found that AA, PA, and BA were the predominant SCFAs in the intestines of little yellow croakers. SCFAs have been shown to act synergistically with thyroid hormones, particularly T3, to regulate enterocyte differentiation [40]. This suggests that SCFAs may be linked to thyroid function. Furthermore, impaired thyroid function has been associated with decreased levels of AA, PA, and BA in rats [41]. Further evidence suggests that patients with autoimmune thyroid diseases exhibit significantly lower levels of propionate and butyrate, alongside elevated levels of isovalerate, with propionate being negatively correlated with free T3 and free T4 levels [42]. Currently, research on SCFA metabolism modulated by hyperthyroid or hypothyroid states in fish remains limited. Our results demonstrated that T3-induced hyperthyroidism significantly increased the levels of AA and CA, while decreasing BA levels. T4-induced hyperthyroidism was associated with reduced levels of BA, IBA, VA, and IVA, while elevating AA levels. Differently, PTU-induced hypothyroidism resulted in significantly lower levels of BA and IVA. Therefore, evidence suggests that the compositions of intestinal SCFA are related to thyroid status in little yellow croakers.

Intestinal microbiota of various fish species is dominated by several phyla, including Proteobacteria, Firmicutes, Bacteroidota, Actinobacteria, and Fusobacteria [43]. The phyla Firmicutes and Bacteroidota are commonly recognized for their beneficial role in maintaining intestinal health, but the up-regulated abundance of Proteobacteria is closely correlated with pathogenicity [44]. In this study, various dietary thyroid-active agents changed the diversity of dominant phyla in little yellow croakers, such as Proteobacteria and Firmicutes. The imbalance of intestinal microbiota has been linked to thyroidal functional status. For instance, patients with Hashimoto's thyroiditis, characterized by elevated free thyroxine (FT4) content (as markers of thyroid function), exhibit a higher abundance of Proteobacteria and Actinobacteria [45]. In contrast, patients with Graves' disease, who have elevated levels of T3, T4, free triiodothyronine (FT3), and FT4, tend to show a lower abundance of Proteobacteria [46]. However, some patients with higher levels of FT3 and FT4 suggest a higher abundance of Proteobacteria and Actinobacteria [45], suggesting that the underlying mechanisms of these differing patterns require further investigation. In the present study, elevated serum T4 levels significantly reduced the abundance of Proteobacteria while increasing the abundance of Actinobacteria. Proteobacteria in little yellow croakers was primarily represented by the genus *Vibrio*, a major group of bacterial pathogens commonly found in marine fish cultures. Research has shown that serum T4 gradually decreases during the natural progression of vibriosis of silver sea bream (*Sparus sarba*) [47]. Additionally, studies indicate that the TH synthesis pathway in some fish species plays a crucial role in regulating infections caused by *Vibrio* spp. [48,49]. Furthermore, we found that Bacteroidota in little yellow croakers was primarily represented by the genera *Sediminibacterium* and *Prevotella*, and elevated serum T4 significantly increased the abundance of Bacteroidota. These findings align with previous research on thyroid dysfunction in rats [50]. Correlation analyses in our study revealed that serum T3 content was significantly associated with the abundances of genera *Vibrio* and *Sediminibacterium*, but serum T4 content showed a reverse association with these two genera. Inhibition of pathogenic bacteria can effectively improve the disease resistance in fish species [48,49]. Thus, a thorough understanding of how T3 and T4 influence these pathogens is crucial for enhancing disease management in aquaculture.

SCFAs play a critical role in maintaining the microbial ecosystem, with their composition significantly influencing interactions within the thyroid–gut microbiota axis [26]. A previous study demonstrated that after *Vibrio splendidus* infection, the content of AA gradually decreased, while IBA content progressively increased in the mid-intestine of *Apostichopus japonicus* [51]. Our study also found a significant correlation between the abundance of the *Vibrio* genus and the levels of AA and IBA. On the other hand, various genera within the phyla Firmicutes and Bacteroidota are known to efficiently produce SCFAs, which in turn modulate the intestinal barrier and energy metabolism [44]. The genus *Sediminibacterium*, a member of the phylum Bacteroidota, has been shown to exhibit altered abundance following infections. For instance, *Vibrio cholerae* infection results in a decrease in the relative abundance of *Sediminibacterium* sp. and an increase in various *Vibrio* ASVs in the intestine of zebrafish (*Danio rerio*), with IBA acting as a key metabolite involved in these interactions [52]. Here, our result revealed that the abundance of the genus *Sediminibacterium* was significantly negatively correlated with IBA content. Both *Vibrio* and *Sediminibacterium* genera are commonly regarded as disease biomarkers [53]. We hypothesize that intestinal AA and IBA might exert a crucial function in modulating the abundance of *Vibrio* under the hyperthyroidism status in little yellow croakers. However, the precise regulatory mechanisms by which AA and IBA influence the microbiota remain to be explored.

The gut microbiota profile can provide insights into the metabolic processes and health status of fish [26]. This study found that the intestinal microbiota primarily contributes to metabolic processes, particularly those related to amino acid and energy metabolism. Moreover, genera *Vibrio* and *Sediminibacterium* can modulate the process of amino acid and energy metabolism [54–56]. Notably, in the T4-treated group, the abundance of *Vibrio* and *Sediminibacterium* was significantly different from that in the other groups, suggesting that these genera are involved in the modulation of amino acid and energy metabolism under hyperthyroid conditions induced by T4. In addition, BugBase analysis predicted a higher abundance of aerobic bacteria and a lower abundance of bacteria with pathogenic and stress-tolerant characteristics in the T4 group. However, the underlying mechanisms driving the changes in the intestinal microbiota following dietary T4 supplementation remain unclear and warrant further investigation.

5. Conclusions

In summary, dietary thyroid-active agent (T3, T4, and PTU) supplementation can inhibit growth and regulate the thyroid status in little yellow croakers. These types of thyroid dysfunction result in differential intestinal SCFA profiles. Specifically, dietary supplementation with T3 or PTU had no significant effect on the richness or diversity of the intestinal microbiota in little yellow croakers, whereas dietary T4 supplementation notably increased microbial diversity. Our findings suggested that the genera *Vibrio* and *Sediminibacterium* played key roles in multiple metabolic pathways within the host intestine. Correlation analyses further indicated that intestinal AA and IBA were characteristic metabolites involved in the alteration of the genus *Vibrio*. These results provide a foundation for investigating the effects of thyroid-disrupting activities on the intestinal SCFA profiles and microbiota composition in marine fish. Additionally, the administration dosage of thyroid-active agents may have different impacts on the thyroid–gut microbiota axis, warranting further experimental investigation.

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