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Received: 7 July 2026 | Revised: 7 October 2026 | Accepted: 20 October 2026

DOI: 10.1111/jfb.70713

REGULAR ARTICLE

JOURNAL OF FISH BIOLOGY fsbi

Effect of dietary supplementation with *Clostridium butyricum* on growth, intestinal microbiota, metabolome and transcriptome in a hybrid fish (*Megalobrama amblycephala*♀ × *Culter alburnus*♂)

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Funding information
National Natural Science Foundation of China, Grant/Award Numbers: 32202906, 32293252, 32373119; Science and Technology Program of Hunan Province, Grant/Award Number: 2024RC3282; Earmarked Fund for Agriculture Reasearch System of China, Grant/Award Number: CARS – 45; Key Research and Development Program of Hunan Province of China, Grant/Award Numbers: 2024JK2034, 2025QY2004

Abstract

This study investigated the effects of dietary supplementation with *Clostridium butyricum* on the growth performance, intestinal microbiota, metabolome and transcriptome of a hybrid fish (*Megalobrama amblycephala*♀ × *Culter alburnus*♂). Experimental fish were diet supplemented with *C. butyricum* at 0 (control group [CG]), 4.02×10^8 (L), 1.66×10^9 CFU/kg (T), respectively, for 8 weeks feeding. The results showed that the growth performance of fish followed the trend of T > L > CG. Compared to group CG, the microbial community structure in group T shifted significantly. The relative abundance of *Fusobacteriota* and *Cetobacterium* was higher while that of *Pseudomonadota*, *Bacillota* and *Plesiomonas* was lower. The predicted functional abundances of biotin metabolism, D-alanine metabolism and propanoate metabolism in the intestinal microbiota were higher in group T than in group CG. Besides, 131 DEMs were identified and mainly enriched in glycerophospholipid metabolism and beta-alanine metabolism. In addition, transcriptome analysis showed 1479 differentially expressed genes (DEGs) were identified and mainly enriched in pentose and glucuronate interconversions, peroxisome and protein digestion and absorption. Correlation analysis showed that the dominant bacterium *Cetobacterium* was positively associated with fish growth performance and glycerophospholipid metabolites. These findings suggested that dietary supplementation with *C. butyricum* reshaped intestinal microbiota

Yunyun Liu, Shandong Chen and Yiting Ma contributed equally to this work.

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structure and influenced lipid, carbohydrate and protein metabolism, as well as the expression of genes related to carbohydrates and lipids, which may provide insights into the microbiota-host interactions that regulate growth in hybrid fish.

KEYWORDS
Clostridium butyricum, gene expression, hybrid fish, intestinal microbiota, metabolism

1 | INTRODUCTION

As live microbial preparations, probiotics have been widely applied in the aquaculture industry to improve growth performance, maintain intestinal health and enhance immunity and disease resistance (Butt et al., 2021). *Clostridium butyricum*, one of the most studied butyrate-producing bacteria, is a spore-forming, Gram-positive, strictly anaerobic bacterium exhibiting exceptional environmental adaptability, including acid tolerance, heat resistance and bile salt resistance (Liao et al., 2015). Like most probiotics, *C. butyricum* has the capacity to improve growth performance by producing metabolites including short-chain fatty acids (SCFAs), enzymes, vitamins and other nutrients and protect intestinal health by regulating immune function, alleviating liver inflammation and maintaining intestinal homeostasis (Cai et al., 2025; Stoeva et al., 2021).

The potential mechanisms of *C. butyricum* as a probiotic in aquaculture species primarily include the following three aspects. First, *C. butyricum* improves feed utilization and promotes the growth of aquaculture animals by producing nutrients (SCFAs, vitamins, alcohols and amino acids) and enhancing digestive enzyme activity. Several studies have reported *C. butyricum* can ferment undigested dietary fibre and utilize diverse carbon sources to generate SCFAs. The production of SCFAs acidifies the intestinal lumen, which in turn inhibits the colonization of potential pathogens, maintains intestinal barrier function and enhances the activity of digestive enzymes such as amylase, lipase and protease (Duan et al., 2018; Shen et al., 2024; Stoeva et al., 2021). Second, *C. butyricum* alters the intestinal microbiota composition and modulates intestinal morphology, which in turn maintains intestinal health and promotes nutrient metabolism. It has been reported that *C. butyricum* supplementation can inhibit the colonization of pathogenic bacteria including *Aeromonas hydrophila*, *Vibrio parahaemolyticus* and *Salmonella enteritidis* and stimulate the proliferation of probiotic bacteria including *Bifidobacterium* and *Lactobacillus*, thus restoring the balance of intestinal microbiota communities (Gao et al., 2013; Pan et al., 2008; Xu et al., 2018). Studies have shown that *C. butyricum* can reinforce the intestinal mucosal barrier of aquaculture species such as sea bass (*Lateolabracidae*), kuruma shrimp (*Marsupenaeus japonicus*) and turbot (*Scophthalmus maximus*) through enhancing mucin secretion, increasing intestinal villus width and length and promoting microvillus proliferation (Bi et al., 2023; Duan et al., 2017; Kong et al., 2023). Finally, *C. butyricum* protects hosts against external stress by regulating the activity of antioxidant enzymes and immune enzymes and modulating the expression of immune genes. Several studies revealed that *C. butyricum* metabolites activate the *Keap1/Nrf2* signalling pathway to increase corresponding gene expression and the activity of antioxidant enzymes (SOD, GSH-Px and CAT) to alleviate oxidative stress (Chen et al., 2025; Wang et al., 2024). Simultaneously, *C. butyricum* increased serum complement C3 and C4 levels, upregulated immunoglobulin M, immune-related genes (IL-10, TGF-β, TLR-2 and MyD-88) and tight junction genes (ZO-1 and occludin), while downregulated intestinal proinflammatory factors (IL-1β, TNF-α, IL-6, IL-17A, IFN-γ1 and IFN-γ2), thereby effectively alleviating the intestinal inflammatory responses (Guo et al., 2025; Meng et al., 2021; Tran et al., 2020).

The intestinal microbiota is an integral component of the intestinal tract, forming a microecological system with specific physiological functions with the host intestine. In recent years, the microbial community within the intestine has been recognized as a metabolic organ. The intestinal microbiota serves as a vital reservoir of enzymes, generating SCFAs, amino acids, glucose and other metabolites through interactions with the host. These substances provide direct nutrition to the intestinal epithelial tissue and the organism. Besides, metabolic signal molecules produced by certain intestinal bacteria, such as 5-HT, can regulate microbial community structure, enhance intestinal function and modulate immune responses (Wilson et al., 2020). Recent studies indicated that fish can regulate intestinal microbiota to improve food digestion, absorption and utilization. For instance, following adaptation to herbivory, grass carp (*Ctenopharyngodon idella*) exhibited significantly increased abundance of cellulase-producing bacteria such as *Clostridium*, *Citrobacter*, *Leuconostoc* and *Actinomyces* (Tran et al., 2017). In farmed fish species such as rainbow trout (*Oncorhynchus mykiss*), European sea bass (*Dicentrarchus labrax*) and common carp (*Cyprinus carpio* L.), the proportion of plant protein in artificial feed was positively correlated with the composition and diversity of enzymes-produced microbial communities (Xie et al., 2021). These studies indicated that intestinal microbiota plays a crucial role in the dietary adaptation of fish.

An allopolyploid hybrid fish was obtained from female blunt snout bream (BSB, *Megalobrama amblycephala*) × male topmouth culter (TC, *Culter alburnus*). This hybrid exhibits herbivorous traits and represents a highly valuable germplasm resource (Xiao et al., 2014). Earlier studies revealed that the intestinal microbiota and gastrointestinal tract of the hybrid fish exhibited greater similarity to the herbivorous parent *Megalobrama amblycephala*. During herbivorous adaptation (approximately 45 days old, 3 cm body length), the mevalonate pathway and genes related to food metabolism such as *sqlea* (involved in fat digestion), *hmgcra* (involved in cholesterol synthesis), *pck1* (involved in gluconeogenesis) were activated. Additionally, the abundance of *C.*

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butyricum, a dominant intestinal bacterium associated with bile acid metabolism, was significantly increased (Li et al., 2018, 2021). These results suggested *C. butyricum* may play a crucial role in the herbivorous adaptation of the hybrid fish. The present study investigated the effects of dietary supplementation with *C. butyricum* on the growth, intestinal microbiota structure, metabolism and gene expression of the hybrid fish. The results may provide a theoretical basis for the application of *C. butyricum* as a dietary probiotic in aquaculture and for improving nutrient utilization in farmed fish.

2 | MATERIALS AND METHODS

2.1 | Preparation of experimental diets

Clostridium butyricum (1×10^9 CFU/g) was obtained from Henan Jinbaihe Biological Technology Co., Ltd. (Henan, China). The experimental basal diet (containing 32.0% crude protein, 4.8% crude fat, 15.0% crude ash, 6.0% crude fibre, 2.0% lysine, 1.0% total phosphorus and 11.5% moisture per kilogram of feed) was supplemented with *C. butyricum* at 0 (control, CG), 0.5 (L) and 2 (T) g/kg of diet, respectively (Li et al., 2022). At the same time, each group of experimental fish was fed with the same amount of fresh duckweed every day (the duckweed was rinsed with purified water and mashed). The final *C. butyricum* concentrations in the three diets were 0, 4.02×10^8 and 1.66×10^9 CFU/kg, which were determined by a Neubauer haemocytometer under the light microscopy. *C. butyricum* was mixed with sterile pure water and then mixed with the basal diet under aseptic conditions, and pelleted into 2-mm diameter. All the diets were kept at room temperature and air-dried under ventilated conditions. During the experiment, the bacterial cell count of the diet was examined daily using the coated MRS plate count method to verify the concentration of the *C. butyricum* and to check for possible contamination (Duan et al., 2017).

2.2 | Experimental setup and sampling

A total of 450 healthy hybrid fish (initial weight 1.34 ± 0.15 g, body length 5.76 ± 0.23 cm) at 45 days of age were randomly assigned to 9 groups of 50 fry per group and placed into ceramic tanks with 1 m in diameter and 1.5 m in height. The groups were set up as control group (CG), L and T, with three replicate tanks in each group. The fish were fed with diets for 8 weeks. Throughout the experimental period, a continuous aeration system was employed in each tank; the pH value was maintained at 7.5–8.0; the dissolved oxygen (DO) concentration was maintained above 5.0 mg L^{-1}, the water temperature ranged from 24.0 to 28.0°C; the total ammonia nitrogen concentration was <0.2 mg L^{-1}, and the natural photoperiods were maintained during the feeding experiment.

At the end of the feeding trial, fish were fasted for 24 h. For each treatment group, 10 fish from each replicate tanks (totalling 30 fish) were randomly collected. These fish were individually weighed and measured for standard length, then anaesthetized with 50 mg L^{-1} MS-222 before dissection. The intestinal tissues and liver tissues were rapidly cut using sterilized scissors and forceps, photographed and immediately placed into petri dishes containing sterile water. After careful removal of surrounding adipose tissue and residual blood, the liver tissue was collected for transcriptome sequencing, and the luminal contents were gently scraped for metabolome sequencing and bacterial 16S rDNA gene sequencing. All omics samples were collected from all three replicate tanks, with no sample mixing. Specifically, 16S rRNA gene sequencing, metabolomic analysis and transcriptomic analysis were performed on four, five and three samples per group, respectively. All samples were stored at −80°C before use.

Calculation formulas: WGR (weight gain rate, %) = (FBW − IBW)/IBW × 100%. SGR (specific growth rate, %/day) = [ln (FBW) − ln (IBW)]/days × 100%.

The low-dose *Clostridium butyricum* group (L) showed no significant difference in growth performance compared with the CG. Therefore, only group T and group CG were selected for bacterial 16S rDNA, metabolome and transcriptome sequencing and analysis.

2.3 | Bacterial 16S rDNA sequencing and analysis

DNA was extracted using the QIAmp Rapid DNA Faecal Mini Kit (QIAGEN), and DNA quality was checked on a 1% agarose gel, and DNA concentration and purity were determined using a NanoDrop 2000 UV–visible spectrophotometer (ThermoScientific, Wilmington, USA). The variable region (V3∼V4) of the bacterial 16S rDNA gene was amplified with primer pairs (341F: 5′-CCTACGGGNGGCWGCAG-3′ and 806R: 5′-GGACTACHVGGGTATCTAAT-3′). Amplicons were evaluated with 2% agarose gels and purified using the AMPure XP Beads (Beckman, CA, USA). Sequencing libraries were generated using Illumina DNA Prep Kit (Illumina, CA, USA) following manufacturer's recommendation. The library quality was assessed with ABI StepOnePlus Real-Time PCR System (Life Technologies, Foster City, USA) and then sequenced on the NovaSeq 6000 platform to generate 2 × 250 bp paired-end reads. As previously described (Chen, Jiang, et al., 2021), clean reads were obtained by filtering raw reads with fastp (v0.18.0). Paired reads merged into raw tags using FLASH (v1.2.11) with a 10-bp minimum overlap and 2% mismatch, followed by stringent filtering to obtain clean tags. Clean tags were clustered into 97% operational taxonomic unit (OTU) via UPARSE (Usearch v11.0.667), chimeras removed with UCHIME, and the most abundant sequence per OTU selected as the representative. Based on effective tag – derived OTU abundance statistics, alpha diversity and beta diversity were analysed. Differences between the two groups were assessed using the Wilcoxon rank-sum test implemented in LEfSe with a significance threshold of $p < 0.05$. Linear Discriminant Analysis (LDA) was then applied to identify discriminative features. Functional annotation of bacterial (16S) using KEGG Pathway was performed with PICRUSt2 software, setting the Wilcoxon rank-sum test significance threshold at ≤0.05 (Douglas et al., 2019).

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2.4 | Metabolome sequencing and analysis

Intestinal tissues (100 mg) were individually ground with liquid nitrogen and the homogenate was resuspended with 500 μL prechilled 80% methanol by well vortex. The samples were processed as previously described (Want et al., 2013). Finally, the supernatant was injected into the LC–MS/MS system for analysis. According to the previously described method (Yang et al., 2022), metabolomics data processing was performed using Linux (CentOS 6.6), R and Python. Differential metabolites were identified by combining OPLS-DA VIP values with univariate *t*-test (*p*-values). The differential threshold was set as VIP ≥1 and FDR-adjusted *p*-value < 0.05. Based on metabolite identification results, the corresponding compound IDs were retrieved from the KEGG database. Following multiple testing correction, pathways with *Q*-values (FDR-corrected *p*-value) ≤0.05 were considered significantly enriched for the differentially expressed metabolites. Pathway enrichment analysis was performed to identify the major biochemical metabolic and signalling pathways associated with these differentially expressed metabolites.

2.5 | Transcriptome sequencing and analysis

Total RNA was extracted from the samples using TRIzol reagent (Invitrogen, USA) following the standard protocol. After RNA quality assessment, mRNA was enriched, purified and fragmented at high temperature. The fragmented mRNA was reverse-transcribed into first-strand cDNA, followed by second-strand synthesis coupled with end repair and A-tailing. Adapters were then ligated, and target fragments were purified with Hieff NGS® DNA Selection Beads. After PCR amplification, the library was sequenced on the Illumina NovaSeq X Plus. Then, the reads were further filtered by fastp (v0.18.0) to obtain high-quality clean reads. Short reads alignment tool Bowtie2 (v2.2.8) was used for mapping reads to ribosome RNA (rRNA) database. The rRNA-mapped reads were removed, and the remaining clean reads were used for assembly and gene abundance calculation. Paired-end clean reads were mapped to the reference genome index using HISAT2 (v2.1.0) with default parameters (Kim et al., 2015). The mapped reads of each sample were assembled by using StringTie (v1.3.1) in a reference-based approach (Love et al., 2014). For each transcription region, an FPKM value was calculated using RSEM to quantify its expression abundance and variations. The differential expression genes were identified via the DESeq2 (v2.13) with a screening threshold of FDR-adjusted *p*-values <0.05 and absolute Fold Change ≥2 (Pertea et al., 2015). After gene function annotation, function enrichment was analysed on the platform Omicsmart (https://www.omicsmart.com) and *p*-value <0.05 was considered to be significant.

2.6 | Correlation analysis

Spearman's rank correlation analysis was performed on growth performance, microbiota, differential metabolites, differentially expressed genes (DEGs) (Sokol et al., 2017). The differentially abundant bacteria and differential metabolites (vip scores >3.2) were selected for correlation analysis. Meanwhile, weighted gene co-expression network analysis (WGCNA) was performed based on DEGss (Material S1) to screen out hub genes correlated with growth traits, which were used for subsequent correlation analysis.

2.7 | Statistical analysis

Data were presented as the mean ± standard deviation (SD). Statistical analysis was performed using Excel and SPSS 20.0. Data were tested for normality and homogeneity of variance prior to analysis. Differences among groups were evaluated using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Statistical significance was considered when *p*-value < 0.05.

3 | RESULTS

3.1 | Effects of *Clostridium butyricum* on hybrid fish growth

After 8 weeks of feeding, the final body weight, final body length, SGR and WGR in group T were significantly higher than those in group CG ($p < 0.05$), while there were no significant differences between group CG and group L ($p > 0.05$) (Table 1).

3.2 | Intestinal microbiota diversity and composition

A total of approximately 327.8 MB of data were obtained from 16S rDNA sequencing, which identified 46 phyla, 403 genera and 6778 OTUs. Principal coordinates analysis (PCoA) analysis based on the Bray–Curtis distance showed that PC1 and PC2 explained 82.31% and 14.31% of the total variation; the two groups were clearly separated along PC1, indicating that dietary supplementation with *C. butyricum* altered the intestinal microbiota structure (Figure 1a). Analysis of the Shannon index revealed bacterial diversity significantly decreased in group T (Figure 1b).

At the phylum level, two groups shared 27 bacterial phyla, with 2 phyla exclusive to group T and 6 exclusive to group CG (Figure 1c). *Pseudomonadota*, *Fusobacteriota*, *Bacillota* and *Bacteroidota* were the dominant phyla across both groups. The relative abundance of *Fusobacteriota* (60.97% ± 6.82%) in group T was significantly higher than that in group CG (23.47% ± 7.19%), while the relative abundances of *Pseudomonadota* and *Bacillota* (32.03% ± 6.61% and 3.11% ± 1.22%, respectively) were significantly lower than those in group CG (62.46% ± 9.61% and 8.97% ± 4.19%, respectively) (Figure 1d). At the genus level, two groups shared 133 bacterial genera, with 62 genera exclusive to group T and 46 exclusive to group CG (Figure 1e). *Cetobacterium*, *Aeromonas*, *Plesiomonas* and *Paraclostridium* were the dominant genera across both groups. The relative abundance of *Cetobacterium*

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TABLE 1 Comparison of growth among three groups of experimental fish.

Item	Group		
	CG	L	T
IBW (g)	1.34 ± 0.15	1.34 ± 0.15	1.34 ± 0.15
IBL (cm)	5.76 ± 0.23	5.76 ± 0.23	5.76 ± 0.23
FBW (g)	6.88 ± 0.45^{b}	7.18 ± 0.50^{ab}	7.57 ± 0.46^{a}
FBL (cm)	11.18 ± 0.54^{b}	11.64 ± 0.50^{ab}	11.94 ± 0.80^{a}
WGR (%)	413.13 ± 33.23^{b}	436.12 ± 37.55^{ab}	464.63 ± 34.50^{a}
SGR (%)	2.92 ± 0.11^{b}	2.99 ± 0.13^{ab}	3.09 ± 0.11^{a}

Note: Values are presented as mean ± SD ($n = 3$, n represents the number of replicate tanks. Ten fish per tank were randomly selected for measurement, and tank means were used for statistical analysis). Different letters within the same row indicate significant differences between groups ($p < 0.05$).
Abbreviations: FBL, average final body length; FBW, final body weight; IBL, average initial body length; IBW, initial body weight; SGR, specific growth rate; WGR, weight gain rate.

in group T (60.97% ± 6.83%) was significantly higher than that in group CG (23.47% ± 7.19%), while the relative abundances of *Plesiomonas* (7.01% ± 1.82%) and *Paraclostridium* (2.30% ± 1.13%) were significantly lower than those in group CG (34.73% ± 3.60% and 7.83% ± 4.40%, respectively) (Figure 1f).

LEfSe analysis identified 19 differentially abundant taxonomic clades (LDA values ≥3.5) across both groups. Bacteria significantly enriched in group T included *Fusobacteriaceae*, *Cetobacterium* and *Aquihabitans*, whereas those enriched in group CG included *Bacteroidales*, *Peptostreptococcales_Tissierellales*, *Paraclostridium* (Figure 2a).

Functional prediction analysis indicated that predicted microbial functions in group T were associated with biotin metabolism, D-alanine metabolism, propanoate metabolism, fructose and mannose metabolism, etc. The predicted microbial functions in group CG were linked to glycine, serine and threonine metabolism, histidine metabolism, fatty acid degradation, etc. (Figure 2b).

3.3 | Intestinal metabolomics analysis

OPLS-DA analysis results showed that samples were clearly separated between groups, with distinct clustering within groups (Figure 3a). The model yielded $R^2X = 0.815$, $R^2Y = 0.995$, $Q^2 = 0.655$. A permutation test was performed to assess the risk of overfitting. The obtained intercepts ($R^2 = 0.98$, $Q^2 = -0.13$) confirmed that there was no overfitting in the OPLS-DA model (Figure S1).

A total of 131 differentially expressed metabolites were identified. In positive ion mode, 83 metabolites were differentially expressed, including 48 increased and 35 decreased metabolites. In negative ion mode, 48 differentially expressed metabolites were detected, including 14 increased and 34 decreased metabolites (Figure 3b). These differential metabolites were predominantly classified as lipid and lipid-like molecules, short peptides and amino acid derivatives. In group T, amino acid derivatives and short peptides were significantly decreased; conversely, lysophospholipids were increased (Figure 3c).

KEGG pathway enrichment analysis revealed the differential metabolites were mainly involved in 11 pathways. Among these, seven pathways manifested upregulation, including choline metabolism in cancer, glycerophospholipid metabolism, efferocytosis, eicosanoid metabolism and arachidonic acid metabolism. Four pathways manifested downregulation, including beta-alanine metabolism and lysine degradation (Figure 3d,e).

3.4 | Transcriptome sequencing and analysis

Compared with group CG, 1479 DEGs were identified in group T, including 1033 upregulated genes and 446 downregulated genes (Figure 4a).

GO functional enrichment analysis was performed to explore the biological functions of DEGs. In the BP category, the DEGs were significantly enriched in diverse metabolic pathways. The most highly significant terms included organic acid metabolic process (GO:0006082), oxoacid metabolic process (GO:0043436) and carboxylic acid metabolic process (GO:0019752). The small molecule metabolic process (GO:0044281) contained the largest number of DEGs (292) with high statistical significance. Moreover, multiple pathways related to fatty acid metabolism, lipid catabolism and amino acid metabolism were markedly enriched, demonstrating the extensive regulation of cellular fundamental metabolism by these DEGs. In the MF category, oxidoreductase activity (GO:0016491), which covered 167 DEGs, was the only significantly enriched term (Figure 4b).

KEGG enrichment analysis revealed that the upregulated DEGs were mainly enriched in pentose and glucuronate interconversions, biosynthesis of cofactors, DNA replication, peroxisome, ascorbate and aldarate metabolism, etc. (Figure 4c), whereas the downregulated DEGs were predominantly enriched in protein digestion and absorption, ECM-receptor interaction, pancreatic secretion, P53 signalling pathway, glioma, etc. (Figure 4d).

To validate the RNA-seq data, we selected 8 DEGs (*abcg8*, *hsd3b7*, *acp2*, *fkbp8*, *vimr2*, *ccl25b*, *fosb*, *amy2a*) for qPCR with three biological replicates per group, each in triplicate. The consistency between the qPCR and RNA-seq results for the eight selected DEGs supported the reliability of the RNA-seq data for these genes and provided partial validation of the transcriptomic data (Figure S2).

3.5 | Co-analysis of the microbiome, metabolome, transcriptome

Correlation analyses were performed to explore potential associations. At the microbiome-metabolome level, positive correlations

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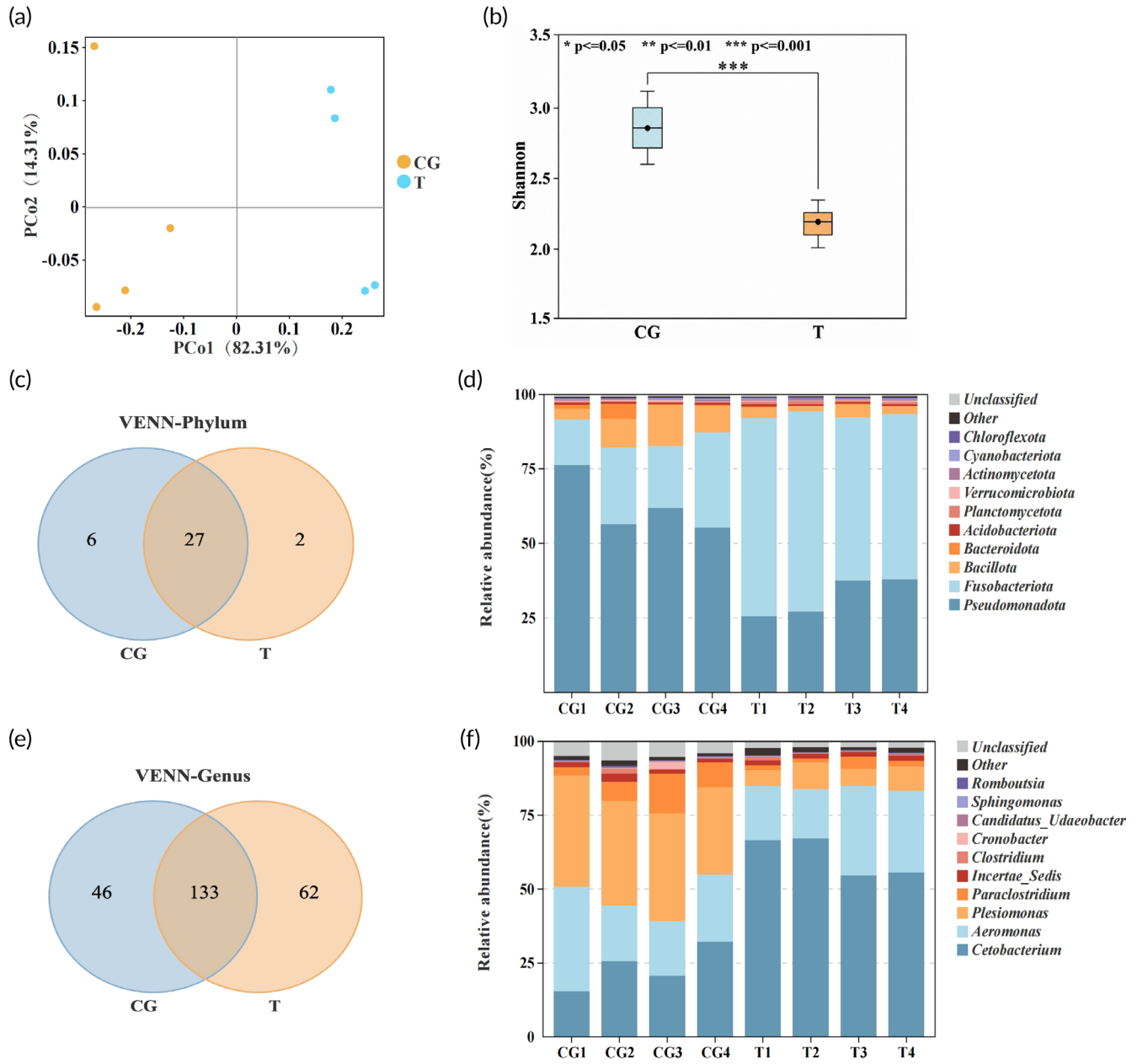


FIGURE 1 Analysis of intestinal microbiota diversity and composition between group CG and group T. (a) PCoA analysis. (b) Shannon diversity of intestinal microbiota. (c) Venn diagram of shared and unique microbiota between group CG and group T at the phylum level. (d) The composition and relative abundance of microbiota at the phylum level. (e) Venn diagram of shared and unique microbiota between group CG and group T at the genus level. (f) The composition and relative abundance of microbiota at the genus level.

were observed for *Cetobacterium*, *Pirellula* and *JSC.12* with lysophosphatidylcholines (LPC). By contrast, *Sphingomonas*, *Plesiomonas*, *Paraclostridium* and *Cronobacter* tended to show negative correlations with these metabolites. Furthermore, *Turicibacter*, *Massilia* and *Candidatus_Solibacter* showed positive associations with short peptides, while *JSC.12* was negatively correlated with short peptides (Figure 5a). At the metabolome-transcriptome level, positive correlations were observed for LPC with genes including *gpia*, *eepd1*, and *abcg8*. Inversely, short peptides tended to show negative correlations with *hps5* and *sord* (Figure 5b). At the microbiota-growth level, *Cetobacterium* abundance was positively associated with growth traits (FBW, SGR) (Figure S3).

4 | DISCUSSION

This study observed that *C. butyricum* supplemented to the diet increased the growth performance of the hybrid fish. This result was consistent with previous studies in sea bass (*Lateolabrax maculatus*), Chinese Perch (*Siniperca chuatsi*) and yellow catfish (*Pelteobagrus fulvidraco*) (Kong et al., 2023; Zhang et al., 2022). For instance, in yellow catfish, dietary supplementation with appropriate concentrations of *C. butyricum* increased intestinal SCFA content, enhanced intestinal protease and alkaline phosphatase activities and markedly improved growth performance (Wang et al., 2023). Furthermore, there was no significant difference in growth between group CG and group L in this

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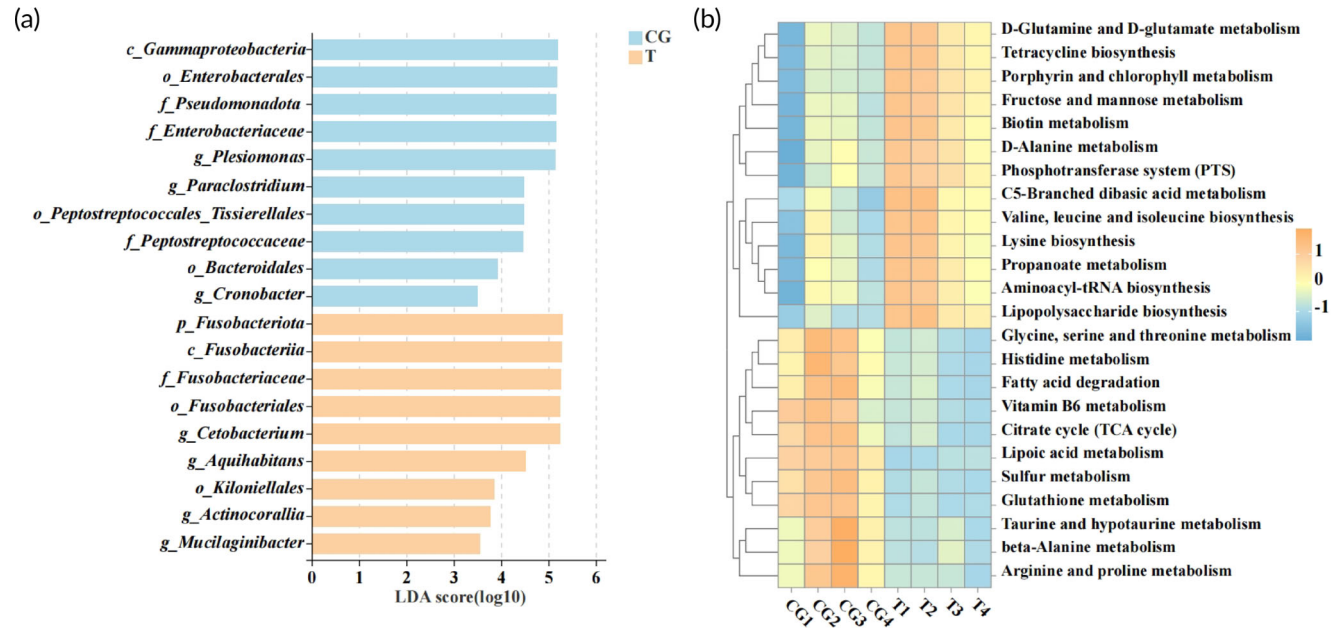


FIGURE 2 Screening of marker microorganisms and functional prediction analysis of dominant microbial communities. (a) Marker microorganisms between group CG and group T. (b) PICRUSt functional prediction of microbiota.

study. Similarly, dietary supplementation with *C. butyricum* did not significantly affect final body weight, growth rate or digestive enzyme activity in hybrid grouper (*Epinephelus lanceolatus* ♂ × *Epinephelus fuscoguttatus* ♀) (He et al., 2017). This phenomenon may be related to variations in probiotic dosage, strain type and fish developmental stage.

Research indicated that moderate intake of probiotics contributed to improving intestinal microbial structure and concomitantly increasing microbial diversity (Ntakirutimana et al., 2023). In the present study, dietary supplementation with *C. butyricum* altered the intestinal microbiota structure of the hybrid fish, as evidenced by a decreased relative abundance of *Plesiomonas* and a concomitant increase in *Fusobacteriota* and *Cetobacterium*. Notably, microbial diversity decreased in group T, implying that *C. butyricum* supplementation inhibited certain bacteria, consequently reducing overall microbial biodiversity (Kong et al., 2023). Previous studies have similarly reported that dietary *C. butyricum* led to a decrease in α-diversity of intestinal microbiota in Large Yellow Croaker (*Larimichthys crocea*), and such alterations were closely linked to juvenile growth and metabolism (Yin et al., 2021). Therefore, the effects of dietary supplementation of *C. butyricum* on intestinal microbiota are influenced by multiple factors, including *C. butyricum* concentration, fish species, developmental stage and rearing conditions.

Plesiomonas, belonging to the phylum *Proteobacteria*, is a common opportunistic pathogen in the intestines of aquatic animals and various mammals. Moreover, an elevated abundance of *Proteobacteria* in the intestine may lead to dysbiosis (Shin et al., 2015). In this study, the relative abundance of *Plesiomonas* in group T significantly decreased, which suggested *C. butyricum* may help maintain intestinal microbial homeostasis and may be beneficial for gut health. Research has confirmed that *Paraclostridium* can secrete cellulase and participate in cellulose degradation (Silva Rabelo et al., 2020). The decreased relative abundance of *Paraclostridium* in group T might be attributed to the elevated intestinal butyrate content following *C. butyricum* supplementation, which consequently inhibited the acid-sensitive *Paraclostridium*. *Cetobacterium* is a common dominant microbiota in freshwater fish. Studies in Nile tilapia (*Oreochromis niloticus*), zebrafish (*Danio rerio*) and yellow catfish (*Pelteobagrus fulvidraco*) have revealed its crucial roles in carbohydrate, protein and lipid metabolism as well as in the regulation of glucose homeostasis and maintenance of intestinal health (Wang et al., 2021; Wang, Tang, et al., 2025; Xie et al., 2022; Zhang, 2023). Furthermore, it has been reported that *Cetobacterium* is capable of producing vitamin B_{12}, which can enhance intestinal barrier tight junctions and improve the stability of the intestinal microbial ecological network to prevent pathogen infection (Qi et al., 2023). In our study, the significant increase in the relative abundance of *Cetobacterium* was observed in group T. The increased abundance of *Cetobacterium* may contribute to the regulation of host nutrient metabolism and the maintenance of intestinal health.

PICRUSt2-based Functional prediction revealed that the biotin metabolism pathway of the intestinal microbiota was significantly enriched in group T compared with group CG. Biotin, as a coenzyme for multiple carboxylases, participates in the metabolism of fats, carbohydrates and proteins in aquatic animals, promoting energy metabolism and aiding cell growth (Li et al., 2010; Liu et al., 2021). Biotin has also been reported to enhance lysozyme and alternative complement pathway activity, thereby improving non-specific immune responses in large yellow croaker (*Larimichthys crocea R*) (Zhang et al., 2016). Our results suggested that the intestinal microbiota may possess greater biotin metabolic potential upon *C. butyricum*

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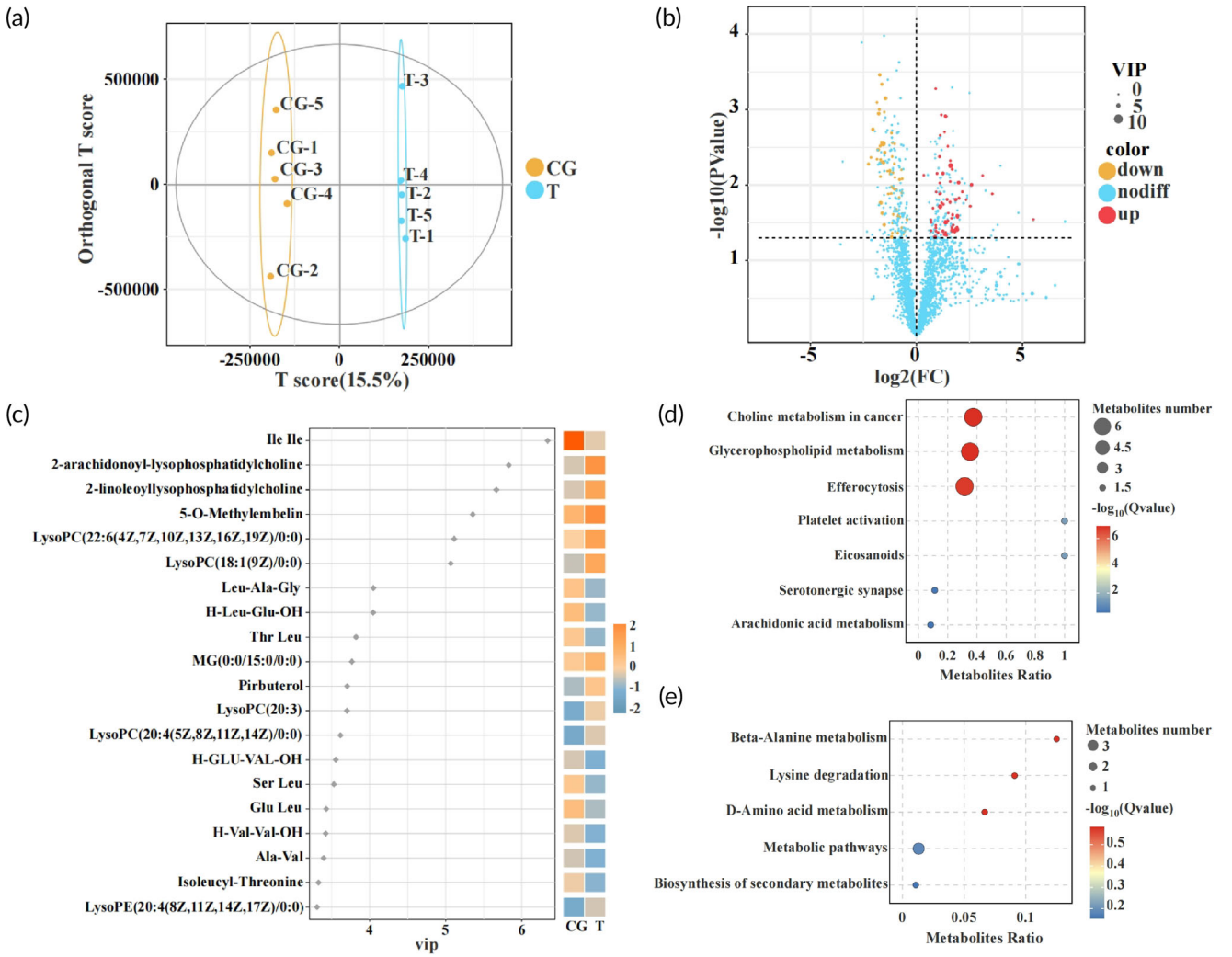


FIGURE 3 Screening and functional analysis of intestinal differential metabolites. (a) OPLS-DA scoreplot. (b) Volcano plot of differential metabolites. (c) Top 20 differentially expressed metabolites between group CG and group T. (d) KEGG enrichment for the upregulated metabolic pathways in group T. (e) KEGG enrichment for the downregulated metabolic pathways in group T.

supplementation. This may relate to the nutrient metabolism and immune function of hybrid fish and offer a direction for future investigation into the link between biotin metabolism and *C. butyricum* supplementation.

Alterations in dietary composition modify nutritional attributes of animal and consequently influence related metabolic adaptations. Simultaneously, an interrelationship also exists between host intestinal microbiota and dietary adaptation. We found that the levels of LPC in the intestine of hybrid fish were significantly higher in group T than in group CG, and the glycerophospholipid metabolism pathway was significantly enriched. The natural algae consumed by herbivorous fish are low in lipids and predominantly contain galactolipids, which serve as a major dietary source of fatty acids (De Caro et al., 2008; Sahaka et al., 2020). Fish primarily absorb fats through intestinal epithelial cells, which take up emulsified triglycerides and fatty acid micelles, ultimately synthesizing very-low-density lipoproteins (VLDL). LPC is a lysophospholipid produced by the hydrolysis of glycerophospholipids. As an amphiphilic molecule, it acts as an emulsifier to aggregate triglyceride and galactolipid breakdown products into micelles, increasing the surface area exposed to digestive enzymes. This may enhance absorption efficiency in low-fat diets and compensate for reduced nutrient density (Liu et al., 2024). Intestinal health fundamentally depends on the integrity of the intestinal barrier. Specific glycerophospholipids not only play a crucial role in maintaining cell membrane structure but also promote tight junctions between intestinal epithelial cells. Consequently, the enhanced glycerophospholipid metabolism may support the rapid turnover and repair of intestinal epithelial cells. Furthermore, metabolites such as lysophosphatidylserine (LPS) play vital roles in lipid metabolism, anti-inflammation and cellular signalling, potentially helping mitigate gut inflammatory responses induced by plant-based diets (Chen, Yi, et al., 2021; Gao et al., 2023; Lee et al., 2013; Liu et al., 2019).

Significantly reduced levels of short peptides were observed in group T, and KEGG enrichment analysis of DEGs revealed significant

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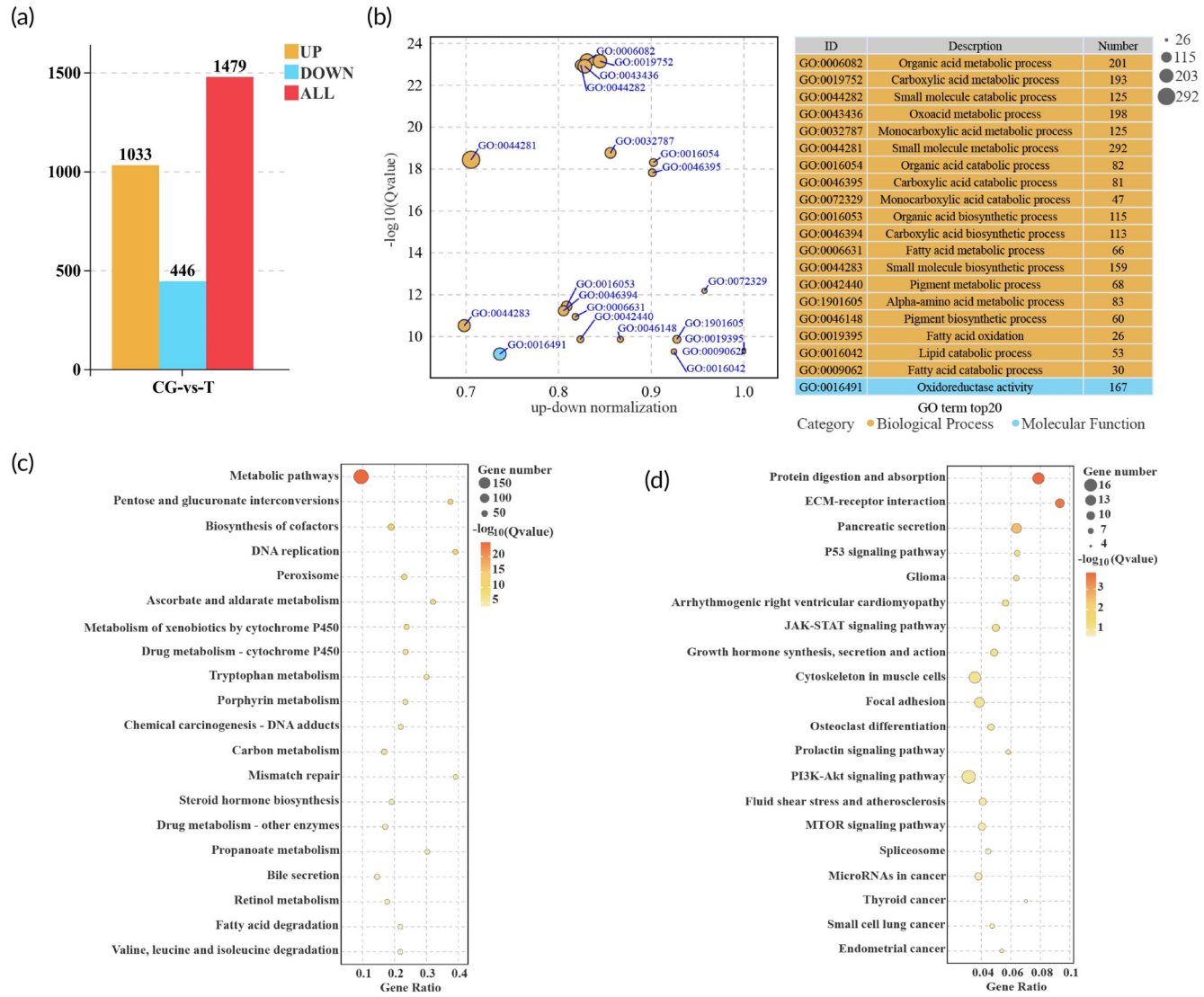


FIGURE 4 Identification and functional enrichment analysis of DEGs in intestinal tissues of CG versus T. (a) The number of DEGs in the three groups. (b) Top 20 GO term in DEGs, Rich_factor is the ratio of the proportion of genes annotated to a pathway in the DEGs to the proportion of genes annotated to that pathway in all genes. (c) TOP 20 pathway in downregulated DEGs. (d) TOP 20 pathway in upregulated DEGs.

downregulation of the protein digestion and absorption pathway. This suggested that intestinal bacteria may participate in protein digestion, driving the terminal hydrolysis of oligopeptides and subsequent release of amino acids (Ringoe et al., 2022; Wang, Li, et al., 2025). Intestinal bacteria can utilize short peptides or amino acids as carbon and nitrogen sources for proliferation. Meanwhile, this process diminishes harmful fermentation substrates, thereby lowering the risk of damage to the intestinal epithelium from metabolic byproducts like ammonia (Marchesi et al., 2016; Yang & Liao, 2019). The reduction in threonine-containing peptide segments may increase the availability of free amino acids for mucin synthesis and the maintenance of gut barrier integrity (Yang & Liao, 2019).

This study showed that *C. butyricum* modulated host intestinal genes and pathways related to carbohydrate and lipid metabolism. In group T, key carbohydrate metabolism genes (*gpia*, *grhprb* and *ins*) were significantly upregulated, and pathways such as carbon metabolism and the pentose and glucuronate interconversions were significantly enriched. This suggested a metabolic shift towards glycolysis and the pentose phosphate pathway, which may provide sufficient biosynthetic precursors for nucleotide synthesis and compensatory proliferation of intestinal epithelial cells (Caruso & Sheridan, 2011; Lin et al., 2009). Consistent with this shift, microbial functional prediction showed enhanced fructose and mannose metabolism and propionate metabolism. This consistency may indicate that intermediate metabolites derived from microbial carbohydrate fermentation could be absorbed and utilized by the host. In addition, genes (*pparg*, *cpt1aa*, *pnpla2* and *hacl1*) involved in fatty acid degradation were upregulated (Huang et al., 2025; Kamoshita et al., 2022; Liu et al., 2023; Xie et al., 2024; Yang et al., 2026), while *aclya* (ATP-citrate lyase a), a key enzyme in de novo lipogenesis, was downregulated (Jia et al., 2024), both of which may help the host maintain energy homeostasis under low-fat, low-nutrient dietary conditions.

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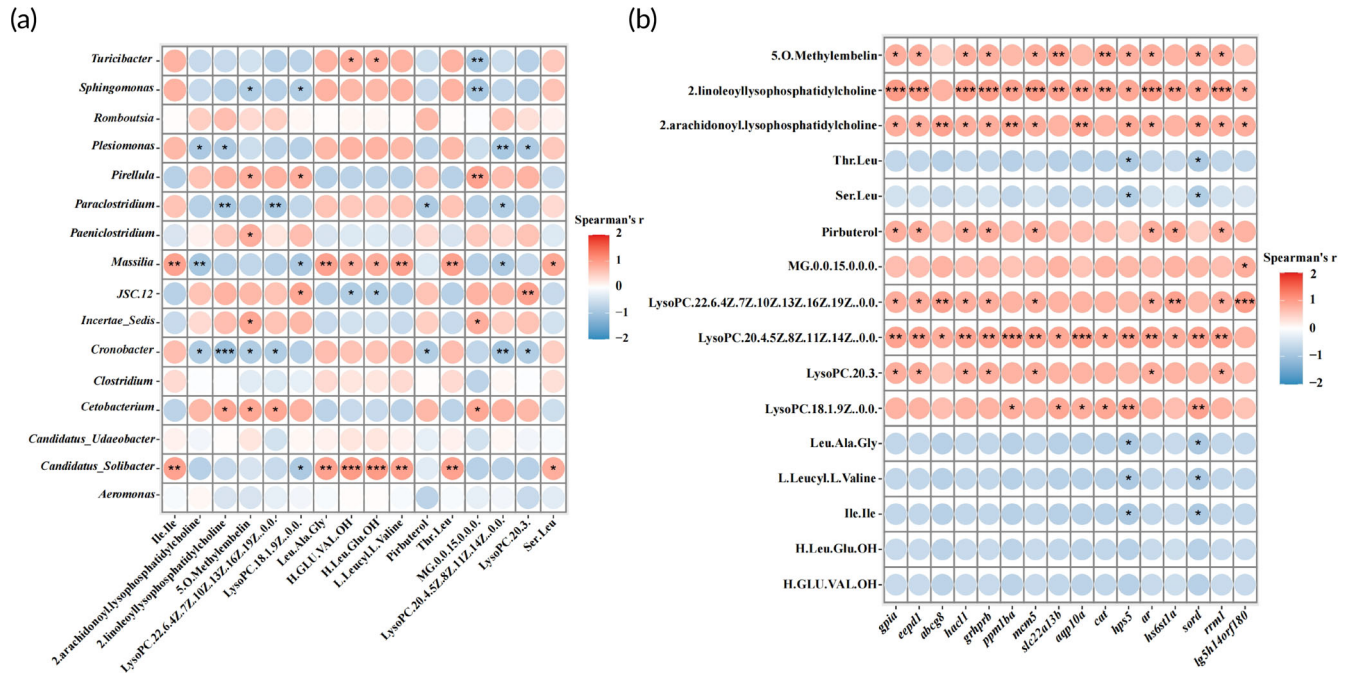


FIGURE 5 Correlation analysis. (a) Correlation analysis of microbial communities and differential metabolites. (b) Correlation analysis of differential metabolites and differentially expressed genes (*p < 0.1, **p < 0.05, ***p < 0.01).

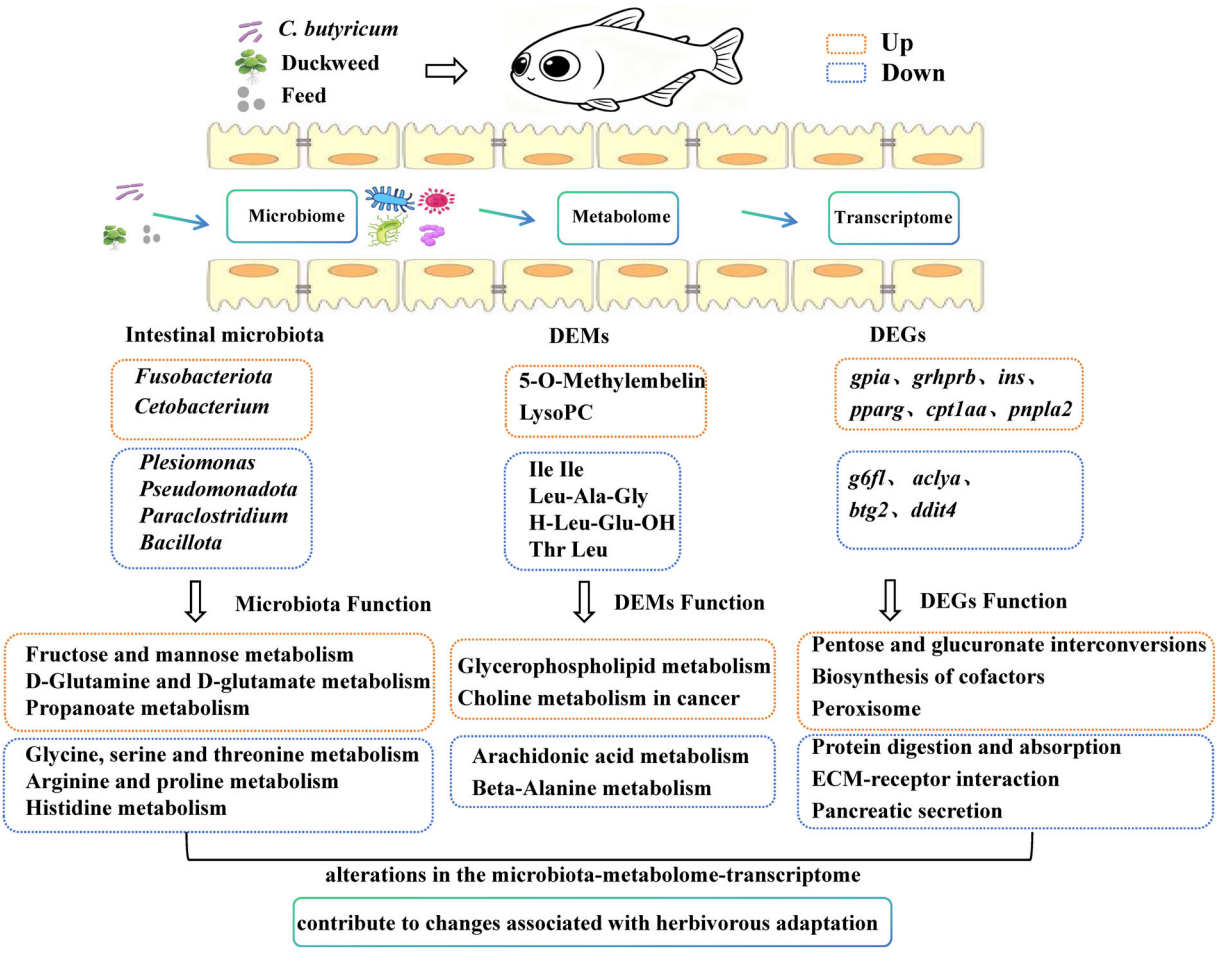


FIGURE 6 Potential mechanism by which *C. butyricum* promotes the formation of feeding habits in hybrid fish.

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Moreover, the significant downregulation of anti-proliferation-related genes (*btg2*, *ddit4*) may relieve the inhibition of the intestinal epithelial cell cycle, further promoting compensatory proliferation of the intestinal epithelium (Faught & Vijayan, 2019; Lim, 2006).

Together, these findings suggested that *C. butyricum*-induced alterations in the microbiota-metabolome-transcriptome may contribute to changes associated with herbivorous adaptation in hybrid fish (Figure 6). However, the underlying mechanism needs further investigation.

5 | CONCLUSION

In summary, dietary supplementation with *C. butyricum* at 1.66×10^9 CFU/kg significantly enhanced growth performance, reshaped the intestinal microbiota and modulated host metabolism in the hybrid fish. The beneficial effect of *C. butyricum* may be achieved by modulating the intestinal microbiota, thereby altering the function of intestinal microbiota, which may in turn influence host metabolism and ultimately lead to host metabolic remodelling. The results of this study may provide a theoretical basis for the application of *C. butyricum* as a dietary probiotic in aquaculture and offer new insights into how the interactions between the host and intestinal microbiota regulate fish growth.

AUTHOR CONTRIBUTIONS

Yunyun Liu: Writing—original draft; visualization; software; investigation; formal analysis; data curation; conceptualization. **Shandong Chen**: Visualization; validation; software; investigation. **Yiting Ma**: Visualization; validation; software. **Wenting Zhu**: Visualization; validation; software. **Hongbo Xi**: Visualization; software; investigation. **Ye Yuan**: Visualization; software; investigation. **Zexun Zhou**: Validation; software; investigation. **Yongchun Li**: Visualization; resources; investigation. **Zhongyuan Shen**: Visualization; data curation. **Chongqing Wang**: Visualization; data curation. **Wuhui Li**: Writing—review and editing; writing—original draft; visualization; supervision; methodology; funding acquisition; formal analysis; conceptualization.

ACKNOWLEDGEMENTS

Bioinformatic analysis was performed using Omicsmart, a dynamic real-time interactive online platform for data analysis (https://www.omicsmart.com).

FUNDING INFORMATION

This work was supported by the National Natural Science Foundation of China (32202906, 32293252, 32373119), the Science and Technology Innovation Program of Hunan Province (2024RC3282), Earmarked Fund for Agriculture Reasearch System of China (CARS – 45) and the Key Research and Development Program of Hunan Province (2024JK2034, 2025QY2004).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The 16S rDNA gene sequences of intestinal bacteria and the transcriptome sequences were deposited in the NCBI Sequence Read Archive (SRA) at https://www.ncbi.nlm.nih.gov/database under accession number PRJNA679638. Data will be made available on request.

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SUPPORTING INFORMATION

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How to cite this article: Liu, Y., Chen, S., Ma, Y., Zhu, W., Xi, H., Yuan, Y., Zhou, Z., Li, Y., Shen, Z., Wang, C., & Li, W. (2026). Effect of dietary supplementation with *Clostridium butyricum* on growth, intestinal microbiota, metabolome and transcriptome in a hybrid fish (*Megalobrama amblycephala*♀ × *Culter alburnus*♂). *Journal of Fish Biology*, 1–13. https://doi.org/10.1111/jfb.70713

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