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Dietary bile acid supplementation alters the gut transcriptome and metagenome and contributes to herbivorous diet adaptation in juvenile allodiploid hybrid fish

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ABSTRACT

The aim of this study was to explore the effects of dietary bile acids (BAs) supplementation on herbivorous diet adaptation in allodiploid hybrid fish derived from blunt snout bream (♀) × topmouth culter (♂). Three experimental diets were formulated: a commercial basal diet (CG group), a basal diet supplement with duckweed (H group), and a basal diet supplemented with duckweed and 600 mg kg^{-1} BA (T group). After 56 days of feeding, the growth parameters increased, and greater intestinal cellulase activity was detected in the T group. Moreover, the T group fish presented a longer and denser small intestinal villi. In general, the characteristics of the H group were intermediate between those of the other two groups (T > H > CG). Compared with CG group fish, 711 specific differentially expressed genes (DEGs) were identified in the intestine of T group fish. The upregulated DEGs were primarily enriched in MAPK signaling pathway, protein digestion and absorption, and mTOR signaling pathway; the downregulated DEGs were predominantly enriched in steroid hormone biosynthesis, fatty acid metabolism and primary bile acid biosynthesis. Besides, several upregulated genes, such as *jak*, *irs*, *egfr*, *prss*, and *cpa* which related to cell proliferation and differentiation, digestion, and metabolism, are associated with the changes in intestinal histomorphology. These changes could promote the intestinal adaptation of an herbivorous diet. The metagenome sequencing results revealed that exogenous BAs significantly changed the structure of the gut microbiota; decreased the abundance of potentially pathogenic bacteria or fungi such as *Proteobacteria*, *Mucoromycota,* and *Pseudomonas*; and increased the abundance of probiotics such as the cellulose-producing bacteria *Micromonospora*. In addition, functional analysis revealed that the abundance of some enzyme families related to cellulose degradation was significantly greater in the T group. These findings suggested that exogenous BAs influences host intestinal gene expression and the structure of the gut microbiota via the gut microbiota-bile acid pathway, which in turn contributes to herbivorous diet adaptation in juvenile allodiploid hybrid fish.

1. Introduction

Aquatic products provide one-fifth of the high-quality animal protein for humans; however, the feed source is one of the most important factors in determining the yield (Boyd et al., 2022). The Grass carp (*Ctenopharyngodon idellus*) and blunt snout bream (*Megalobrama amblycephala*) are important commercial fish in China. As herbivorous fish, they are increasingly emphasized for their food, which is mainly derived from plants in nature. Interestingly, several studies have demonstrated that genes encoding cellulose-digesting enzymes are not identified in the genomes of grass carp and blunt snout bream, and that the ability of these to digest plant food relies mainly on the microbiome

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in their intestine (Liu et al., 2017; Tran et al., 2017; Wang et al., 2015; Wei et al., 2018). The cellulase-producing microbes, such as *Clostridium*, *Citrobacter*, *Enterobacter*, *Aeromonas*, and *Actinomyces*, are observed dominant microbiota in the gut of grass carp and blunt snout bream after herbivorous diet adaptation (Tran et al., 2017; Li et al., 2023). Obviously, the gut microbiome plays an important role in diet digestion, in turn, the fish (host) also affected their intestinal microbes enrichment. However, little research focuses on the host-microbes interactions that contribute to herbivorous diet transition and adaptation during the early life cycle of fish.

BAs are the amphoteric compounds synthesized from cholesterol in the liver, which play significant roles in the absorption of lipids and maintenance of cholesterol balance (Hofmann and Hagey, 2008). BAs in the gastrointestinal tract interact with dietary fiber to alter the bioavailability and bioaccessibility of secondary metabolites (Singh et al., 2019). Meanwhile, they have also been identified as potent signaling molecules (Kiriyama and Nochi, 2019). This includes the nuclear receptor FXR, which upon binding with BAs, regulates a variety of metabolic processes such as glucose, BAs, and lipid metabolism (Wen et al., 2021). Furthermore, there is an intensive interaction between the gut microbiota and bile acids metabolism (Wahlström et al., 2016). BAs exert a direct influence on the gut microbial landscape, fostering the proliferation of bile acid-metabolizing bacteria and suppressing the growth of bile-sensitive bacterial populations (Guo et al., 2022). Additionally, it can indirectly regulate the microbiota through the activation of FXR (Wahlström et al., 2016). In turn, the gut microbiota can regulate the bile acid pools through the production of secondary BAs, which contribute to the regulation of bile acid-dependent digestive, absorptive, and signaling functions, as well as other physiological processes (Guo et al., 2022). This bidirectional relationship between BAs and the gut microbiota exemplifies the intricate interdependence of the gut microbiota–bile acid–host axis (Winston and Theriot, 2019).

The hybrid fish, derived from blunt snout bream (BSB, herbivory, ♀) and topmouth culter (*Culter alburnus*, TC, carnivory, ♂) is an allodiploid fish and exhibits bisexually fertile (Xiao et al., 2014). Interestingly, previous studies have demonstrated that the gut microbiota communities and gastrointestinal tracts of the hybrid fish are more biased toward herbivores (Li et al., 2018). In addition, after herbivorous diet adaptation, the hybrid fish gut presented decreased expression levels of *fxr* and increased expression levels of *cyp7a1*, and steroid biosynthesis and the mevalonate pathway were activated (Li et al., 2021b), similar to the changes in gene expression after herbivorous diet adaptation in grass carp (Wang et al., 2015). However, to date, little research has been conducted on the diet adaptation of newly formed hybrid fishes. In this study, to better understand the effects of dietary BA supplementation on herbivorous diet adaptation, intestinal histomorphology, digestive enzyme activities, intestinal transcriptome, and metagenome in different feed groups were comparatively analyzed. The results may help elucidate the function of the gut microbiota-bile acid pathway in herbivorous diet adaptation, which provides a theoretical basis for the efficient utilization of plant proteins and ecologically healthy aquaculture in farmed fish.

2. Materials and methods

2.1. Ethics statement

All the experimental procedures were conducted following the standards and ethical guidelines established by the Animal Ethical Review Committee of Hunan Normal University, Changsha, China.

2.2. Preparation of experimental diets

In this study, three different feed groups were set up, namely, the control group (CG), experimental Group 1 (H group), and experimental Group 2 (T group), which were fed a basal diet, a basal diet and duckweed, and a basal diet supplemented with 600 mg kg^{-1} bile acid and duckweed, respectively. After collection, the duckweed was cleaned with ultrapure water to remove invertebrates and broken up before being fed. The main ingredients of the basal diet were fish meal, soybean meal, rapeseed meal, rice bran, wheat meal, fish oil, mineral mixture and vitamin premix. The basal diet formulation and analyzed chemical compositions are shown in Table S1. The BA used in this study was cholic acid (Sangon Biotech, Shanghai, China), and the dosage of the BAs was selected as previously described (Yao et al., 2021). After all ingredients were manually mixed, the dry floating pellets of 0.5 mm diameter were made using a twin-screwed extruder (HQ, Zhaoqing, China). The diets were dried at 25 °C for 24 h and stored at −20 °C until use.

2.3. Experimental fish, feeding, and sampling

The fertilized embryos, larvae, and juveniles from hybridizing female BSB and male TC were all reared at Hunan Normal University, China.

A total of 450 healthy juvenile fish (40 days old; initial body weight, IBW = 0.50 ± 0.05 g) were randomly selected and distributed to nine identical tanks ($n = 50$, 2 m × 1 m × 1 m), with three replicates per feed group, and one-third of the water was replaced daily. Before the feeding experiment, the fish were fasted for 24 h. The fish in each tank were artificially fed twice a day (9,30 and 16:30) to visual satiation for 8 weeks. In addition, the H and T groups were fed an adequate number of crushed duckweed. Throughout the experimental period, a continuous aeration system was employed in each tank, the pH was maintained at 7.5–8.0, the dissolved oxygen (DO) concentration was maintained above 5.0 mg L^{-1}, and the water temperature ranged from 24.0 to 28.0 °C, the total ammonia nitrogen (TAN) concentration was less than 0.2 mg L^{-1}, and the natural photoperiods were maintained during the feeding experiment.

At about 24 h after the last feeding, each group fish was anesthetized with 50 mg L^{-1} MS-222, and the final body weight (FBW) and length were measured. Moreover, the weight gain rate (WGR), average final length (AFL), and specific growth rate (SGR) were calculated. Then, ten fish from each group were randomly selected and sacrificed, and a piece of anterior intestine from each group fish was clipped and fixed in a 4 % paraformaldehyde solution for histological analysis. The remaining intestines were quickly stripped. Subsequently, the intestinal mucosa layers and the contents were carefully scraped and collected for metagenome. The intestines were washed in 1× PBS and collected for transcriptome sequencing. All the samples were immediately frozen in liquid nitrogen after collection and transferred to −80 °C for storage until analysis.

2.4. Histological analysis of the intestinal tract

Histological analysis was performed as described in previous studies (Li et al., 2021b). The section samples were observed and photographed via Leica DM2500 (Leica, Solms, Germany). The thickness of the flat knitting machine (CM), the thickness of the longitudinal muscle (LM), the width of the small intestine villi (VW), the height of the small intestine villi (VH), and the number of goblet cells (GCs) and small intestine villi were measured, counted, and calculated.

2.5. Measurement of intestinal digestive enzyme activity

The intestinal contents from each feed group ($n = 5$) were used to assay indicators of cellulase, α-amylase, protease, and lipase activities. The experiments were conducted in accordance with the standard experimental protocol of commercial kits (Nanjing Jiancheng Bioengineering Institute, China). All assays were repeated three times.

2.6. Transcriptome sequencing and analysis

The total RNA of the intestinal samples ($n = 9$) was extracted using TRIzol reagent (Invitrogen, USA) in a standard experimental protocol. After the concentration and integrity of the RNA were tested and qualified, the library construction was conducted via the Hieff NGS® Ultima Dual-mode mRNA Library Prep Kit for Illumina® (No.13533ES96). Subsequently, the library was sequenced using the PE 150 sequencing platform (Illumina NovaSeq 6000 platform). The raw reads were filtered via fastp (version 0.23.0) to obtain clean reads. A total of 54.49 Gb of clean data were obtained. The complete clean data were uploaded to the NCBI Sequence Read Archive (SRA) database (accession number PRJNA679638). As previously described (Li et al., 2021b; Ren et al., 2019), after the clean data were mapped onto the reference genome (genome assemblies ASM986986v1 and ASM986977v1) via HISAT2 (version 2.0.4), the number of mapped reads in each gene was calculated with some in-house Perl scripts. The transcripts were assembled via StringTie (Version 1.3.4d) (Kim et al., 2015; Pertea et al., 2015). Quantification of gene expression levels was conducted via the FPKM method (Mortazavi et al., 2008). The differential expression genes (DEGs) were identified via the DESeq2(version 2.13) with a screening threshold of p-values <0.05 and absolute Fold Change ≥2. After gene function annotation, function enrichment was analyzed on the platform BMKCloud (www.biocloud.net) and p-value <0.05 was considered to be significant. The co-expression analysis was performed on the platform OmicShare (www.omicshare.com).

2.7. Validation of transcriptome via quantitative real-time PCR

Eight DEGs were selected for quantitative real-time PCR (qPCR) and the primers used are presented in Table S2. The relative expression of genes was calculated using *β-actin* as an internal reference gene via the $2^{-\Delta\Delta Ct}$ method.

2.8. Gut microbiota metagenome sequencing and data analysis

Total DNA was extracted using the CTAB method in a standard experimental protocol. After the concentration and integrity of the DNA were tested and qualified, the library construction was conducted via the VAHTS® Universal Plus DNA Library Pren Kit for Illumina (ND617-C3–02). Subsequently, the library was sequenced using the PE 150 sequencing platform (Illumina NovaSeq 6000 platform). The raw reads were filtered via fastp (version 0.23.0) to obtain clean reads, which were then subjected to further processing to remove any contamination from the host genome via the bowtie2 (version 2.2.4). Metagenome assembly was performed via the MEGAHIT (version 1.1.2) software (Li et al., 2015). The assembly results were evaluated via the software QUAST (version 2.3). Gene identification was subsequently conducted via MetaGeneMark (version 3.26) software (Zhu et al., 2010) with the default parameters. The redundant genes were removed via the MMseqs2 (version 12-113e3) software in order to construct a catalogue of non-redundant genes and the parameter thresholds were set at 95 % similarity and 90 % coverage (Steinegger and Söding, 2017).

The non-redundant genes were aligned to several commonly used databases (including Nr, GO, KEGG, eggNOG, Pfam, and SwissProt databases) to obtain annotation information contained in the corresponding databases via Diamond (version 2.0.15) software with the threshold of e-value <1e-5 (Buchfink et al., 2015). In addition, the protein sequences of non-redundant genes were aligned to the dbCAN database to obtain the annotation information for the carbohydrate-activated enzymes (CAZys) via HMMER (version 3.0) software (Cantarel et al., 2009), and the abundance calculations were referred to the previous studies (Li et al., 2024).

Species composition and relative abundance information was obtained at the taxonomic level from phylum to species, based on annotations obtained from the Nr database. The differences in microbial communities and the identification of biomarkers among the groups were analyzed via Linear discriminant analysis (LDA) and LEfSe software (Segata et al., 2011). The relationships between enzymes (including cellulases, proteases, and lipases) and dominant microbial communities (genus) were analyzed via redundancy analysis and canonical correspondence analysis (RDA/CCA analysis) (Park et al., 2021). Function enrichment analysis were conducted on the platform BMKCloud (www.biocloud.net) and p-value <0.05 was considered to be significant.

To investigate the relationship between changes in intestinal gene expression and intestinal microbiota after dietary supplementation BAs and enhanced herbivory adaptations in hybrid fish, an integration analysis of DEGs, differential microbial communities, and shared significantly enriched KEGG pathways identified in gut transcriptomes and metagenomes was performed.

2.9. Statistical analysis

The data are presented as the mean ± the standard error of the mean (SEM). Statistical analyses were conducted via SPSS 20.0 and the data were checked for normality and homogeneity of variance before analysis. All the data were analyzed via one-way ANOVA and Duncan's multiple comparisons. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Growth performance analysis

Table 1 indicated that dietary BA supplementation significantly enhanced the growth performance of juvenile hybrid fish ($P < 0.05$). The parameters of FBW, WGR, SGR, and AFL of the T group were significantly increased in comparison to those of the CG and H groups ($P < 0.05$).

3.2. Intestinal histomorphology analysis

The intestinal histomorphology is presented in Fig. S1 and Table 2. The results revealed that CM was significantly greater in the T group than in the CG and H groups ($P < 0.05$); LM and VH both presented a trend of T > H > CG, and there was a significant difference between the CG and T groups ($P < 0.05$). The number of GCs was T > H > CG, and there was a significant difference among all the groups ($P < 0.05$). No significant difference was observed in VW among the three groups of fish ($P > 0.05$).

3.3. Intestinal digestive enzyme activity analysis

The results of the enzyme activity analysis are presented in Fig. 1.

Table 1
The effects of dietary BA supplementation on growth performance in juvenile hybrid fish[1].

Item	Groups		
	CG	H	T
IBW (g)[2]	0.50 ± 0.05	0.50 ± 0.05	0.50 ± 0.05
FBW (g)[2]	1.91 ± 0.20[b]	2.06 ± 0.18[b]	2.91 ± 0.29[a]
WGR (%)[3]	282.86 ± 40.65[b]	312.86 ± 35.61[b]	481.33 ± 58.29[a]
SGR (%/day)[4]	2.39 ± 0.18[b]	2.53 ± 0.16[b]	3.14 ± 0.17[a]
AFL (cm)[5]	6.57 ± 0.41[b]	6.88 ± 0.21[b]	7.71 ± 0.41[a]

[1] Values were reported as mean ± SEM ($n = 10$). [a, b] Values with different letters within the same row are significantly different ($P < 0.05$).
[2] IBW (g) is initial body weight, and FBW (g) was final body weight.
[3] WGR (weight gain rate, %) = (FBW - IBW) / IBW × 100 %.
[4] SGR (specific growth rate, %/day) = [ln (FBW) – ln (IBW)] / days × 100 %.
[5] AFL (cm) was average final length.

Table 2
The effects of dietary BA supplementation on the intestinal histomorphology in juvenile hybrid fish[1].

Item	Groups		
	CG	H	T
CM thickness (μm)[2]	47.26 ± 1.55^{b}	47.01 ± 0.89^{b}	52.50 ± 1.03^{a}
LM thickness (μm)[2]	16.61 ± 1.92^{b}	18.13 ± 0.78^{ab}	19.39 ± 0.57^{a}
VH (μm)[2]	279.34 ± 10.07^{b}	282.48 ± 5.60^{ab}	299.31 ± 9.00^{a}
VW (μm)[2]	68.83 ± 2.73	68.58 ± 0.94	68.18 ± 2.27
small intestine villi number	20.03 ± 0.96^{b}	22.33 ± 1.53^{ab}	23.33 ± 2.08^{a}
The number of GC [2]	121.00 ± 7.94^{c}	141.67 ± 7.57^{b}	157.67 ± 3.51^{a}

[1] Values were reported as mean ± SEM (n = 3). [a, b, c] Values with different letters within the same row are significantly different ($P < 0.05$).
[2] CM: flat knitting machine; LM: longitudinal muscle; VH: the height of small intestine villi; VW: the width of small intestine villi; GC: goblet cell.

The results showed that significantly higher intestinal cellulase activity and significantly lower lipase activity in the T group in comparison to the CG and H groups ($P < 0.05$), and no difference was detected between the CG and H groups ($P > 0.05$). The intestinal protease activity was significantly higher in the T and H groups than in the CG ($P < 0.05$). Although the intestinal α-amylase activity among the groups tended to decrease in the order of T > H > CG, no significant difference was observed between the groups of fish ($P > 0.05$).

3.4. DEG identification and functional enrichment

A total of 54.49 Gb clean data were obtained (Table S3). A total of 1156 DEGs were identified in the H group compared with the CG group, including 763 upregulated and 393 downregulated genes. Similarly, 1230 DEGs were identified in the T group compared with the CG group, comprising 779 upregulated and 451 downregulated genes (Fig. 2A). Table 3 shows the top 10 up-regulated and top 10 down-regulated DEGs in H compared to CG, and the top 10 up-regulated and top 10 down-regulated DEGs in T compared to CG. The specific DEGs between CG and T were identified, and there were detected 360 upregulated and 351 downregulated genes (Fig. 2B and C).

GO function enrichment analysis revealed that the most upregulated DEGs were related to cytoplasm, binding, protein binding, extracellular region, and response to external stimulus. In contrast, the most downregulated DEGs were enriched in obsolete cytoplasmic part, catalytic activity, small molecule metabolic process, oxidoreductase activity, and oxidation-reduction process (Table S4 and Fig. S2). KEGG enrichment analysis revealed that the upregulated DEGs were enriched mainly in MAPK signaling pathway, protein digestion and absorption and mTOR signaling pathway (Fig. 2D), whereas the downregulated DEGs were enriched predominantly in steroid hormone biosynthesis, fatty acid metabolism and primary bile acid biosynthesis (Fig. 2E).

The expression patterns of all the co-expressed genes could be divided into 8 patterns, among which three trends were significant ($P < 0.05$), and 1302,1038 and 628 genes were enriched, respectively (Fig. 3A). Interestingly, we identified several important genes that were specifically expressed in the T group, such as *irs*, *jak*, *egfr*, *prss*, and *cpa*, whose expression was upregulated, and *srebp1*, *elovl6*, *cpt1*, *apoe*, *cyp7a1*, *cyp8b1*, *cyp27a1*, and *hmgcr*, whose expression was downregulated (Supplementary material 1).

3.5. qPCR validation

To validate the RNA-seq data, we selected 8 DEGs (*elovl6*, *cyp7a1*, *srebp1*, *hmgcr*, *cpa*, *irs*, *prss*, and *cpt1*) for qPCR. The qPCR results were consistent with the RNA-seq data, confirming the reliability of the RNA-seq data (Fig. 3B).

3.6. Gut microbiota

The Ace and Shannon indices tended to decrease in the order of T > H > CG (Fig. 4A and B), suggesting that dietary supplementation with duckweed and BA increased the diversity of the gut microbiota. The relative abundances of the top 10 microorganisms at the phylum and genus levels are presented in Fig. 4C and D, respectively. At the phylum level, the most dominant microorganisms were *Proteobacteria*, *Mucoromycota*, *Actinobacteria*, *Firmicutes,* and *Basidiomycota*; at the genus level, the top 5 microbiota were *Acinetobacter*, *Micromonospora*, *Rhizophagus*,

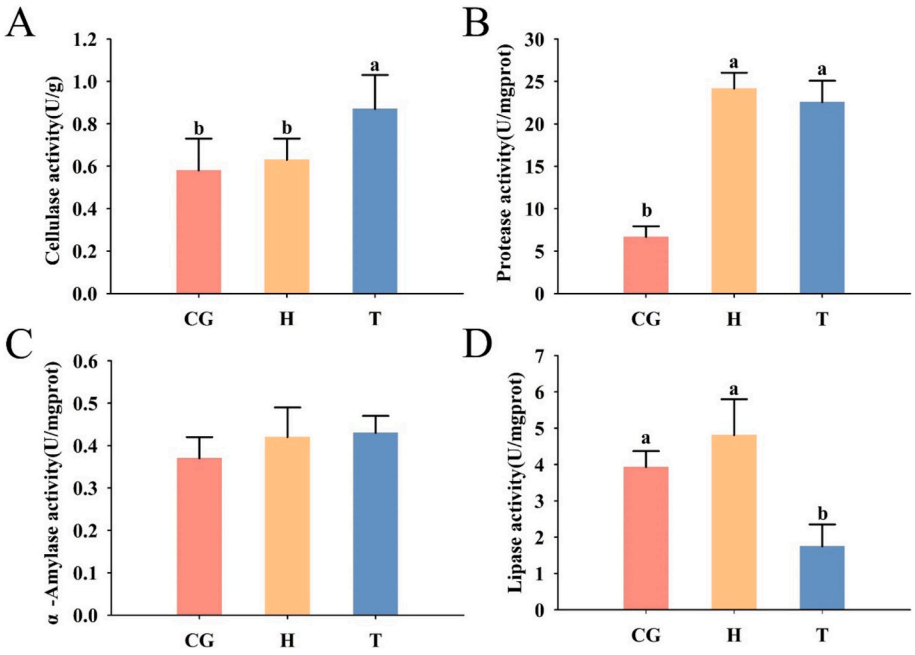

Fig. 1. The effect of dietary BA supplementation on the activity levels of cellulase, protease, α-amylase, and lipase in the intestine. (A) Cellulase activity. (B) Protease activity. (C) α-Amylase activity. (D) Lipase activity. [a, b] Values with different letters in the same figure are significantly different ($P < 0.05$).

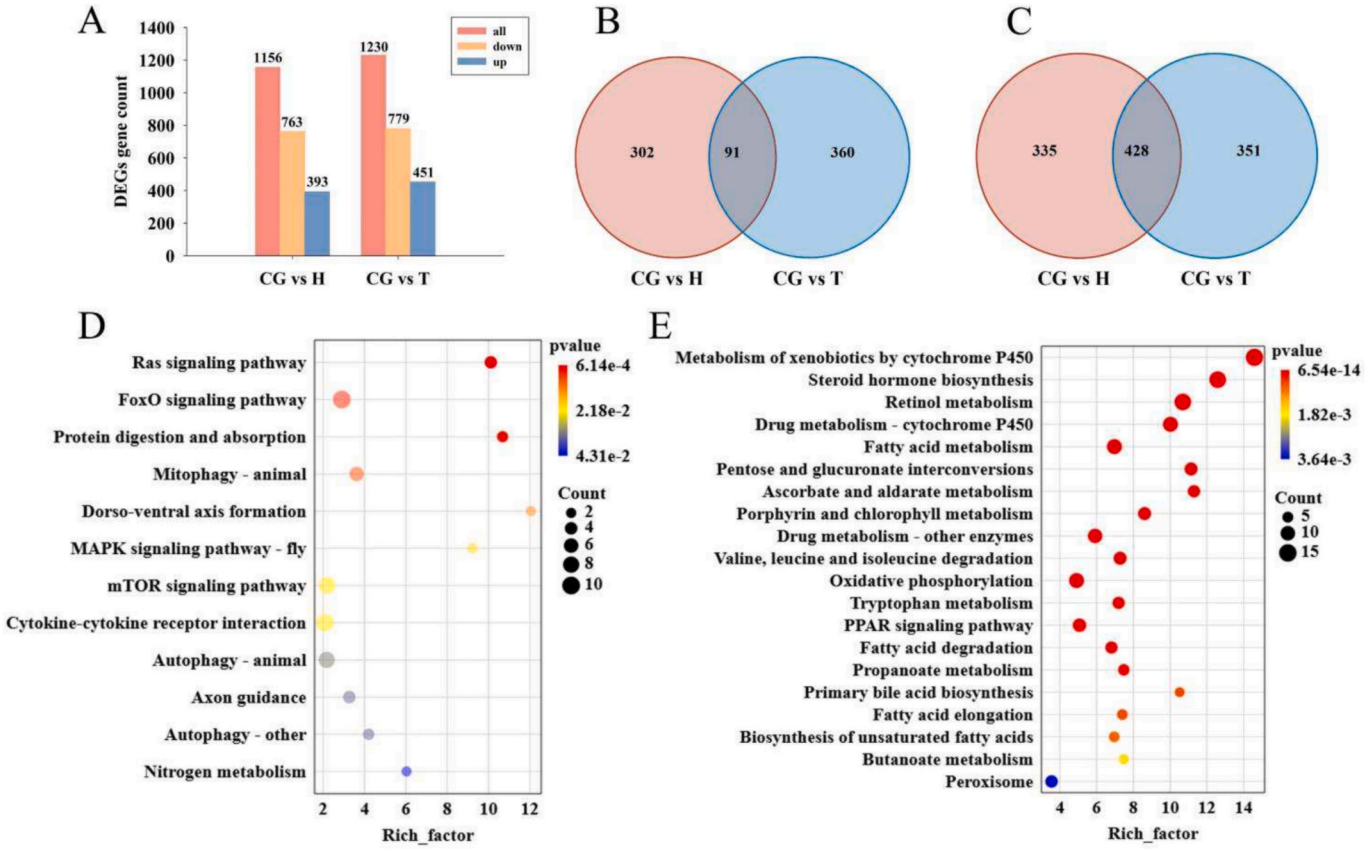

Fig. 2. Identification and KEGG enrichment analysis of DEGs in intestinal tissues of different feed groups. (A) The number of DEGs in the three groups, red represents the total DEGs, yellow represents the downregulated genes, and blue represents the upregulated genes. (B) CG vs T specific up-regulated DEGs identified by Venn diagram. (C) CG vs T specific down-regulated DEGs identified by Venn diagram. (D) TOP 20 pathway in up-regulated DEGs. (E) TOP 20 pathway in down-regulated 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
the top 10 up-regulated and down-regulated DEGs in H compared with those in CG and in compared with those in CG.

CG vs H			CG vs T		
KO	Gene	Log2(Fold Change)	KO	Gene	Log2(Fold Change)
K09228	KRAB	13.33	K06785	JAM3	14.48
K04613	V2R	8.62	K00432	gpx	9.75
K10415	DYNC1I	7.17	K06511	CD84	9.7
K09047	CREB5	6.71	K22614	NOD3	8.03
K01020	CHST3	6.71	K22017	MUC4	7.70
K19655	SPATA7	6.68	K01370	CASP1	7.65
K08790	STK38	6.59	K08019	RAPGEF5	7.54
K22388	NAB	6.55	K05512	CCL19	7.17
K04496	CTBP	6.43	K06058	DTX	7.04
K14802	DRS2	6.31	K21918	KCTD	6.39
K12800	NLRP3	−12.38	K09228	KRAB	−9.67
K14480	APOL	−10.76	K16634	RNH1	−9.61
K10639	HEI10	−7.47	K12800	NLRP3	−9.14
K06267	HMMR	−7.24	K13907	CST	−8.75
K04257	OLFR	−7.14	K10798	PARP	−8.64
K08202	OCTN	−6.67	K20865	NLRP12	−8.36
K01365	CTSL	−6.65	K09228	KRAB	−8.22
K03985	PLAUR	−5.92	K12800	NLRP3	−8.18
K23531	CABP	−5.22	K11508	CENPP	−7.81
K01047	PLA2G	−5.13	K12004	TRIM14	−7.65

Pseudomonas, and *Epulopiscium*. The relative abundances of *Acinetobacter*, *Rhizophagus*, and *Pseudomonas* gradually decreased, and the relative abundances of *Micromonospora* and *Epulopiscium* gradually increased with the dietary supplementation with duckweed and BA.

From the phylum-to-genus level, a total of 51 specific biomarkers with a LDA score exceeding 3 were identified in the intergroup comparisons (CG vs T, H vs T, and CG vs H) (Fig. 5A-C). Compared with the CG, the H group presented 6 specific biomarkers, and the T group presented 31 specific biomarkers, indicating that BA significantly affects the species composition of the gut.

RDA/CCA analysis revealed that cellulase activity was positively correlated with the *Micromonospora* and *Pseudomonas* in the T group (Fig. 5D). Similarly, protease activity was positively correlated with the *Micromonospora*, *Enterococcus,* and *Epulopiscium* in the T and H groups. Additionally, lipase activity was positively correlated with the majority of the dominant microbial communities observed in the CG and H groups.

The analysis of the genes encoding CAZy revealed that the T group was characterized by a greater abundance of CAZy genes encoding enzymes that degrade cellulose in comparison to the CG and H groups (Table 4). The top 10 KEGG pathways and the top 30 KOs that enriched with the most functional genes with significant differences were screened (Fig. 6A and B). The significant pathways were glycerophospholipid metabolism; ubiquitin mediated proteolysis; degradation of aromatic compounds; and naphthalene degradation in the T group, whereas mismatch repair; pantothenate and CoA biosynthesis; alanine, aspartate and glutamate metabolism; and phenylalanine, tyrosine and tryptophan biosynthesis were enriched in the CG group. Overall, the H group can be considered to fall between the other groups.

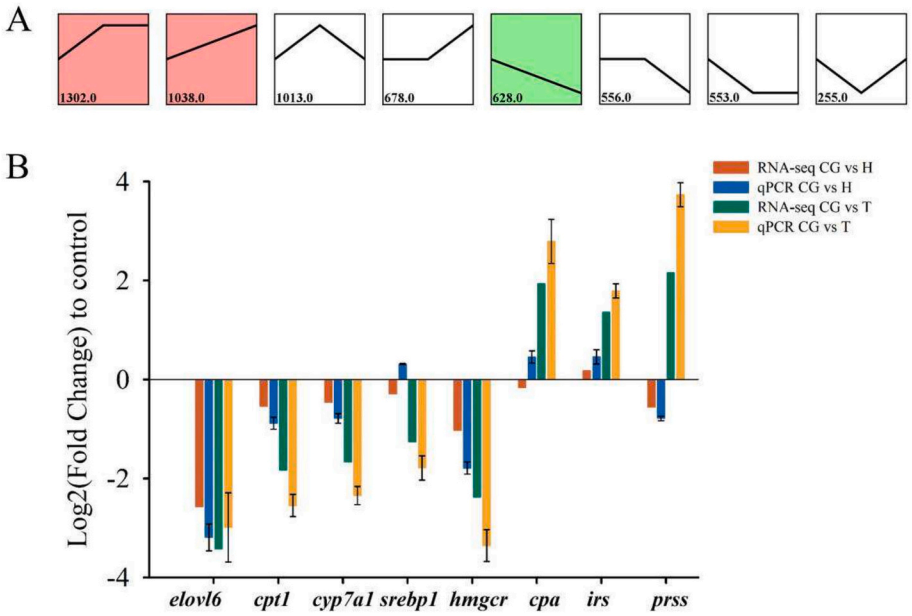

Fig. 3. Result of trend analysis for all the co-expressed genes and validation of RNA-Seq using RT-PCR. (A) Eight expression patterns of the genes. (B) Validation of RNA-Seq results with qPCR.

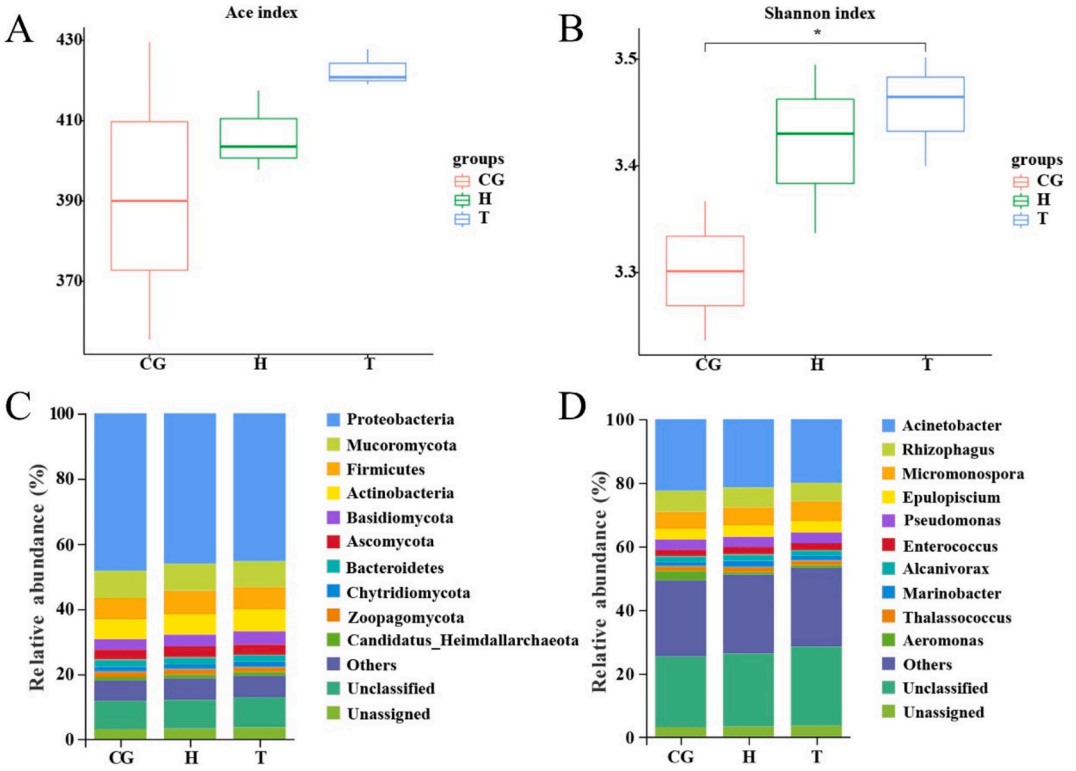

Fig. 4. Alpha diversity indices and relative abundance of the gut microbiota in juvenile hybrid fish from different feed groups. (A) Ace index. (B) Shannon index. (C) The relative abundance of the TOP 10 gut microbiota at the phylum. (D) The relative abundance of the TOP 10 gut microbiota at the genus. CG, basal diet; H, basal diet and duckweed; T, basal diet supplemented with BA and duckweed.

3.7. Integration analysis of gut transcriptome and metagenome

Fig. 7 showed the potential interactions between the gut microbiota and host intestinal genes, and their potential function in contributing to herbivorous diet adaptation. Modifications in the function of the gut microbiome and the expression of host intestinal genes influence the levels of enzymes, short-chain fatty acids (SCFAs), cytokines (CK), and other metabolites (Fig. 7). These modifications positively affect the herbivorous diet adaptations of the fish and contribute to growth and development, and digestive and metabolism functions.

4. Discussion

In this study, dietary supplementation with 600 mg kg^{-1} BA

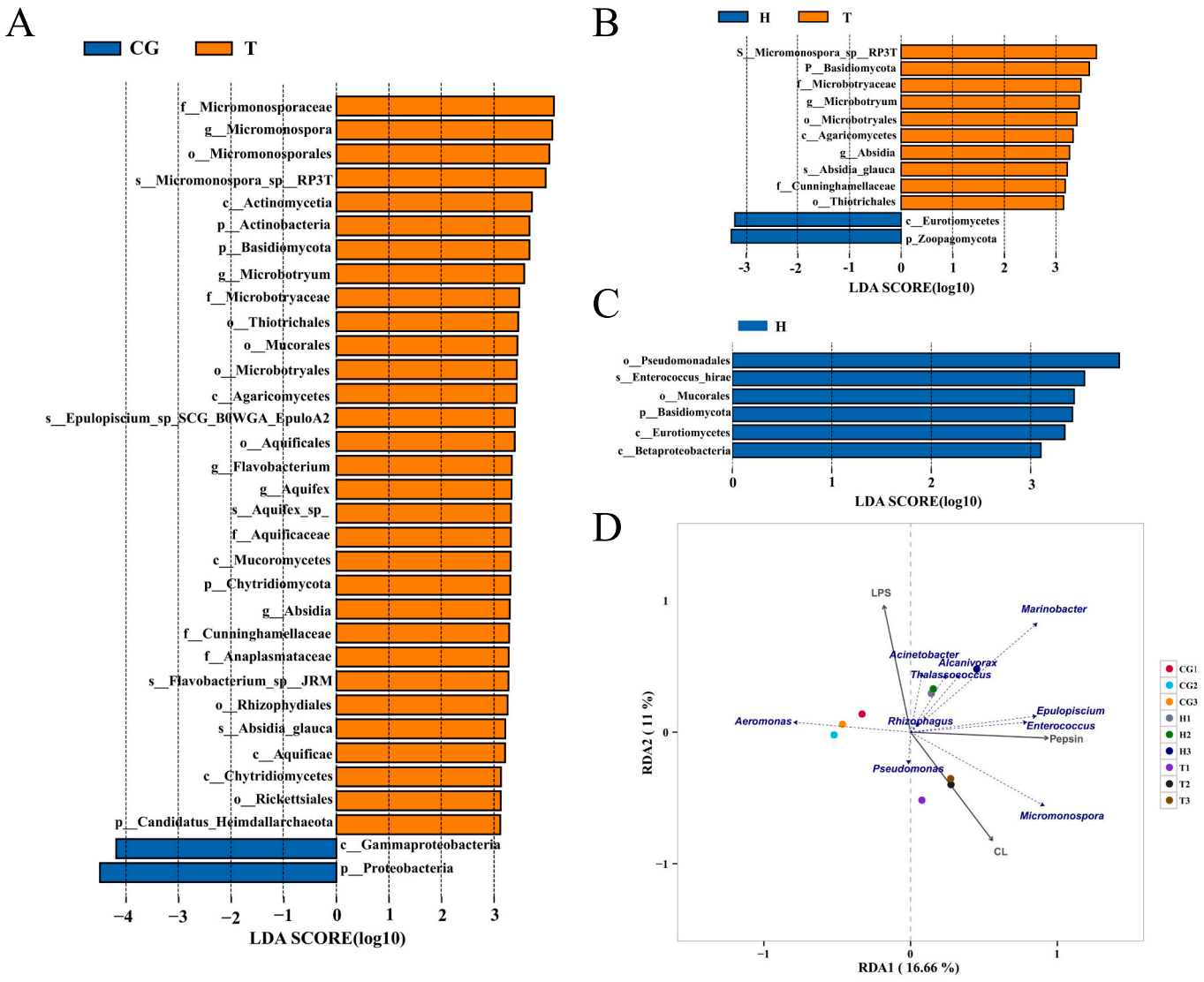

Fig. 5. Identification and correlation analysis of biomarker microbes in the juvenile hybrid fish from different feed groups. Linear discriminant analysis (LDA) coupled with effect size (LEfSe) analysis of the gut microbiota between CG, H, and T. (A) CG vs T. (B) H vs T. (C) CG vs H. (D) Redundancy analysis/Canonical correspondence analysis (RDA/CCA) showing the correlation of three intestinal enzymes and dominant microbial taxa among the three groups of hybrid fish.

Table 4
Relative abundance statistics of cellulose-related CAZy families.

CAZy Family	Groups		
	CG	H	T
AA10	8.67 ± 2.51	9.00 ± 1.72	9.33 ± 2.31
GH1	106.33 ± 26.08^{b}	261.33 ± 29.50^{b}	577.00 ± 155.09^{a}
GH3	17.67 ± 2.52^{c}	37.67 ± 6.81^{b}	51.67 ± 6.43^{a}
GH5	13.67 ± 3.06	13.33 ± 5.51	21.00 ± 2.65

Values were reported as mean ± SEM (n = 3). $^{a, b, c}$ Values with different letters within the same row are significantly different ($P < 0.05$).

significantly increased growth performance indices (WGR, SGR, and AFL) in juvenile hybrid fish compared with those in the CG and H groups (Table 1). Similar results were obtained for largemouth bass (*Micropterus salmoides*), rice field eel (*Monopterus albus*), and grass carp (*Ctenopharyngodon idella*) (Lei et al., 2023; Peng et al., 2019; Yin et al., 2021). The growth-promoting effect of BAs has been demonstrated to correlate with increased digestive enzyme activity. However, the present study showed that 600 mg kg^{-1} BA had no significant effect on amylase or protease ($P > 0.05$) (Fig. 1). In tongue sole (*Cynoglossus semiliaevis*) and thinlip mullet (*Liza ramada*), supplementation with appropriate levels of BAs increased amylase and protease activity, while in largemouth bass (*Micropterus salmoides*) observed the opposite results (Guo et al., 2024; Li et al., 2021a). Notably, exogenous BA significantly increased the intestinal cellulase activity but decreased the lipase activity (Fig. 1). This phenomenon was also detected in thinlip mullet (*Liza ramada*), and the changes of digestion enzymes may increase the digestibility of food (Abdel-Tawwab et al., 2023). Thus, the effects of dietary BA supplementation on intestinal digestive enzymes are related to differences in a variety of aspects, such as fish species, developmental stages, and feeding conditions (Jiao et al., 2023).

The results of the intestinal histomorphology revealed that the intestinal tract of hybrid juvenile fish thickened, the small intestinal villi lengthened, the intestinal wall muscles became more developed, and the number of goblet cells increased during the process of herbivory adaptation to adapt to changes in dietary habits (Table 2 and Fig. S1). These changes could increase intestinal peristalsis, enhance intestinal grinding and digestive capacity, and improve adequate digestion and absorption

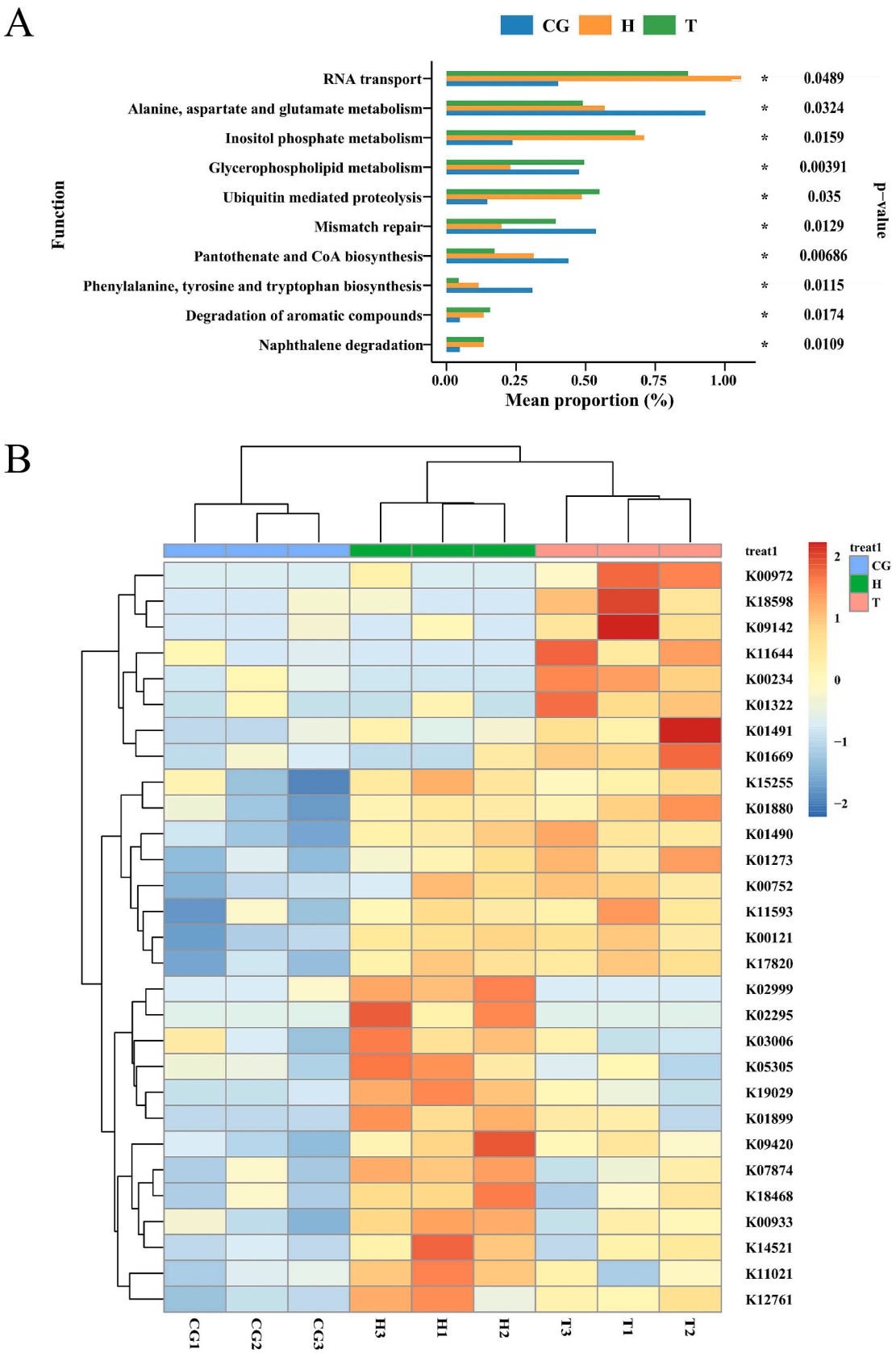

Fig. 6. Identification and analysis of functional genes of gut microbiota in juvenile hybrid fish from different feed groups. (A) KEGG enrichment of differential functional genes. (B) TOP 30 KOs enrichment and abundance heat map of differential functional genes.

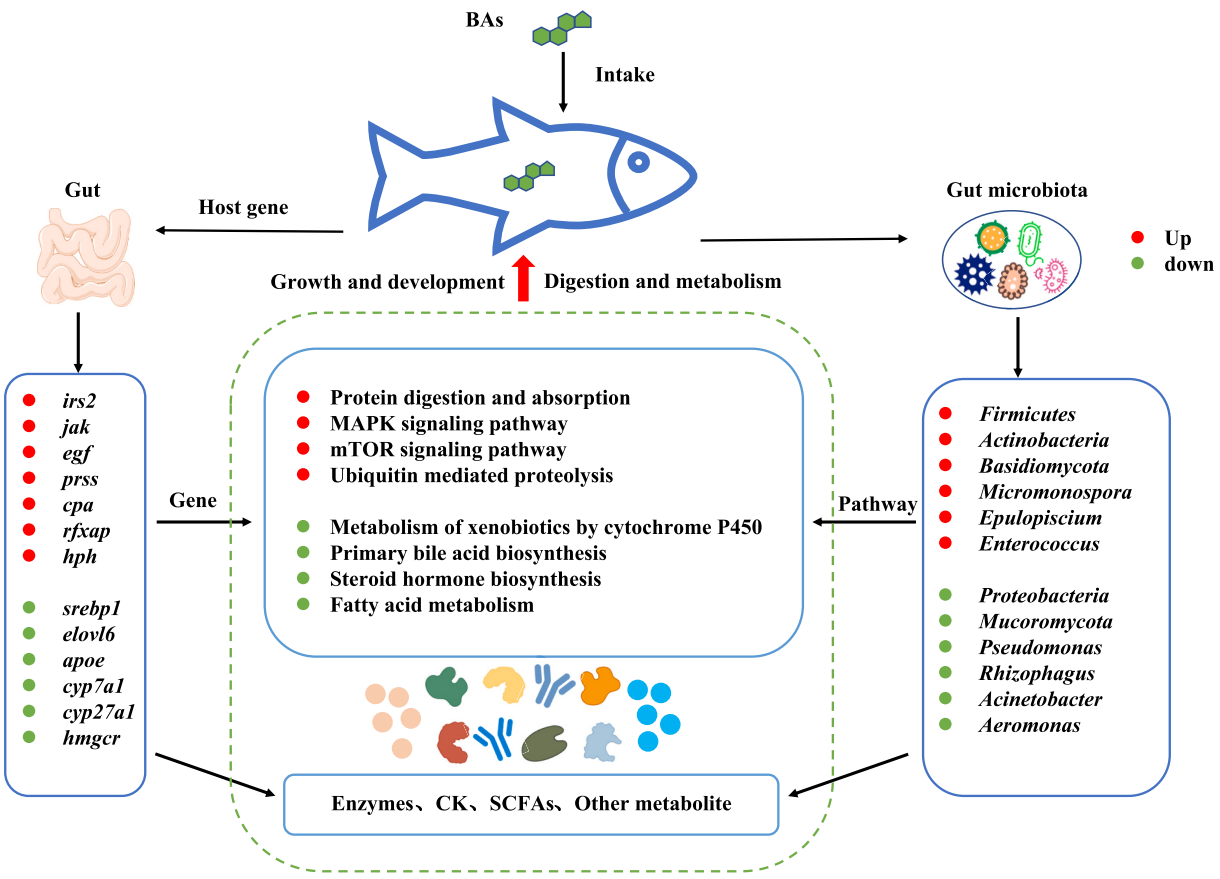

Fig. 7. Integration analysis of gut transcriptome and metagenome.

of plant food (Gustafsson and Johansson, 2022), whereas dietary BA supplementation may significantly accelerate this process.

Several genes including *irs*, *jak*, and *egfr,* which are associated with cell proliferation and growth, were significantly higher expressed in the intestinal tissue of the T group fish. In addition, the expression of the genes *prss* and *cpa*, which are associated with protein digestion and absorption, was specifically upregulated in the T group, in which PRSS plays a key role in the activation of other digestive enzyme and the hydrolysis of proteins (Dam et al., 2020). Those genes were also identified higher expression in grass carp during herbivorous diet transition (He et al., 2015). Besides, the upregulated DEGs were significantly enriched in some pathways that related to cell proliferation and growth, and nutrient digestion and absorption (Fig. 2D) (Fang and Richardson, 2005; Liu and Sabatini, 2020). The above findings indicate that dietary BA supplementation can increase the expression levels of the corresponding signaling pathways by increasing the expression of key genes, thereby promoting the growth of the gut and the digestion and absorption of nutrients in juvenile hybrid fish, thus enabling them to adapt to herbivory and contributing to the improvement of growth performance.

Recent studies have demonstrated dietary BA supplementation can reduce the expression of lipid synthesis genes and, promote lipid oxidative degradation genes (Yao et al., 2021; Zhang et al., 2022). In this study, there detected the lipid synthesis genes, *srebp1,* and *elovl6* were significantly downregulated, while the lipid oxidative degradation genes, *cpt1,* and *apoe* were also downregulated in the T group. This may result from the fish diet changing from artificial feed to duckweed, and selective gene expression occurs in the organism in response to the change in food intake. Studies in Atlantic salmon (*Salmo salar* L.) and European seabass (*Dicentrarchux labrax*) have also demonstrated that organisms may selectively express genes to regulate their metabolic pathway in response to varying dietary conditions (Król et al., 2016; Leduc et al., 2018).

BAs, represent the primary route of cholesterol excretion from the body and directly affect the body's cholesterol and BAs metabolism (Hofmann and Hagey, 2008). Dietary BA supplementation usually downregulates the expression of the bile acid synthesis genes *cyp7a1* and/or *cyp8b1* (Yin et al., 2021; Yu et al., 2019). This study also detected dietary BA supplementation reduced the expression of *cyp8b1*, *cyp7a1*, and *hmgcr*. The result suggested that exogenous BA may affect cholesterol and BAs metabolism in the hybrid fish by reducing the expression of *cyp7a1* (the rate-limiting enzyme for BA synthesis), *hmgcr* (a key enzyme for cholesterol synthesis), and *cyp8b1* (regulate the ratio of CA and CDCA) (Chiang, 2009).

The gut microbiome plays an important role in the health and metabolism of fish. For example, *Pseudomonas* and *Mucoromycota* usually cause bacterial diseases; *Firmicutes*, *Micromonospora,* and *Actinobacteria* can produce beneficial secondary metabolites in fish (Barka et al., 2015; Litvak et al., 2018). In this study, dietary BA supplementation increased the diversity and abundance of the gut microbiota. Meanwhile, the abundance of potentially pathogenic bacteria such as *Mucoromycota* and *Proteobacteria* was reduced, and the abundance of cellulase-producing bacteria such as *Actinobacteria* and *Micromonospora* was increased (Fig. 4) (Chen et al., 2020; Tran et al., 2017). In addition, a number of biomarkers associated with the degradation of abundant and complex polysaccharides degradation, including *Epulopiscium*, *Micromonospora*, *Rhizophydiales* and *Chytridiomycota* (Letcher et al., 2008; Ngugi et al., 2017), were identified in the T group. This may facilitate the adaptation of the hybrid fish to herbivorous diets. Besides, dietary BA supplementation increased the abundance of certain enzyme families associated with cellulose degradation (Table 4), including GH1, GH3, GH5, and AA10 (Eijsink et al., 2019; Li et al., 2024). In addition, the functional genes significantly enriched in carbohydrate metabolism, protein hydrolysis, energy utilization, and genetic information

processing and handling, suggesting that dietary BAs affected the structure of the gut microbiota, and the dynamic changes of those microbiota may involve in adapting to the herbivorous diet and growth performance improvement (Fig. 6) (Kashinskaya et al., 2018; Parris et al., 2019).

In recent years, the gut microbiota and the host have been recognized as an interacting symbiosis (Lynch and Hsiao, 2019), and the dietary habits of the host can exert a significant influence on the composition of the gut microbiota (Kolodziejczyk et al., 2019). This means that juvenile hybrid fish undergoing herbivorous diet adaptation may alter the composition of the gut microbiota, including through enrichment of cellulase-producing flora, among others. In addition, host gut gene expression is altered to varying degrees during this process. The collective impact of these disparate elements gives rise to modifications in the function of the gut microbe-host symbiosis (Fig. 7). However, the potential mechanism of microbe-host interactions contributing to diet transition and adaptation needs further investigation.

5. Conclusion

In summary, our results showed that dietary supplementation with 600 mg kg^{-1} BA significantly enhanced growth performance, improved intestinal digestive ability, and promoted the adaptation of allodiploid hybrid fish to plant foods. The effect of BAs on herbivorous diet adaptation in hybrid fish may be achieved by promoting the expression of genes related to intestinal growth, and digestion and absorption, and enriching with probiotics and reducing pathogenic flora, thereby altering the function of the gut microbiome. The results of this study may provide a theoretical basis for how to efficiently utilize plant proteins in farmed fish as well as for ecologically healthy farming.

CRediT authorship contribution statement

Zexun Zhou: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ye Yuan:** Visualization, Validation, Software, Investigation. **YunYun Liu:** Visualization, Validation, Software. **Shandong Chen:** Visualization, Software, Investigation. **Yongchun Li:** Visualization, Software, Investigation. **Yan Miao:** Validation, Investigation. **Shi Wang:** Visualization, Resources, Investigation. **Zhongyuan Shen:** Visualization, Data curation. **Lei Zeng:** Visualization, Data curation. **Li Ren:** Visualization, Data curation. **Chang Wu:** Writing – review & editing, Data curation. **Qizhi Liu:** Writing – review & editing, Data curation. **Qinbo Qin:** Writing – review & editing, Data curation. **Wuhui Li:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Shaojun Liu:** Writing – review & editing, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2025.742506.

Data availability

Data will be made available on request.

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