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Analyzing the influence of *Clostridium butyricum* on liver health in spotted sea bass (*Lateolabrax maculatus*) via transcriptomics and metabolomics

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Abstract
Clostridium butyricum (CB) has received much attention as a probiotic; however, few studies have focused on its effects on liver health. Here, we studied the influence of CB on the liver health of spotted sea bass through transcriptomics and metabolomics studies and preliminarily explored its molecular mechanisms. This study showed that CB significantly reduced hepatic aspartate aminotransferase (AST) activity and increased alkaline phosphatase (AKP) and acid phosphatase (ACP) activity ($P < 0.05$). CB has demonstrated significant effects in strengthening liver immunity and can increase hepatic amylase and trypsin activities and promote hepatic catabolism of carbohydrates and amino acids. Integration of the liver transcriptomics and metabolomics showed altered transcript levels of mainly gluconeogenic, lipogenic, and amino acid metabolic pathways. It regulated the abundance of metabolic biomarkers such as arachidonate, crotonyl-CoA, and D-glucose 1-phosphate. Our findings support that CB can reduce liver damage in spotted sea bass, enhance liver immunity, and improve liver metabolism.

Keywords *Clostridium butyricum* · Spotted sea bass · Liver · Transcriptomics · Metabolomics

Introduction

Clostridium butyricum (CB), as a gram-positive anaerobic bacterium, is highly resistant to the external environment and can be found in natural soil, fermented dairy products, and the intestinal tract of animals. Within the animal gut, CB secretes amylase to enhance carbohydrate digestion and absorption, produces butyric acid to improve gut immunity, and inhibits the growth of pathogenic bacteria in the intestines (Sun et al. 2020). Currently, the beneficial properties of CB as a feed additive have been widely recognized. Research has indicated that CB can improve the gut microenvironment, reduce the incidence of liver inflammation, and enhance intestinal immunity by regulating liver and immune genes

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(Yamamoto et al. 2022). Not only can CB improve the inflammatory microenvironment at the genetic level, but it can also enhance liver function by impacting liver metabolism (Mencarelli et al. 2013; Mun et al. 2022). Furthermore, CB also enhances the antioxidant function in animals. When added to the diet of hybrid groupers, it was found to improve growth performance, antioxidant capacity, and increase the abundance and diversity of beneficial gut bacteria (Yang et al. 2023). CB can also enhance the intestinal mucosal barrier of turbot, reduce intestinal inflammatory factors, and thus improve gut health (Bi et al. 2023). Therefore, CB has various functions such as promoting growth, enhancing immunity, improving antioxidant capacity, and modifying the gut microbiota.

Currently, the primary research is centered around investigating the effects of CB on the intestinal system. However, there is a relative scarcity of studies examining its impact on the liver of fish. However, as an important organ in the fish body, the liver plays a crucial role in metabolism, detoxification, immune response, excretion, and digestion processes (Manco and Itzkovitz 2021). During aquaculture, the liver is susceptible to various environmental factors, dietary intake, and pathogenic microorganisms, which can lead to liver metabolism and immune function disorders, ultimately reducing the growth performance of cultured animals and even resulting in animal deaths. For instance, external environmental stimuli can trigger oxidative stress and inflammatory responses, leading to liver damage and adverse effects on its physiological functions (Gan et al. 2022). Hormones within the animal body can influence the liver by regulating metabolic homeostasis and stress responses, ultimately resulting in the occurrence of fatty liver and hepatic inflammation (Chan et al. 2020). Furthermore, changes in external environmental conditions and the influence of pathogenic microorganisms may lead to an increase in the production of reactive oxygen species, ultimately resulting in liver oxidative damage (Abasubong et al. 2018; Slaninova et al. 2009). In summary, it is of significant importance to explore the alleviation and treatment methods for liver damage and functional impairment in fish. Therefore, it is of great significance to investigate the impact of *Clostridium butyricum* on the liver of sea bass through transcriptomics and metabolomics analyses.

In conclusion, this study analyzed the effect of CB on spotted sea bass from the perspective of liver health, which can further enhance our understanding of the role of CB. This study designed different levels of CB feed and explored the effects of CB on the liver health of sea bass through transcriptome and metabolome analyses. The research results can further provide a theoretical basis for the application of CB, thereby promoting the high-quality development of the aquaculture industry.

Materials and methods

Diet formulation

CB (1×10^9 cfu/g) was added into the basal diet of spotted sea bass with 0 (CC) for the control group, and 0.1% (CB1), 0.2% (CB2), 0.3% (CB3), 0.4% (CB4), and 0.5% (CB5) for the experimental group and blended with flour, and the additive CB was purchased from Henan Jinbaihe Biotechnology Co., Ltd. The formulation and main components of the basic diet are shown in Table 1. The feed ingredients were superfine crushed through 60 mesh sieves, and the ingredients were mixed well according to the ratio of quantification. The feed was extruded by a twin-screw extruder with a 3-mm particle size, dried in a

Table 1 The formulation and main components of the basic diet

Ingredients	Contents (%)
Fish meal	49.0
Soybean meal	23.5
Flour	15.0
Yeast powder	3.0
Fish oil	3.0
Soybean oil	2.0
Lecithin	1.0
Mineral premix (1)	0.6
Vitamin premix (2)	0.8
Choline	0.6
$Ca(H_2PO_4)_2$	1.2
Antioxidant	0.3
Nutrient composition	
Crude protein	46.13
Crude fat	9.94
Ash	9.23
Crude fiber	1.58
Moisture	8.09
Nitrogen-free extract	26.45
Gross energy (kJ/kg)[4]	16.56
Protein-energy ratio (mg prot/kJ)	27.85

(1) Mineral premix contains $MnSO_4 \cdot 4H_2O$ 50 mg/kg, $MgSO_4 \cdot H_2O$ 4000 mg/kg, $CuSO_4 \cdot 5H_2O$ 20 mg/kg, $ZnSO_4 \cdot H_2O$ 150 mg/kg, $CoCl_2$ (1%) 100 mg/kg, $FeSO_4 \cdot H_2O$ 260 mg/kg, KI 100 mg/kg, and Na_2SeO_3(1%) 50 mg/kg

(2) Vitamin premix contains thiamine 25 mg/kg, riboflavin 45 mg/kg, pyridoxine hydrochloride 20 mg/kg, VB_{12} 0.1 mg/kg, VK_3 10 mg/kg, inositol 800 mg/kg, niacin 200 mg/kg, pantothenic acid 60 mg/kg, folic acid 20 mg/kg, biotin 1.2 mg/kg, VD_3 5 mg/kg, and α-tocopherol 120 mg/kg. VA acetate 32 mg/kg and ethoxy quinoline 150 mg/kg

ventilated oven at 55 °C for approximately 12 h, and then stored in a refrigerator at −20 °C for storage.

The nutrient composition of the basal diet was determined according to the Chinese state standard. In brief, the crude protein, crude fat, ash, crude fiber, and moisture were analyzed with reference to GB6432-2018, GB6433-2006, GB6438-2007, GB6434-2006, and GB6435-2014, respectively, as described in detail in the previous research (Yang et al. 2019).

Experimental procedure

Spotted sea bass were purchased from a nursery in Zhangzhou, and the aquaculture experiment was completed at the aquaculture farm of Jimei University. Purchased juvenile fish were temporarily reared in 1600-L-cylindrical PVC drums for 2 weeks to observe the swimming condition and feeding of the fish. Samples of the muzzle, gills, and body surface

were examined to ensure that the fish were free of disease, injury, parasites, vigor, and feeding. A total of 450 fish of the same size (initial mean weight of 9.58 ± 0.05) were randomly divided into 6 groups of 3 replicates each. A total of 18 tanks were allocated for the experiment. In addition, the incubation period was 54 days, with two regular feedings per day (8:30 am and 5:30 pm). Meanwhile, 30–40% of the water would be replaced daily to maintain the stability of water quality. During the period, the water temperature was 25 ± 0.1 °C. Nitrite concentration was 0.020 ± 0.005 mg/L; ammonia concentration was 0.18 ± 0.01 mg/L; pH was 8.0 ± 0.3; and dissolved oxygen concentration was 7.0 ± 0.3 mg/L. The water quality was maintained at the same time.

Sample collection

At the end of the culture, the spotted sea bass were fasted for 24 h. After fasting, the fish in each tank were anesthetized with 150 mg/L of eugenol (Holloway et al. 2004). A total of eleven fish were randomly selected from each tank. The liver of each fish was separated, and the attached adipose tissue was rinsed with 0.86% chilled saline, placed in liquid nitrogen for rapid freezing, and then transferred to a −80 °C refrigerator for storage. Three fish liver tissues were selected from each bucket and homogenized with 0.86% saline in the ratio of 1:9 using a tissue crusher and then centrifuged in a low-temperature centrifuge at 2500 r/min for 10 min to obtain the supernatant for the subsequent determination of physiological and biochemical indexes and digestive enzyme activities. CC and CB3 livers (three replicates) were selected based on our previous study for transcriptomic and metabolomic sequencing analysis (Kong et al. 2023).

Physiological and biochemical indicators

Serum alanine aminotransferase (ALT), alkaline phosphatase (AKP), aspartate aminotransferase (AST), acid phosphatase (ACP), malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT) were measured using assay kits supplied by Nanjing Jiancheng Technology Co.

Take liver tissue and 0.86% physiological saline in a ratio of 1:9, homogenize using a tissue homogenizer, and then centrifuge at 2500 r/min for 10 min using a low-temperature centrifuge. The α-amylase, trypsin, and lipase assay kits used are provided by Nanjing Jiancheng Technology Co., Ltd. (Nanjing, China) (Catalog Nos: AMS: C016-1-1, LPS: A054-1-1, and TRS: A080-2).

Alanine aminotransferase (ALT), alkaline phosphatase (AKP), aspartate aminotransferase (AST), acid phosphatase (ACP), and malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), amylase (AMS), trypsin (TRS), and lipase (LPS) were determined using commercially available kits provided by Nanjing Jiansheng Science and Technology Co. Ltd., Nanjing, China (Catalog No.: ALT: C009-2-1; AKP: A059-2; AST: C010-2-1; ACP: A060-2; MDA: A003-1; SOD: A001-3; CAT: A007-1-1; AMS: C016-1-1; and LPS: A054-1-1; TRS: A080-2).

RNA extraction and hepatic transcriptomics analysis

Total RNA was isolated from three individual liver samples of each group. Furthermore, reverse transcription and library construction were performed by Beijing Biomarker Technologies Co., Ltd. (Beijing, China). According to the manufacturer's instructions,

construction of 6 sequenced libraries (3 replicates, 2 treatment groups, and 1 tissue sample) were constructed for Illumina® sequencing (NEB, USA) using the NEBNext® Ultra™ RNA Library Preparation kit. The quality of the built libraries was evaluated using the Agilent Bioanalyzer 2100 system. After a qualified quality assessment, sequencing was performed on an Illumina Hiseq 2000 platform. Differential expression analysis of two groups was performed using the DESeq R package (1.10.1) with the screening criteria of fold change (FC) ≥ 1.5 and q-value < 0.05. Analysis was performed using the Gene Ontology database (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG).

Untargeted hepatic metabolomics analysis

Liver samples were extracted using a methanol solution, and supernatants were prepared according to the previously reported method (Wang et al. 2022a). The supernatants were mixed to prepare quality control (QC) samples. Metabolomic assay analysis by Beijing Biomarker Technologies Co. An LC/MS system was used with a Waters ACQUITY UPLC high-speed steel T3 column (1.8 μm 2.1 × 100 mm). The solvent gradients were varied as follows: 98% A (0 min), 98% A (0.25 min), 2% A (10 min), 2% A (13.0 min), 98% A (13.1 min) and 98% A (15 min).

The collected raw data were processed with Progenesis QI software. The KEGG, HMDB, and LIPID MAP databases were searched for classification and pathway information of the identified compounds. Based on the grouping information, the diversity of differences was calculated and compared, and the t-test was used to calculate the significance P value of the differences for each compound. OPLS-DA modeling was performed, and 200 permutation tests were performed to verify the reliability of the model. The VIP values of the model were calculated by multiple cross-validations. Differential metabolites were screened using a combination of a diversity of differences, P-values, and VIP values from the OPLS-DA model. The screening criteria were $FC > 1$, P-value < 0.05, and $VIP > 1$. A hypergeometric distribution test was used to calculate the differential metabolites with KEGG pathway enrichment significance.

Real-time quantitative PCR

Ten differentially expressed genes (5 upregulated and 5 downregulated) were randomly selected from each group for the validation of Illumina sequencing results. Based on the assembly results, the unigene fragment sequences of the selected genes were obtained, and primers were designed and synthesized by Hunan Accurate Biotechnology Co., Ltd. β-Actin was used as an internal reference gene (Table 2) (Liu et al. 2020). RNA was reverse transcribed to cDNA using a reverse transcription kit (Item No. RC112-50 Vazyme Co., Ltd., Nanjing, China), and the concentration and quality of cDNA products were checked using an ultramicro spectrophotometer. The concentration of all cDNA products was adjusted to 0.20 μM for subsequent qPCR assays. qPCR assays were performed using a SYBR Green Pro Taq HS Premix qPCR Master Mix kit (item no. AG11701, Accurate Biotechnology Co., Ltd., Hunan, China) with a reaction volume of 20 μL. The qPCR amplification procedure was pre-denaturation: 95 °C for 30 s; 40 cycles of reactions: 95 °C for 5 s, 60 °C for 30 s, and 60 °C for 30 s. The lysis curves were analyzed in a LightCycler 480 II after the amplification was completed. Calculation of differential expression of differentially expressed genes using $2^{-\Delta\Delta Ct}$.

Table 2 Primer sequences for real-time RT–qPCR

Gene name	Primer sequence (5′–3′)	Annealing temperature (°C)	Product size (bp)	Amplification efficiency (%)	R^2
TRINITY_DN224	F: GCATTAACCGCCAACATGAGC R: AAGTTCAAGCCACCAACTGC	60	127	106.44	0.9994
TRINITY_DN19443	F: TCACCCTACTTGCCCTTACCC R: GCCGCATCAGTCTTCATTACCAG	60	135	106.74	0.9818
TRINITY_DN3768	F: TTCTACACAGCAGAGATTGCCAG R: TTTTCCTTGCACAGGCCGAA	60	140	101.69	0.9580
TRINITY_DN13113	F: CATGCAGATGTAAATACGCCCAT R: GTCAACATCCCGAAGTCCGAT	60	174	97.79	0.9859
TRINITY_DN1596	F: TCAGCCAGTCAGAGCACGTC R: TCTTTCTCCAGCTGCAGTCTCG	60	199	102.09	0.9701
TRINITY_DN14018	F: CATGTCACCCACAGTTGGCAG R: TTGAAATGGGCTACTCCGTCAC	60	157	102.48	0.9637
TRINITY_DN26158	F: TTCTGCCTTTACCCAGTAGCG R: TATGCATTCAGCCTCGAGCTT	60	90	92.87	0.9885
TRINITY_DN1615	F: TTGCTTCTCCTCAGCGGGTA R: GACACTTGGCCTGTTCCCT	60	191	106.14	0.9971
TRINITY_DN2013	F: CTCACTGCTAGCTCCACGTTC R: AACCCCAAACACCTAGTGACC	60	137	99.42	0.9697
TRINITY_DN204	F: ACTCCTTCCATTTTCTCATGTGC R: GAGAAACCAATGCGGTCCATCC	60	144	107.08	0.9623
β-actin	F: CAACTGGGATGACATGGAGAAG R: TTGGCTTTGGGGTTCAGG	60		97.20	0.9974

F means forward primer, while *R* means reverse primer

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using SPSS22.0 software; data with significant differences were subjected to Duncan's multiple comparisons and

selected as significant at $P< 0.05$, and the results of the test were expressed as *mean* ± standard deviation (*mean* ± *SD*).

Results

Effects of CB on the biochemical indicators

According to the results shown in Table 3, compared to the control group, the addition of CB to the feed significantly increases the activity of liver AKP and ACP in spotted sea bass ($P < 0.05$). Furthermore, these activities showed a gradual increase with the increasing dosage of CB. The liver AKP and ACP activities reached their highest levels when the CB dosage was 0.3%. This indicates that CB has a positive impact on the non-specific immune function of spotted sea bass's liver. However, the addition of CB to the feed did not have a significant effect on the antioxidant capacity of spotted sea bass's liver ($P > 0.05$). Regarding the levels of ALT, no significant difference was observed in the liver between the control group and the CB group. However, CB was found to effectively reduce liver AST activity compared to the control group ($P < 0.05$), indicating a potential protective effect of CB on the liver (Fig. 1).

Effects of CB on the activity of hepatic digestive enzymes

Table 4 shows the effects of CB supplementation in the diet on the activity of liver digestive enzymes. Compared to the control group, the addition of CB to the feed significantly increased the TRS activity in the liver of spotted sea bass, with the highest activity observed when the CB addition was 0.1% ($P > 0.05$). However, while CB increased the activity of AMS and LPS in the liver of spotted sea bass, the increase was not statistically significant compared to the control group ($P < 0.05$).

Effects of CB on the hepatic transcriptomics

RNA Seq analysis was applied to liver samples from the CC and CB3 groups. A total of 5.79 clean data points were obtained in each sample after quality checks. By selecting BLAST parameters with E-values no greater than 1e−5 and HMMER parameters

Table 3 Effects of CB on the activity of liver non-specific immune and antioxidant capacity

GROUP	SOD (U/mg prot)	MDA (nmoL/ mg prot)	CAT (U/mg prot)	AKP (U/g prot)	ACP (U/g prot)
CC	286.9 ± 100.71^{a}	1.02 ± 0.02^{a}	6.68 ± 0.71^{a}	60.13 ± 2.15^{a}	5.14 ± 0.23^{a}
CB1	325.61 ± 68.19^{a}	0.8 ± 0.07^{a}	6.33 ± 0.80^{a}	62.33 ± 3.03^{ab}	5.52 ± 0.30^{ab}
CB2	274.35 ± 16.14^{a}	1.04 ± 0.2^{a}	6.15 ± 1.10^{a}	65.21 ± 2.76^{b}	5.81 ± 0.17^{b}
CB3	307.8 ± 44.64^{a}	1.03 ± 0.22^{a}	6.39 ± 0.70^{a}	66.07 ± 1.72^{b}	5.85 ± 0.24^{b}
CB4	322.09 ± 54.26^{a}	1.13 ± 0.12^{a}	6.00 ± 0.24^{a}	61.65 ± 1.94^{ab}	5.22 ± 0.28^{a}
CB5	242.11 ± 53.31^{a}	1 ± 0.26^{a}	5.86 ± 0.24^{a}	62.23 ± 2.62^{ab}	5.25 ± 0.11^{a}

Different superscript letters in a column indicate significant differences ($P < 0.05$)

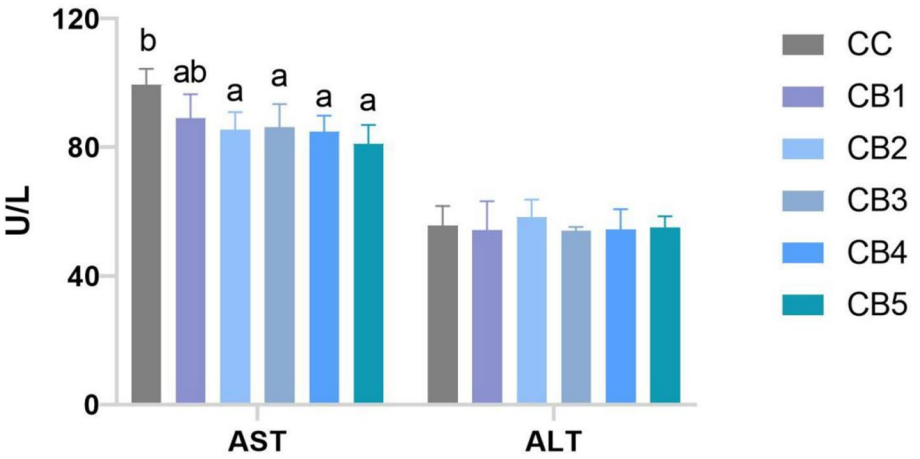

Fig. 1 Liver AST and ALT activity. Note: Different letters on the bar graphs indicate significant differences ($P < 0.05$)

Table 4 Effects of CB on the activity of hepatic digestive enzymes

GROUP	AMS (U/mg prot)	LPS (U/g prot)	TRS (U/mg prot)
CC	0.13 ± 0.01^{ab}	4.08 ± 0.64^{a}	800.53 ± 60.12^{a}
CB1	0.12 ± 0.01^{a}	4.86 ± 1.14^{a}	1011.16 ± 143.39^{b}
CB2	0.13 ± 0.03^{ab}	4.5 ± 0.53^{a}	859.1 ± 139.41^{a}
CB3	0.12 ± 0.02^{a}	5.25 ± 0.36^{a}	851.74 ± 72.48^{a}
CB4	0.2 ± 0.03^{b}	4.89 ± 0.95^{a}	941.27 ± 32.59^{a}
CB5	0.13 ± 0.04^{ab}	4.74 ± 1.35^{a}	828.44 ± 81.35^{a}

Different superscript letters in a column indicate significant differences ($P < 0.05$)

with E-values no greater than 1e−10; 21,383 unigenes with annotation information were finally obtained. These transcripts were compared with the NR, Swiss-Prot, COG, KOG, eggNOG4.5, and KEGG databases, with 20785, 10447, 3809, 12722, 18023, and 17012 unigenes annotated, respectively, among which 2366 unigenes were co-annotated. The total number of genes annotated in the four databases was 21,383.

The homology of the assembled unigene was compared with that of other species. The homology of spotted sea bass with *Morone saxatillis* and *Larimichthys crocea* was closer than that of other species. According to the sample correlation heat map analysis, the R2 between different samples of the same group was greater than 0.8, indicating that the experimental biological replication was reliable (Fig. 2).

The differentially expressed genes of the two groups were taken together, and the FPKM values of the genes were clustered to homogenize the rows (Z score). The heat map was plotted as shown in the results of clustering among the samples indicated that there were significant differences in gene expression between the different groups. For the same group, the differentially expressed genes between different samples in the same group were basically consistent.

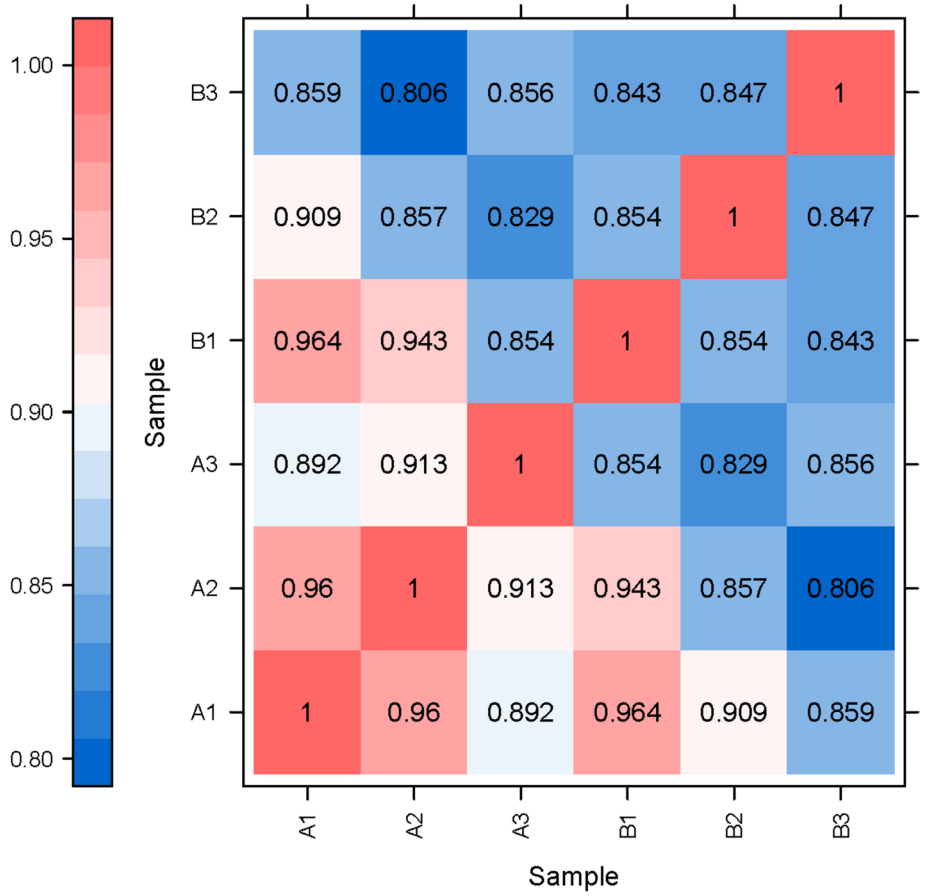

Fig. 2 Correlation heat map of the sample. CC: A1, A2, and A3; CB: A1, A2, and A3

To verify the effect of *Clostridium butyricum* addition on the liver of spotted sea bass, *FDR* and *FC* were used to screen for differentially expressed genes with $FC \geq 1.5$ and $FDR < 0.05$. A total of 149 differentially expressed genes were obtained in the liver tissue. Among them, 84 were downregulated genes, and 65 were upregulated genes. As clearly shown in the volcano plot, the left and upper dots are the downregulated DEGs. As clearly shown in the volcano plot, the points on the left and above are the downregulated DEGs. The volcano plot also shows the main changes in DEGs in the CB3 group of flowering bass compared to the control group.

We performed GO function enrichment analysis. Three major categories were included, namely, biological process, cell component, and molecular function. A total of 112 terms were enriched. As shown in the biological process category, the main enrichment was in the single-organism process, cellular process, metabolic process, and biological regulation. In the cellular component category, the main enrichment was in the cell, cell part, organelle, and membrane. In the cellular component category, the main enrichment was in binding, catalytic activity, and molecular function regulator. KEGG enrichment analysis of differentially expressed genes was performed on 6 samples

between the CC and CB3 groups. 4-2 C shows that we selected the top 20 pathways for differential gene enrichment; the larger the rich factor, the higher the enrichment; and the lower the *q* value, the more significant the enrichment. The analysis revealed that the pathways of CC and CB3 with upregulated differential genes were mainly enriched in metabolism-related pathways (biosynthesis of amino acids, arginine biosynthesis, arginine, and proline metabolism, D-glutamine and D-glutamate metabolism, glycine, serine, and threonine metabolism, alanine, aspartate, and glutamate metabolism, and alpha-linolenic acid metabolism) (Fig. 3A). The pathways that were mainly enriched for

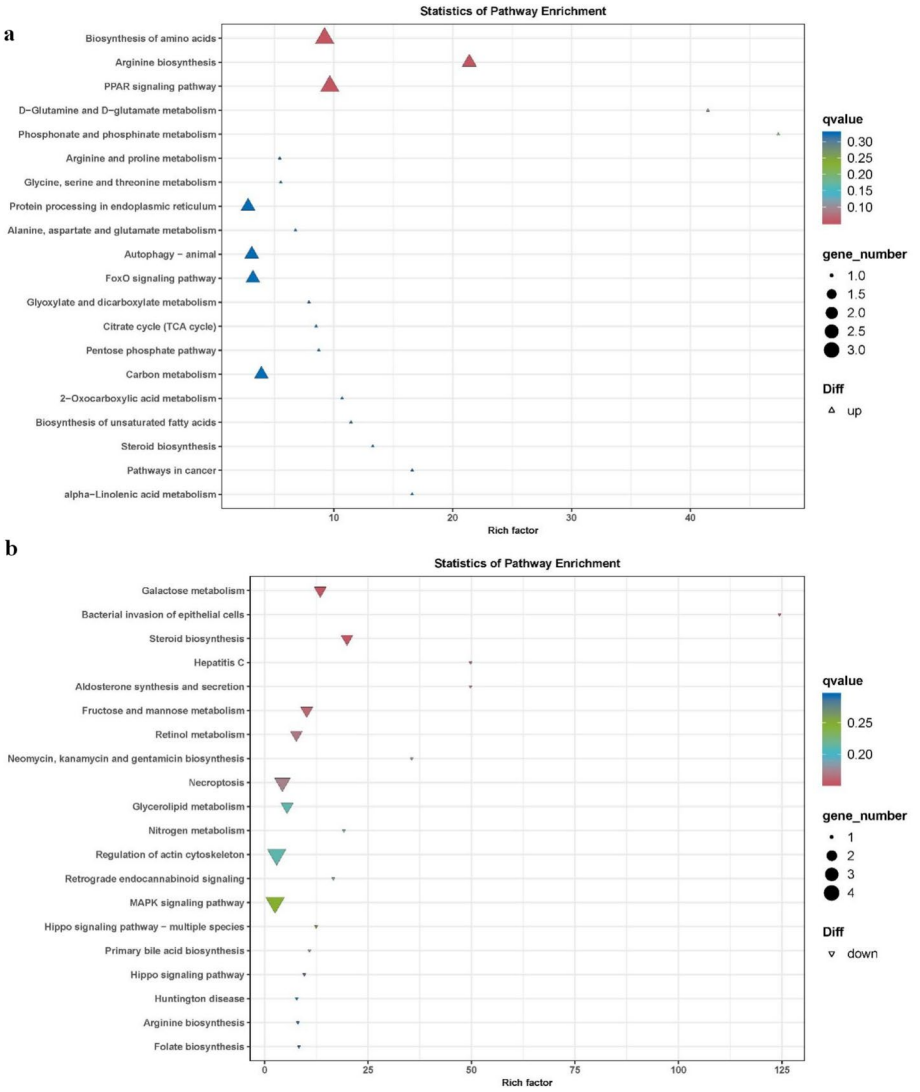

Fig. 3 Upregulated differentially expressed gene KEGG pathway enrichment scatter plot (**a**); downregulated differentially expressed gene KEGG pathway enrichment scatter plot (**b**)

the upregulation of differentially expressed genes were hepatitis C, necroptosis, Huntington disease, aldosterone synthesis, and secretion (Fig. 3B).

Effects of CB on the hepatic metabolomics

To further investigate the effects of CB on the hepatic physiology and metabolic responses in spotted sea bass, liver metabolomic profiling was performed using UHPLC-MS/MS. A total of 11,809 peaks were detected in the metabolomic dataset (6365 for ESI+ and 5444 for ESI−). OPLS-DA was employed to explore the differences between the CC group and the CB group. The score plot demonstrated significant metabolic differentiation between the two groups. In both positive and negative ion modes, R2Y represents the model's explanation rate, while Q2Y represents the model's predictive ability. The closer R2Y and Q2Y values are to 1, the more stable and reliable the model is considered, indicating its suitability for screening differential metabolites. Generally, a model is considered effective when Q2Y > 0.5 and excellent when Q2Y > 0.9. In the positive ion mode, the R2Y and Q2Y values were 0.994 and 0.59, respectively, while in the negative ion mode, the values were 0.997 and 0.675, indicating that the constructed model has good fitness and predictive ability.

The volcano map was used to show the overall distribution of the differential metabolites, and furthermore, the differential metabolites were screened using FC and VIP. Metabolites meeting both $FC > 1$ and $VIP > 1$ were considered differential metabolites. The results showed that there were 248 differential metabolites, among which 157 metabolites were upregulated, and 91 metabolites were downregulated in expression (Fig. 4). These metabolites are mainly steroids and their derivatives, lipids and lipid-like molecules, nucleotides and their analogs, amino acids, organic acids, and monosaccharides. To gain more insight into the different changes in hepatic metabolites by CB supplementation, a KEGG-based metabolic pathway enrichment analysis was performed. The DA score reflects the overall changes of all metabolites in a metabolic pathway. A score of 1 indicates upregulation of all annotated metabolites in the pathway, while −1 indicates downregulation of all annotated metabolites in the pathway. The DA score shows that the downgraded pathways are the renin−angiotensin system and ubiquinone and other terpenoid−quinone biosynthesis. The main upward adjustment pathways were carbohydrate metabolism (starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism, and galactose metabolism), lipid metabolism (fatty acid degradation and regulation of lipolysis in adipocytes), immune system (Fc gamma R-mediated phagocytosis, platelet activation, and Fc epsilon RI signaling pathway), and endocrine system (oxytocin signaling pathway and GnRH signaling pathway) (Fig. 5).

Combined transcriptomics and metabolomics analysis

Through the joint analysis of differential metabolites ($VIP > 1$, $FC > 1$) and DEGs ($FC \geq 2$ and $FDR < 0.01$), 8 pathways were co-enriched. They are glycolysis/gluconeogenesis, fatty acid metabolism, amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism, galactose metabolism, biosynthesis of unsaturated fatty acids, necroptosis, and carbon metabolism (Fig. 6).

Considering the integrated transcriptomics and metabolomics analyses, CB was hypothesized to enhance the immunity of the flowering bass organism through pathways that regulate carbohydrate metabolism and lipid metabolism. To further validate

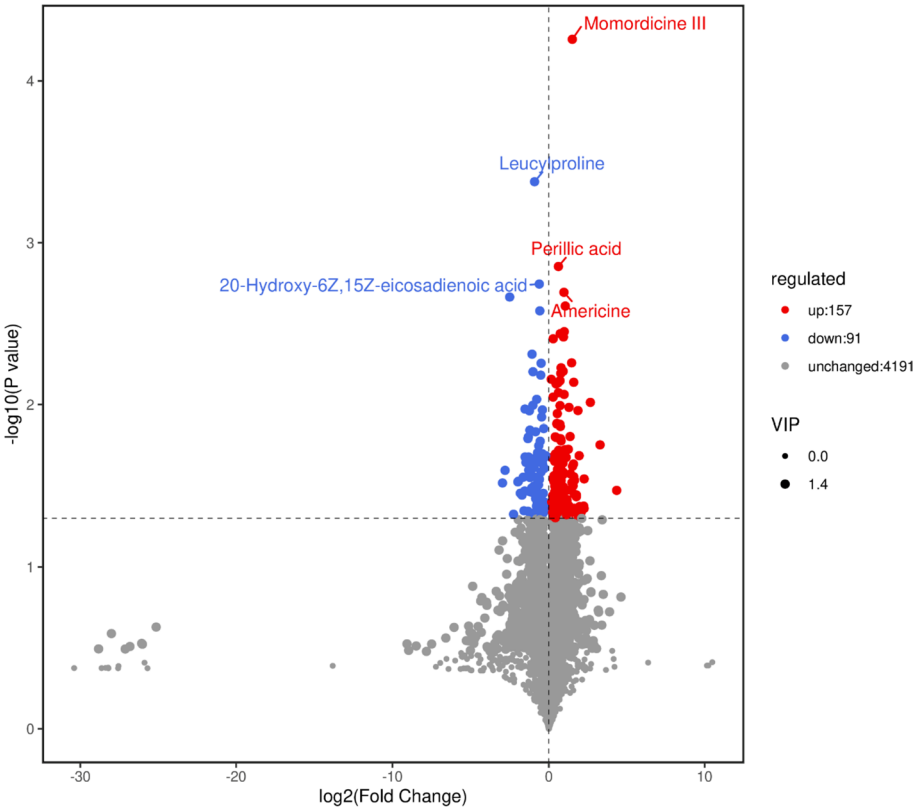

Fig. 4 Volcanic map of differential metabolites. Note: Each dot in the volcano plot represents a metabolite. The blue points in the graph represent downregulated different expressed metabolites; the red points represent upregulated different expressed metabolites; and the gray points represent metabolites detected but not significantly different. In addition, the top 5 characterized metabolites after sorting by *P* value are selected and labeled in the graph

this result, KEGG pathway xml analysis was performed. Multiple genes and multiple metabolites may exist in a pathway, and a gene or metabolite may also belong to the same pathway on multiple pathways. The relationship between each differentially grouped pathway, gene, and metabolite can be visualized using the igraph R package (Fig. 7). We can clearly see the main pathways regulated by CB in the liver metabolism of *C. perfringens*. Upregulation of NLRP3 through the necroptosis signaling pathway leads to increased metabolism of arachidonate, affecting biosynthesis of the unsaturated fatty acid signaling pathway. Acyl-CoA 6-desaturase upregulation affects the fatty acid metabolism pathway, leading to increased crotonyl-CoA metabolism. Carbon metabolism causes glucokinase downregulation through four glucose metabolism pathways, leading to increased D-glucose 1-phosphate metabolism. Upregulation of chitinase also causes an increase in D-glucose 1-phosphate metabolism through the amino sugar and nucleotide sugar metabolism pathways.

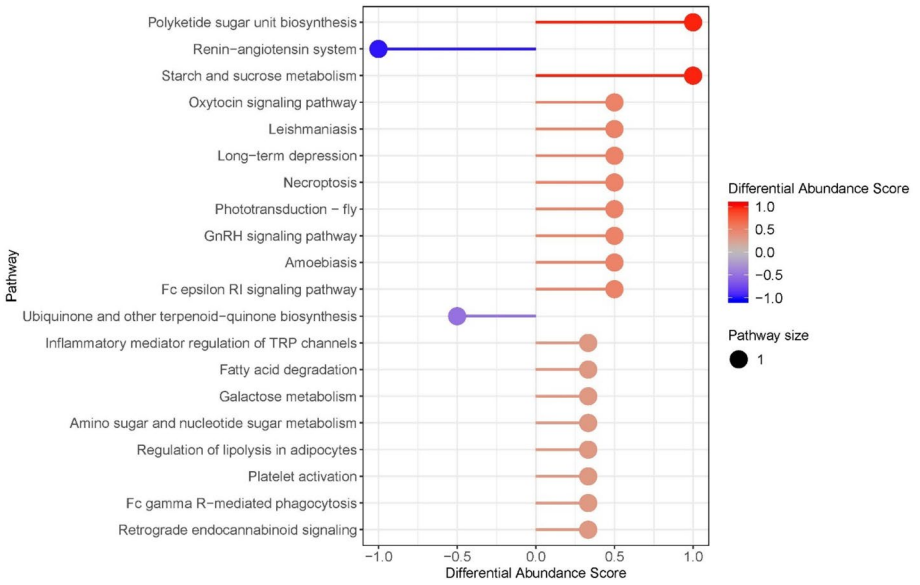

Fig. 5 Differential abundance scores. Note: The length of the line segment represents the absolute value of the DA score, and the size of the dots at the end of the line segment represents the number of differential metabolites in the pathway. The dots distributed on the left side of the axis and the longer the line segment, the more inclined the overall expression of the pathway is to be downregulated; the dots distributed on the right side of the axis and the longer the line segment, the more inclined the overall expression of the pathway is to be upregulated. The larger the dot, the more metabolites there are. The colors of the line segments and dots reflect the *P* value size. The closer the line is to red, the smaller the *P* value, and the closer the line is to blue, the larger the *P* value

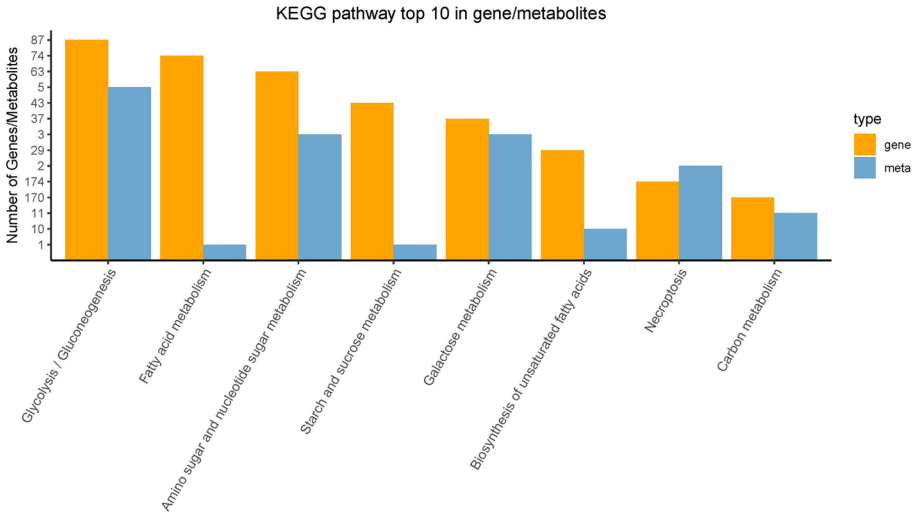

Fig. 6 The top 10 pathways containing the most genes/metabolites. Note: Each bar in the figure represents a KEGG pathway, and different colors indicate different histologies, with yellow indicating genes in the transcriptomics and blue indicating metabolites in the metabolomics

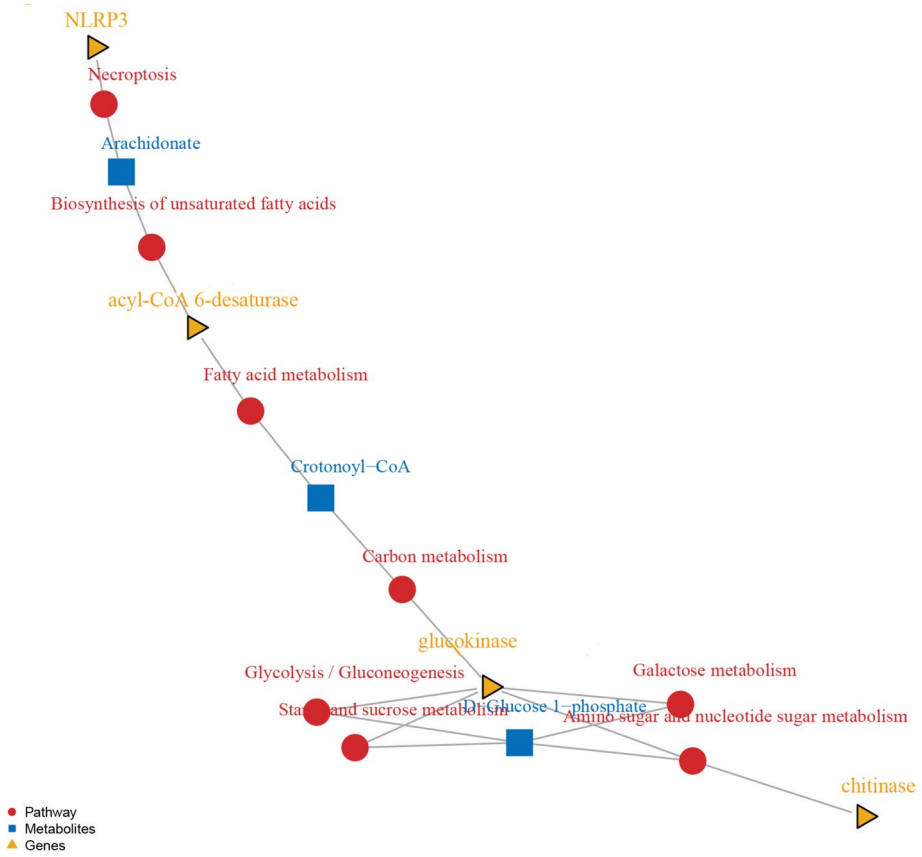

Fig. 7 Pathway and differential gene/metabolite KGML network diagram. Note: Different shapes and colors in the diagram indicate different types: red circles for KEGG pathway, blue squares for metabolites, and yellow triangles for genes

Real-time fluorescence quantitative PCR verification

From the differentially expressed genes of the control group and the experimental group, 10 differentially expressed genes were randomly selected for RT–qPCR, and the transcriptomic sequencing results were verified. The results are shown in Fig. 8. The change trend of gene expression determined by RT–qPCR was consistent with the results of RNA-seq, indicating the reliability of the transcriptomic sequencing analysis results.

Discussion

In this study, we investigated the beneficial effects of CB on the liver health of spotted sea bass. Supplementation with 2 and 3 g/kg CB per day can improve the immune capacity of the liver, regulate the digestive ability of the liver, and improve the liver damage of CB-fed spotted sea bass, which is consistent with the added dose of growth promotion. ALT

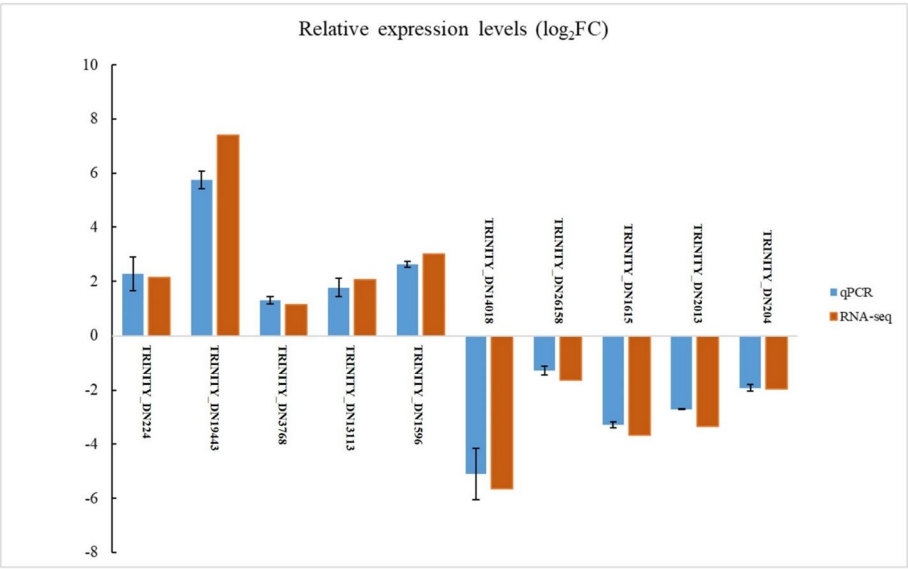

Fig. 8 qRT–PCR results for some differentially expressed genes (DEGs) induced by CB

and AST are important indicators of liver damage in fish (Yalcin et al. 2011). Studies have shown that probiotics can reduce the activities of ALT and AST in animals. However, the effect of CB on the liver function of fish is rarely reported. In this study, the dietary addition of CB significantly decreased the liver AST activity of spotted sea bass. In addition, similar results have been verified in tilapia (Zhang et al. 2021). Non-specific immunity plays an important role in the immune defense of fish, and AKP and ACP are important indicators that reflect the level of non-specific immunity of fish. In a study of *Litopenaeus vannamei*, it was found that CB could significantly improve AKP and ACP activities (Li et al. 2019). In a study of *Miichthys miiuy*, it was found that CB could increase the activity of AKP in serum (Song et al. 2006). The results of this experiment showed that the levels of AKP and ACP in the liver after CB supplementation were significantly higher than those in the CC group ($P < 0.05$), and it was noteworthy that the activities of AKP and ACP reached their peak when the supplemental level reached 3 g/kg. These results indicate that CB supplementation has a good protective effect on liver function and liver injury.

The liver is the main organ that secretes digestive enzymes, including AMS, LPS, and TRS (Ye et al. 2003). Amylase is a general term for a class of enzymes that hydrolyze starch and sugars, and its activity and content determine the ability of fish to decompose and digest sugars in feed (Douglas et al. 2000). Lipase is a hydrolytic enzyme that can hydrolyze triglycerides into glyceryl monooleate and fatty acids, which can be absorbed and utilized by the body (Oku et al. 2006; Sae-Leaw and Benjakul 2018). Trypsin allows the decomposition of proteins into smaller peptides, amino acids, and small amounts of fatty acids (Whitcomb and Lowe 2007). The broken-down amino acids can be synthesized by the liver into new proteins or used for energy metabolism. The results of this experiment showed that the activity of AMS in the liver of spotted sea bass was significantly increased when the supplemental level was 0.4% ($P < 0.05$). When the supplementary level of CB was 0.1%, the activity of AMS in the liver was significantly increased. TRS activity was significantly increased ($P<0.05$), indicating that adding appropriate CB to the diet could

improve the absorption and utilization of carbohydrates and protein in spotted sea bass. The LPS activity of spotted sea bass continued to increase with the addition of CB, and there was no significant difference among components. This result suggests that adding CB increases the liver's breakdown of carbohydrates and amino acids.

Liver transcriptomics studies can help further understand CB's improvement of liver-related metabolic processes in spotted sea bass. Multi-omics analysis is an important means to explore the regulatory mechanism of CB on the liver, such as the key pharmacodynamic substance basis and possible molecular mechanism of the extract of Horny medium against liver cancer (Liu et al. 2023). Through transcriptomics and metabolomics analyses, this study found that the regulatory effect of CB on the liver can play a role in metabolism and related transcription factors through a variety of related signaling pathways. This result is in line with the results of a previous study on the combined effect of CB and other probiotics (Lu et al. 2023). In this study, researchers found that CB has a positive regulatory effect on cirrhosis. However, this study focused on the regulatory mechanism of probiotics in the gut and did not explore the regulatory mechanism of probiotics in the liver. We explored the regulatory mechanism of CB in the liver by means of transcriptomics and metabolomics. GO terms, and KEGG pathways showed that compared with the control group, the biosynthesis capacity of arginine in the CB feeding group was significantly upregulated. Arginine is a key amino acid in the urea cycle. Arginine is important for maintaining nitrogen balance in the body (Anderson et al. 1984). Arginine also plays an important role in the promotion of immunity (Popovic et al. 2007). This study suggests that CB plays a positive role in maintaining homeostasis in the body. Upregulation of the metabolic pathway of linolenic acid, an essential fatty acid, was also observed in the KEGG pathway, which not only plays a role in promoting growth. It has also been shown to enhance immunity in animals (Desale et al. 2021). This study suggests that CB may play an active role in the liver in a way that regulates linolenic acid. The PPAR pathway is not only thought to be partly responsible for the inflammatory response but also ultimately activates the NF-κB pathway. Cytokines, soluble extracellular proteins, or glycoproteins, are key intercellular regulators involved in adaptive inflammatory host defense, cell growth, differentiation, and more. When cells in the body are stimulated and activated, cytokines are released (Tang et al. 2018). The PPAR signaling pathway was also found to be elevated in this study and activated PPARγ inhibited the production of inflammatory factors (TNF-α, IL-1β, IL-2, and IL-6) and showed anti-inflammatory effects (Wang et al. 2022b). According to the above results, CB cannot only participate in the lipid regulation of the liver but also play a role in protecting the liver.

In the metabolomics results, we also observed upregulation of polyketone unit biosynthesis, and polyketone metabolites have obvious antiviral effects (Girija et al. 2022), suggesting that the antiviral capacity of the liver is improved with the participation of *Clostridium butyrate*. At the same time, we observed a significant upregulation of the renin-angiotensin system, which is a major player in blood pressure homeostasis (Sparks et al. 2014), suggesting that the liver's ability to maintain blood pressure homeostasis is enhanced with CB. Second, we also observed significant differences between the CB group and the control group in sucrose metabolism, which plays a key role in development, stress response, and yield formation, mainly through the production of a range of sugars as metabolites to promote growth and synthesis of essential compounds (including proteins, cellulose, and starch). It also acts as a signal that regulates the expression of microRNAs, transcription factors, and other genes, as well as crosstalk with hormonal, oxidative, and defense signals (Ruan 2014). At the same time, in the metabolic group, we found significant differences in fatty acid degradation, galactose metabolism, galactose metabolism,

and regulation of lipolysis in adipocytes, indicating that CB actively participates in carbohydrate and lipid metabolism in the liver. The transcriptomics and metabolomics jointly analyzed the regulatory mechanism of CB in the liver. They found that it is involved in the carbohydrate metabolism pathway (glycolysis/gluconeogenesis, amino sugar, and nucleotide sugar metabolism, starch and sucrose metabolism, galactose metabolism, and carbon metabolism) and biosynthesis of unsaturated fatty acids. Significant differences have been observed in the regulation of fatty acid metabolism and the immune pathway necroptosis. CB can be upregulated in the liver through activation of NLRP3 and chitinase. In the end, CB causes an increase in D-glucose 1-phosphate metabolism, which often represents rapid fattening of the fish. This study suggests that CB is involved in liver glucose metabolism and may promote the growth of fish by improving glucose metabolism.

As an essential fatty acid, arachidonic acid plays an important role in regulating energy metabolism and cell structure. It can be used as an energy source to reduce the consumption of protein. With the development of research, it has been confirmed in *Anguilla japonica* (Shahkar et al. 2016), *Acipenser sinensis* (Wu et al. 2021), *Gadus morhua* (Wu et al.,2021), and *Lateolabrax japonicus* (Xu et al. 2018). While enhancing immunity, ARA can also protect the liver, improve the body's antioxidant function, and ultimately enhance the body's anti-stress function. It has been reported that in addition to SLP, pmf produced during butyric acid fermentation in the human gut can also convert crotonyl-CoA to butyryl-CoA, thus producing energy (Louis and Flint 2009). This mechanism was first identified in *Clostridium kluyveri* (Seedorf et al. 2008). The action of CB on the liver may be consistent with this mechanism.

In summary, CB can participate in the regulation of lipid metabolism and carbohydrate metabolism in the liver. CB enhances the metabolic capacity and immune function of the liver. The results show that CB has good potential as a fish feed and can play a positive regulatory function on the liver of fish.

Conclusion

This study shows that CB has a significant effect on reducing AST levels in the liver, enhancing liver immunity, and can increase the activity of liver amylase and trypsin, and promoting the breakdown of carbohydrates and amino acids in the liver. The integration of the liver transcriptomics and metabolomics suggests that it mainly changes the transcriptional levels of glucose metabolism, lipid metabolism, and amino acid metabolism pathways and regulates the abundance of metabolic biomarkers such as arachidonate, crotonyl-CoA, and D-glucose 1-phosphate. The present work provides a theoretical basis for the use of CB, which is a probiotic that has regulatory effects on the liver transcriptomics and metabolism of spotted sea bass, and the intake of CB is beneficial to the liver health of spotted sea bass. However, in future studies, the use of CB in vivo needs to be described in more detail, and key genes and pathways involved in CB's effect on the liver need to be analyzed and validated in a targeted manner.

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Author contribution Lumin Kong and Zhongbao Li conceived and designed the experiments.Lumin Kong, Sishun Zhou, Hao Lin, Jianrong Ma, Zhongying Long, Huihui Qin, Zhangfan Huang, Longhui Liu and Yi Lin performed the experiments.Lumin Kong performed the analyses.Lumin Kong analyzed the data, wrote

the paper, and prepared figures and tables.Lumin Kong and Zhongbao Li discussed the result together. Zhongbao Li reviewed drafts of the paper.

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Data availability The original contributions presented in the study are included in the article/supplementary material, and further inquiries can be directed to the corresponding author.

Declarations

Competing interests The authors declare no competing interests.

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