

Original Research Articles

Effects of heat-inactivated *Lactobacillus acidophilus* AC on growth performance and intestinal health of *Larimichthys crocea*

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Capsule

Heat-inactivated *L. acidophilus* AC improved intestinal morphology and metabolism in juvenile *L. crocea*, with the 1×10^{10} CFU/g dose showing stronger overall benefits.

Abstract

The overuse of antibiotics in aquaculture presents significant challenges, including the emergence of drug-resistant pathogens, environmental contamination, and food safety risks. Probiotics have emerged as a promising alternative, with heat-inactivated probiotics gaining prominence for their enhanced safety and stability. This study investigates the effects of heat-inactivated *Lactobacillus acidophilus* AC on the growth performance and intestinal health of juvenile yellow croaker, *Larimichthys crocea*. Heat-inactivated *L. acidophilus* AC was prepared at two concentrations— 1×10^{10} CFU/g (ABI1 group) and 1×10^{11} CFU/g (ABI2 group)—and incorporated into the basal diet. These were compared to a control group (NC group) in a 56-day feeding trial. The results demonstrated that the intestinal morphology was significantly improved ($P < 0.001$). The diversity of the intestinal microbiota community in the ABI2 group showed a decreasing trend ($P < 0.05$). Furthermore, the intestinal metabolism was significantly influenced by heat-inactivated *L. acidophilus* AC. In summary, heat-inactivated *L. acidophilus* AC has been demonstrated to optimize growth by optimizing the structure of the intestinal microbiota and promoting the production of beneficial metabolites. This study provides a scientific basis for the application of heat-inactivated probiotics in aquaculture.

INTRODUCTION

The global aquaculture industry has expanded rapidly to meet rising seafood demand, leading to intensified farming practices and increased stocking densities.¹ However, diseases frequently occur in intensive aquaculture systems, and although antibiotics provide short-term relief, they exacerbate antibiotic resistance and environmental pollution, creating an urgent need for sustainable alternatives.² Consequently, identifying sustainable alternatives to antibiotics has become essential for the long-term viability of aquaculture.

Probiotics have gained prominence as viable substitutes for antibiotics and are widely utilized in aquaculture.³ Among probiotic candidates, lactic acid bacteria, particularly *L. acidophilus* AC, are extensively studied for their beneficial effects.⁴ Live *L. acidophilus* AC has been shown to

enhance growth, boost immunity, and increase disease resistance in various aquatic species.⁵ Similarly, heat-inactivated *L. acidophilus* AC exhibits potentiality in promoting growth, regulating immune function, and improving gut health.⁶ For example, Zhang et al.⁷ reported that heat-inactivated lactic acid bacteria significantly enhanced growth performance and disease resistance in tilapia (*Oreochromis niloticus*), while Ren et al.⁸ observed improved intestinal health in Pacific white shrimp (*Litopenaeus vannamei*) through modulation of gut microbiota and metabolites.

The large yellow croaker (*Larimichthys crocea*) is a marine aquaculture species of significant economic value along China's southeastern coast; however, the development of aquaculture has long been constrained by issues such as slow growth and frequent disease outbreaks. Although many studies have demonstrated that heat-inactivated *L. acidophilus* AC promotes growth in aquatic ani-

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mals, few studies have investigated whether the addition of heat-inactivated *L. acidophilus* AC to *L. crocea* feed has distinct probiotic effects. Furthermore, research on the mechanism of heat-inactivated *L. acidophilus* AC remains insufficient. Against this backdrop, this study evaluated the effects of heat-inactivated *L. acidophilus* AC on the growth performance of juvenile *L. crocea*, and through the integration of metabolomics and microbial community analysis to elucidate the mechanisms. The results are expected to provide practical guidance for the application of heat-inactivated *L. acidophilus* AC in *L. crocea* aquaculture and promote a shift toward sustainable, antibiotic-free aquaculture practices.

MATERIALS AND METHODS

PREPARATION OF HEAT-INACTIVATED *L. ACIDOPHILUS* AC SUSPENSION AND EXPERIMENTAL DIETS

L. acidophilus AC, previously isolated from the intestine of *L. crocea*,⁹ was confirmed to exhibit probiotic properties in our laboratory. Hemolysis tests verified its lack of pathogenicity. Glycerol-preserved *L. acidophilus* AC was revived, and single colonies were inoculated into Lactobacillus-selective broth. Cultures were incubated at 37°C for 48 hours in a constant-temperature shaker. Bacterial concentration was measured as optical density (OD) at 600 nm and adjusted to 1×10^{10} CFU/g and 1×10^{11} CFU/g for the experimental groups. The suspensions were heat-inactivated in a 70°C water bath for 10 minutes. Inactivation was confirmed using the dilution plate method: aliquots of the heat-treated suspension were spread onto MRS agar plates (three replicates per group) and incubated anaerobically at 37°C in an anaerobic jar. Plates were inspected for colony growth at 12, 24, and 36 hours to ensure complete inactivation.

Experimental diets were prepared by uniformly spraying the heat-inactivated *L. acidophilus* AC suspension onto a basal diet (Jianma brand *L. crocea* compound feed: crude protein $\geq 47\%$, crude fat $\geq 5\%$). The control diet (NC group) was sprayed with an equivalent volume of phosphate-buffered saline (PBS). Diets were dried at 37°C in an oven, coated with a 10% sodium alginate solution for stability, and stored at 4°C. Fresh diets were prepared every three days to maintain the quality.

EXPERIMENTAL ANIMAL HUSBANDRY MANAGEMENT

The feeding trial was conducted at the Changzhi Island marine aquaculture base (Zhoushan Peninsula Aquaculture Co., Ltd.). Juvenile fish were acclimated in seawater cages for two weeks, fed the basal diet. Nine cages (1m $\times$ 1m $\times$ 1m) were each stocked with 30 healthy fish (average initial weight: 102.8 ± 3.2 g). These cages were randomly assigned to three dietary treatments, resulting in three replicate cages per treatment. The treatments were: (1) NC group (basal diet + PBS), (2) ABI1 group (basal diet + 1×10^{10} CFU/g heat-inactivated *L. acidophilus* AC), and (3) ABI2 group (basal diet + 1×10^{11} CFU/g heat-inactivated *L. aci-*

dophilus AC). Fish were housed in 1 $\times$ 1 $\times$ 1 m cages and fed twice daily (05:00 and 17:00) at approximately 4% of body weight for 56-d. Water quality was as follows: temperature 18.1–25.9°C, salinity 26–30‰, nitrite <0.005 mg/L, ammonia nitrogen <0.2 mg/L, and dissolved oxygen >6.5 mg/L.

SAMPLE COLLECTION

Following the 56-d trial, fish were fasted for 24 h. The number, body length and weight in each group was recorded for subsequent growth indexes analysis. Twelve fish were randomly selected from each cage, and three of them were used for the whole body composition analysis, three fish were dissected to obtain muscle, liver and viscera that used to calculate the muscle body composition analysis, the hepatosomatic and the viscerosomatic indexes. In addition, six fish were dissected, and the intestinal tissues from three of them were fixed in 4% paraformaldehyde for histological examination. The remaining three intestines were placed in sterile cryovials and frozen in liquid nitrogen for intestinal microbiome and metabolome analysis.

GROWTH PARAMETER ANALYSIS

Growth parameters were calculated using the following equations.

$$\text{Weight Gain Rate (WGR, \%)} = 100 \times (W_t - W_0) / W_0$$

$$\text{Specific Growth Rate (SGR, \% / d)} = 100 \times (\ln W_t - \ln W_0) / t$$

$$\text{Condition Factor (CF, g / cm}^3\text{)} = 100 \times W_t / L^3$$

$$\text{Hepatosomatic Index (HSI, \%)} = 100 \times W_h / W_t$$

$$\text{Viscerosomatic Index (VSI, \%)} = 100 \times W_v / W_t$$

Where: W_0 = initial body weight (g), W_t = final body weight (g), L = body length (cm), W_h = liver weight (g), W_v = visceral weight (g), and t = experimental duration (days).

INTESTINAL HISTOLOGICAL ANALYSIS

Fixed intestinal samples were processed by Hangzhou Haoke Biotechnology Co., Ltd. for paraffin embedding and hematoxylin-eosin (HE) staining. Sections were scanned using a digital whole-slide pathology scanner (KF-FL-020) and analyzed with Visiopharm software to quantify, villus length and goblet cell numbers.

INTESTINAL MICROBIOTA ANALYSIS

Total DNA was extracted from intestinal samples using the MP FastDNA Spin Kit (MP Biomedical, USA). The V3–V4 region of the 16S rRNA gene was amplified with primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGAC-TACHVGGGTWTCTAAT-3'). PCR products were purified, quantified, and sequenced on the Illumina NovaSeq 6000 platform. Raw reads were filtered, trimmed, and merged to generate operational taxonomic units (OTUs) for diversity, differential abundance, and correlation analyses.

Table 1. Effects of heat-inactivated *L. acidophilus* AC on growth performance of juvenile *L. crocea*

Items	Groups			P-value
	NC	ABI1	ABI2	
Initial weight (g)	13.00 ± 1.55	11.20 ± 2.12	11.87 ± 1.66	0.613
Final weight (g)	38.60 ± 11.83	37.07 ± 9.47	39.51 ± 10.22	0.666
WGR (%)	195.55 ± 78.03	238.02 ± 88.28	243.61 ± 119.71	0.117
SGR (%/d)	1.86 ± 0.54	2.11 ± 0.49	2.11 ± 0.57	0.122
CF (g/cm ³)	0.97 ± 0.10 ^b	1.08 ± 0.13 ^a	1.01 ± 0.11 ^b	0.002
HIS (%)	2.21 ± 0.10	2.26 ± 0.881	1.97 ± 0.74	0.507
VSI (%)	5.85 ± 1.58	5.66 ± 0.95	5.64 ± 1.43	0.851

The same data labeled with no letter or the same letter indicates that the difference is not significant ($P > 0.05$), and different letters indicate that the difference is significant ($P < 0.05$).

INTESTINAL NON-TARGETED METABOLOMICS ANALYSIS

Intestinal samples stored at -80 °C were sent to Beijing Biomarker Technologies Co., Ltd. for non-targeted metabolomics analysis. The liquid chromatography-mass spectrometry (LC-MS) system included the Waters Acquity I-Class PLUS ultra-high-performance liquid chromatography tandem system and the Waters Xevo G2-XS QToF high-resolution mass spectrometer. Dual-channel data acquisition was performed using MassLynx V4.2. Peak extraction and alignment were performed using Progenesis QI software. Data identification utilized the online METLIN database, public databases, and Biomarker's internally constructed library via Progenesis QI software. Theoretical fragmentation identification was also conducted to ensure that the deviation of parent ion mass numbers was less than 100 ppm and the deviation of fragment ion mass numbers was <50 ppm. Data analysis was performed using the Biomarker Cloud platform (<http://www.biocloud.net>).

STATISTICAL ANALYSIS

Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test in SPSS 27.0. Results are expressed as mean ± standard deviation, with statistical significance set at $P < 0.05$.

RESULTS

EFFECTS OF HEAT-INACTIVATED *L. ACIDOPHILUS* AC ON THE GROWTH PERFORMANCE OF JUVENILE *L. CROCEA*

As presented in Table 1, the WGR and SGR increased with the higher concentrations of heat-inactivated *L. acidophilus* AC, though these differences were not statistically significant compared to the NC group ($P > 0.05$). Notably, the CF in the ABI1 group was significantly higher than that in the NC group ($P < 0.05$). No significant differences were observed in the HSI or VSI across all groups ($P > 0.05$).

EFFECTS OF HEAT-INACTIVATED *L. ACIDOPHILUS* AC ON INTESTINAL TISSUE OF JUVENILE *L. CROCEA*

Quantitative analysis (Fig. 1) confirmed that compared to the NC group, the villus length was significantly greater in the ABI1 group ($P < 0.05$), while the number of goblet cells per villus was significantly higher in the ABI2 group ($P < 0.05$).

EFFECTS OF HEAT-INACTIVATED *L. ACIDOPHILUS* AC ON THE INTESTINAL MICROBIOTA OF JUVENILE *L. CROCEA*

Alpha diversity analysis (Table 2) showed no significant differences in Chao1, ACE, or Shannon indices among the NC, ABI1, and ABI2 groups ($P > 0.05$). However, the Simpson index was significantly lower in the ABI2 group ($P < 0.05$), indicating a decrease in microbial diversity. Principal Component Analysis (PCA, Fig. 2) revealed differences in microbial community structures in the ABI1 and ABI2 groups compared to the NC group.

At the phylum level (Fig. 3), the dominant intestinal microbiota in *L. crocea* included Proteobacteria, Bacteroidota, Actinobacteriota, Acidobacteriota, Chloroflexi, and Firmicutes. With increasing concentrations of heat-inactivated *L. acidophilus* AC, Actinobacteriota abundance increased, while Firmicutes abundance decreased. The relative abundance of Gemmatimonadota was significantly higher in the ABI1 group ($P < 0.05$). At the genus level (Fig. 3), dominant genera included *unclassified_Bacteria*, *unclassified_Micrococcaceae*, *unclassified_Gemmatimonadaceae*, *Sphingomonas*, *unclassified_Vicinamibacterales*, and *unclassified_Microscillaceae*. Higher concentrations of heat-inactivated *L. acidophilus* AC were associated with increased abundance of *unclassified_Micrococcaceae*, *Sphingomonas*, *unclassified_Microscillaceae*, *unclassified_Vicinamibacteraceae*, *Devosia*, and *unclassified_Sphingomonadaceae*, while *unclassified_Vicinamibacterales* decreased.

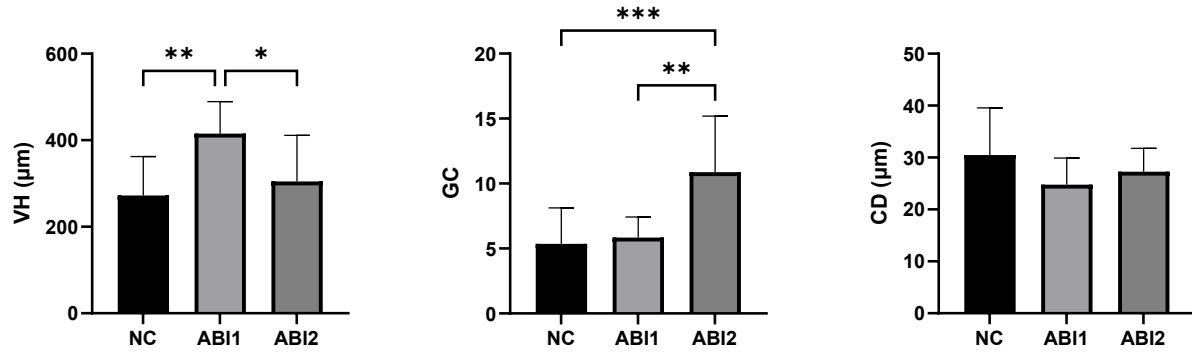


Fig. 1 Quantitative analysis of intestinal tissues of juvenile *L. crocea*. VH: villus height; GC: goblet cell quantity per villi. Values presented are mean $\pm$ SD of three individual fish (N = 3). * represent significant difference.

Table 2. Alpha diversity indexes of the intestinal flora of juvenile *L. crocea*

Items	Groups			P-value
	NC	ABI1	ABI2	
Chao1	2909.14 $\pm$ 134.57	2723.87 $\pm$ 299.39	2454.96 $\pm$ 148.79	0.093
ACE	2908.21 $\pm$ 136.00	2721.32 $\pm$ 299.41	2453.77 $\pm$ 146.86	0.093
Simpson	0.9985 $\pm$ 0.0001 ^a	0.9983 $\pm$ 0.0002 ^{ab}	0.9980 $\pm$ 0.0001 ^b	0.017
Shannon	10.45 $\pm$ 0.05	10.31 $\pm$ 0.19	10.15 $\pm$ 0.08	0.070

The same data labeled with no letter or the same letter indicates that the difference is not significant ($P > 0.05$), and different letters indicate that the difference is significant ($P < 0.05$).

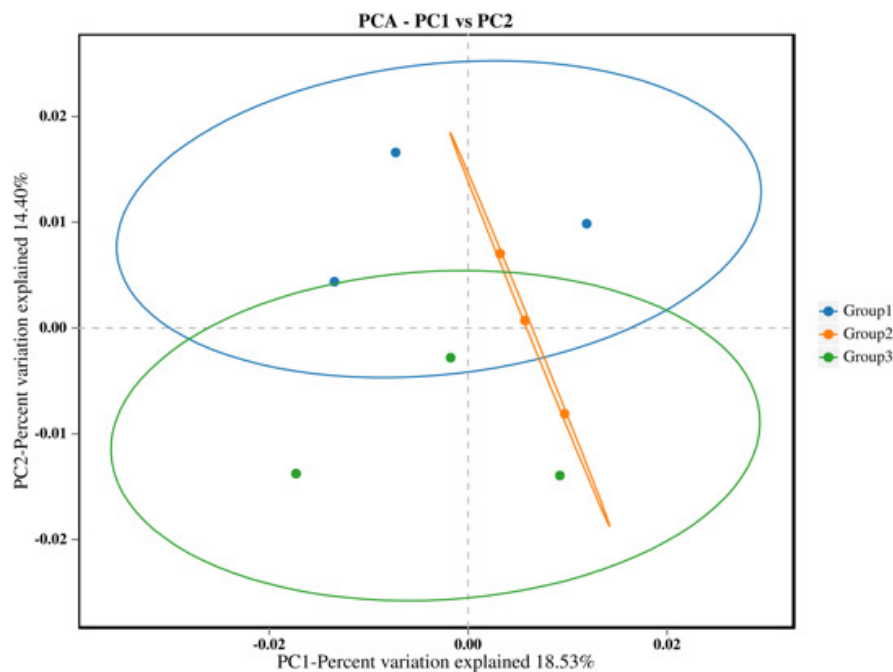
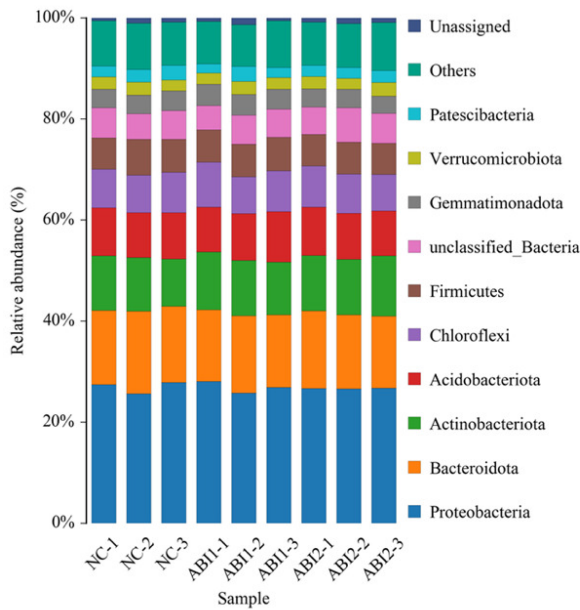


Fig. 2. PCA analysis of the intestinal flora of juvenile *L. crocea*. Group1: NC Group; Group2: ABI1 Group; Group3: ABI2 Group.

A



B

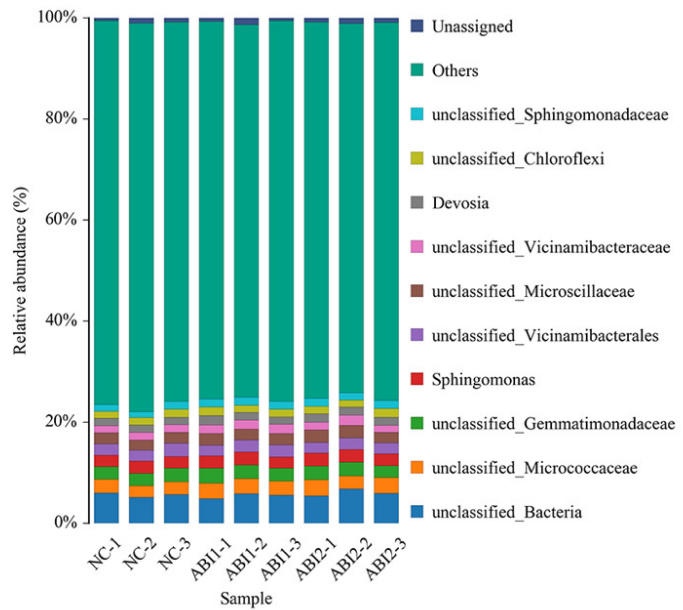
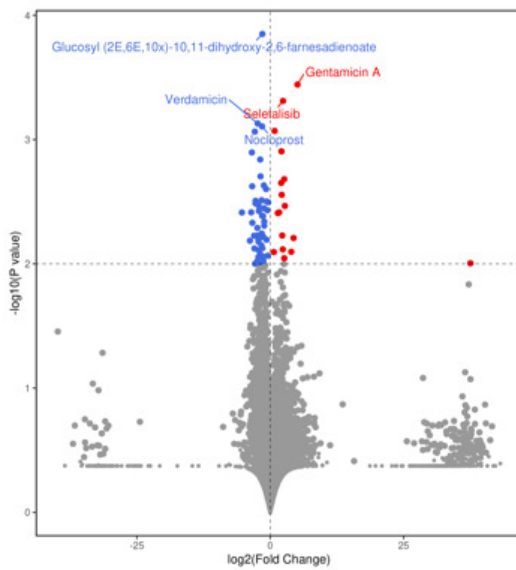


Fig. 3. Analysis of the composition of the intestinal flora of juvenile *L. crocea*. (A) Bacterial composition based on phylum level; (B) Bacterial composition based on genus level.

ABI1 vs. NC



ABI2 vs. NC

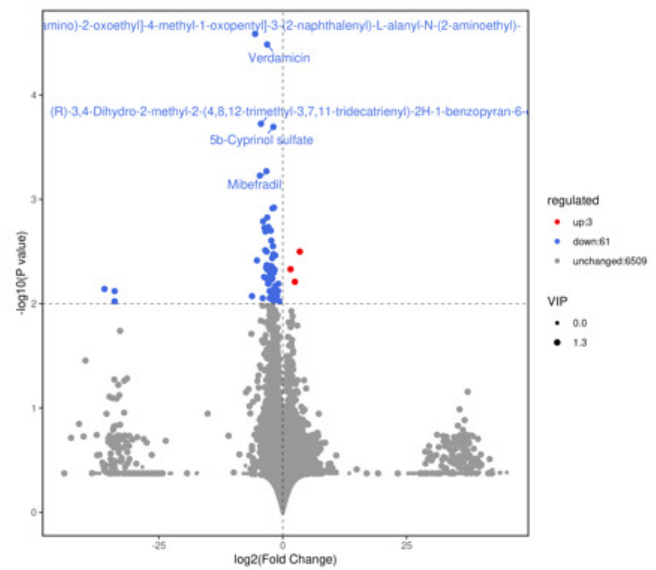


Fig. 4. Volcano map of differential metabolite

EFFECTS OF HEAT-INACTIVATED *L. ACIDOPHILUS* AC ON THE INTESTINAL METABOLITES OF JUVENILE *L. CROCEA*

DIFFERENTIAL METABOLITE ANALYSIS

From the volcano plots, where each data point corresponds to a specific metabolite. The horizontal coordinate

(x-axis) represents the fold change in expression levels of metabolites between different groups ($\log_2$ Fold Change), and the vertical coordinate (y-axis) represents the level of statistical significance ($-\log_{10}$ P-value). The size of the points is positively correlated with the variable importance in projection (VIP) value of the OPLS-DA model, meaning that the larger the point, the stronger the discriminating

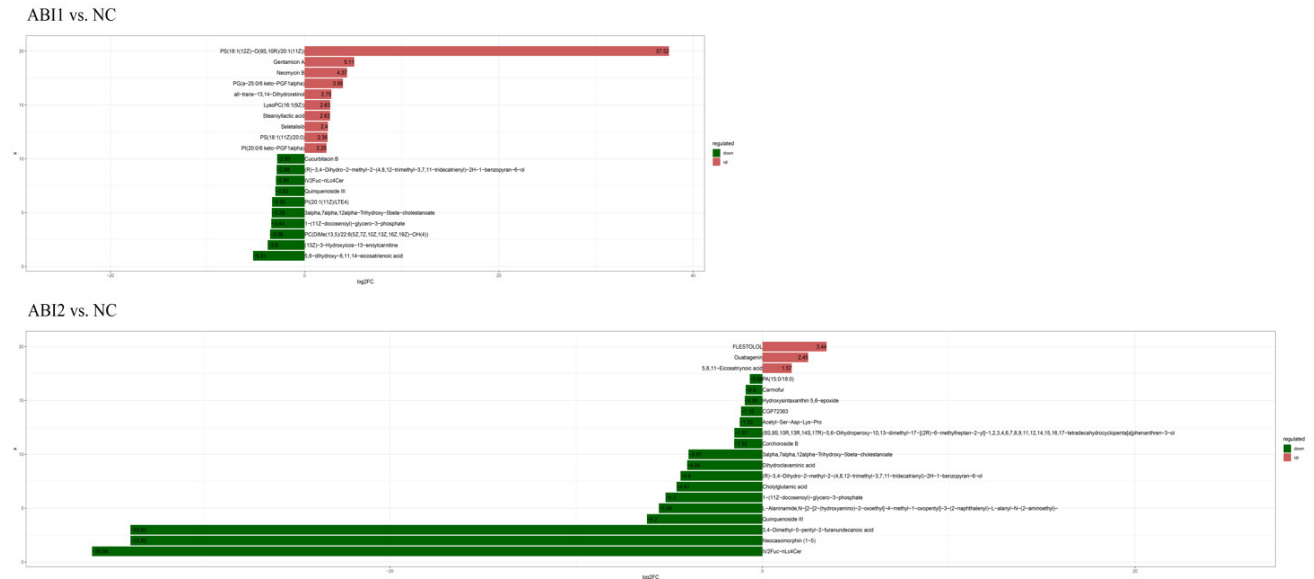


Fig. 5. Difference fold analysis

ability of the metabolite in the model, and the higher the reliability of the screening results. Blue marks indicate down-regulated differentially expressed metabolites, red marks indicate up-regulated differentially expressed metabolites, and gray represents metabolites that were detected but no significantly different. To further highlight key metabolites, the five metabolites with the highest significance based on *P*-values were labeled in the figure.

Volcano plots (Fig. 4) demonstrated the significant metabolic shifts. Compared to the NC group, the ABI1 group exhibited 17 significantly upregulated and 48 significantly downregulated metabolites. Key upregulated metabolites included Gentamicin A and Seletalisib, while Glucosyl (2E,6E,10x)-10,11-dihydroxy-2,6-farnesadienoate, Verdamicin, and Nocloprost were notably down-regulated. In the ABI2 group, 3 metabolites were significantly upregulated and 61 downregulated, with Verdamicin, (R)-3,4-Dihydro-2-methyl-2-(4,8,12-trimethyl-3,7,11-tridecatrienyl)-2H-1-benzopyran-6-ol, and 5b-Cyprinol sulfate among the most downregulated.

DIFFERENTIAL METABOLITE FOLD CHANGE ANALYSIS

Fold change analysis (Fig. 5) showed that in the ABI1 group, PS(18:1(12Z)-O(9S,10R)/20:1(11Z)) exhibited the greatest upregulation (37.52-fold), while 5,6-dihydroxy-8,11,14-eicosatrienoic acid showed the largest down-regulation (5.31-fold). In the ABI2 group, FLESTOL was the most upregulated (3.44-fold) metabolite, and IV2FucnLc4Cer was the most downregulated (35.98-fold) metabolite.

KEGG FUNCTIONAL ANNOTATION AND ENRICHMENT ANALYSIS

The KEGG pathway enrichment analysis of differential metabolites, as shown in Fig. 6, indicates that the differ-

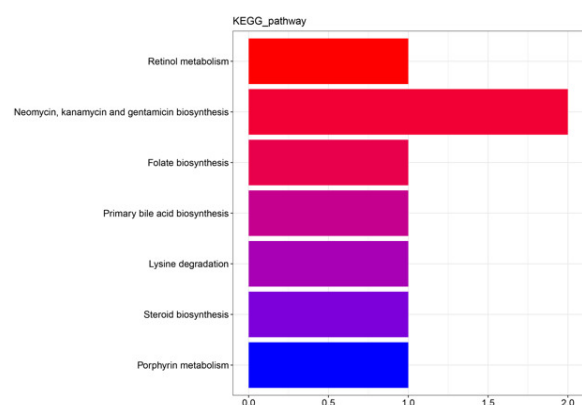
ential metabolites between the ABI1 and NC groups were enriched in retinol metabolism, neomycin, kanamycin and gentamicin biosynthesis, folate biosynthesis, primary bile acid biosynthesis, lysine degradation, steroid biosynthesis, and porphyrin metabolism. At the same time, the differential metabolites between the ABI2 and NC groups were enriched in drug metabolism-other enzyme systems, ubiquinone and other terpenoid-quinone biosynthesis, primary bile acid biosynthesis, steroid biosynthesis, porphyrin metabolism, and steroid hormone biosynthesis. It can be seen that the differential metabolites of both experimental groups compared to the control group were enriched in three pathways: primary bile acid biosynthesis, steroid biosynthesis, and porphyrin metabolism.

DISCUSSION

EFFECTS OF HEAT-INACTIVATED *L. ACIDOPHILUS* AC ON THE GROWTH PERFORMANCE AND INTESTINAL TISSUE STRUCTURE OF JUVENILE *L. CROCEA*

The morphological structure of the intestines directly influences fish health and growth by affecting digestion and absorption, making it a key factor in improving growth performance in aquaculture. Intestinal villus length, and number of goblet cells are key structural indicators that influence fish growth and digestive function. Longer intestinal villi increase the surface area available for nutrient absorption, thereby significantly improving growth rates and feed conversion efficiencies.^{10,11} Intestinal goblet cells secrete mucus to maintain mucosal integrity and protect intestinal tissues from environmental and microbial stressors.^{12, 13} In this study, feeding a diet supplemented with heat-inactivated *L. acidophilus* AC significantly improved intestinal morphology, as evidenced by increased villus length and goblet cell count in the ABI1 group. These changes suggest enhanced intestinal structural development and bar-

ABI1 vs. NC



ABI2 vs. NC

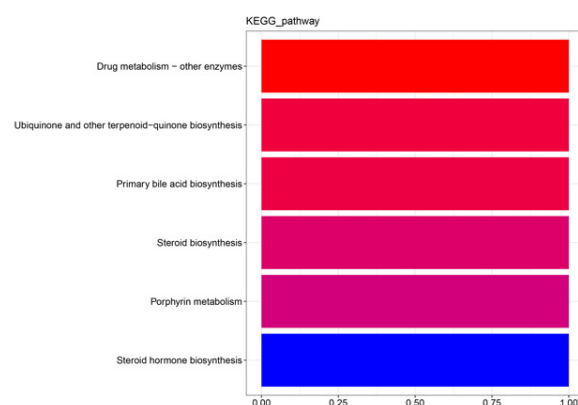


Fig. 6. KEGG enrichment analysis of differential metabolites

rier function. At the genus level, the abundance of *Sphingomonas* in the experimental groups was slightly higher than that in the control group. Previous studies have shown that supplementation with *Sphingomonas* can improve intestinal morphology (by increasing the VH/CD ratio in the ileum) and increase the abundance of beneficial bacteria,¹⁴ such as *Lactobacillus*, that produce short-chain fatty acids (SCFAs). SCFAs promote epithelial cell proliferation and maintain barrier integrity.¹⁵ Although SCFA levels were not directly measured, the improvements in intestinal structure and enhanced barrier function observed in this study may be associated with this bacterial strain.

The CF in Group ABI1 increased significantly. This index is positively correlated with crude fat content and reflects fish growth and health status,¹⁶ suggesting that heat-inactivated *L. acidophilus* AC may indirectly improve fattening by influencing fat digestion and absorption. In this study, although weight gain and specific growth rate showed an upward trend with increasing supplementation levels, the differences were not significant. Therefore, heat-inactivated *L. acidophilus* AC does not appear to directly enhance growth performance but rather mediates these effects indirectly by improving CF and tissue structure and enhancing intestinal health.

EFFECTS OF HEAT-INACTIVATED *L. ACIDOPHILUS* AC ON THE INTESTINAL MICROBIOTA OF JUVENILE *L. CROCEA*

The results showed that heat-inactivated *L. acidophilus* AC did not significantly affect the Chao1 index, ACE index, and Shannon index of the intestinal microbiota of *L. crocea*, but it did significantly affect the Simpson index. The Chao1 and ACE indices mainly reflect species richness, and there were no significant differences in these indicators among the groups, indicating that heat-inactivated *L. acidophilus* AC treatment did not change the total number of microbial species in the intestine. The Simpson's index reflects community evenness; a lower value indicates lower diversity.⁴ Although this index was significantly lower in the ABI2 group than in the NC group, the difference was minimal,

suggesting that high-concentration heat-inactivated *L. acidophilus* AC only slightly affected microbial community evenness and did not cause significant changes in community structure; however, it may still play a positive role in maintaining the balance of the gut microbiota.

The intestinal microbiota of fish plays a critical role in digestion, immunity, disease resistance, growth, and development.¹⁷ In this study, Proteobacteria was the dominant phylum in both the control and experimental groups, which is consistent with previous studies on marine fish.¹⁸ Furthermore, in the intestines of *L. crocea* supplemented with heat-inactivated *L. acidophilus* AC, the relative abundance of Actinobacteria showed an increasing trend. Since microorganisms belonging to this phylum have been shown to enhance disease resistance in fish and maintain microbial balance, an increase in their abundance may be beneficial to the host.¹⁹ As mentioned earlier, at the genus level, *Sphingomonas* may enhance intestinal barrier function in fish by supporting intestinal epithelial development and microbial interactions; therefore, an increase in its relative abundance is also beneficial to the host.

EFFECTS OF HEAT-INACTIVATED *L. ACIDOPHILUS* AC ON THE INTESTINAL METABOLITES OF JUVENILE *L. CROCEA*

Metabolomic analysis revealed significant treatment-specific metabolic changes in the ABI1 group, with Seletalisib and various phospholipid metabolites (including PS(18:1(12Z)-O(9S,10R)/20:1(11Z)), PI(20:0/6 keto-PGF1alpha), PS(18:1(11Z)/20:0) and LysoPC) showing significant upregulation. As components of the cell membrane, these phospholipids play a key role in maintaining cell integrity and signal transduction.²⁰⁻²² Seletalisib has been shown to enhance disease resistance in zebrafish.²³ 5,6-dihydroxy-8,11,14-eicosatrienoic acid, an eicosanoid, is a member of the eicosanoid family and plays a role in inflammatory and immune responses. Therefore, we conclude that the upregulation of seletalisib and various phospholipid molecules in the ABI1 group reflects enhanced membrane integrity, improved signal transduction, and in-

creased resistance to disease, whereas 5,6-dihydroxy-8,11,14-eicosatrienoic acid (an eicosanoid) indicates lower levels of inflammation. These metabolic characteristics are consistent with the improvements in intestinal function.

In the ABI2 group, 5 β -Cyprinol, 3 α ,7 α ,12 α -Trihydroxy-5 β -cholestanoate 1-(11Z-docosenoyl)-glycero-3-phosphate, and Cholyglutamic acid were significantly downregulated metabolites. As bile salts or bile acid derivatives, they are directly involved in metabolic processes such as lipid digestion and absorption.²⁴⁻²⁷ 5,8,11-Eicosatrienoic acid is a polyunsaturated fatty acid analog involved in the regulation of inflammation, influencing cell membrane fluidity, and lipid metabolism in fish.²⁸ Its significant upregulation may enhance the inflammatory response. Overall, the results from the ABI2 group may indicate that lipid metabolism is affected at higher supplementation levels, which could negatively affect growth performance and health status.

In KEGG pathways, compared with the NC group, the differential metabolites in both the ABI1 and ABI2 groups were enriched in three common pathways: primary bile acid biosynthesis, steroid biosynthesis, and porphyrin metabolism pathways. Primary bile acids are essential for the digestion and absorption of lipids,²⁷ steroids, which maintain cellular structural stability as membrane components,²⁹ and porphyrins, which are involved in redox reactions and energy metabolism³⁷. These changes in metabolic pathways suggest that heat-inactivated *Lactobacillus acidophilus* AC primarily exerts its effects by regulating metabolism and homeostasis rather than by directly promoting growth performance.

CONCLUSION

This study demonstrates that adding *L. acidophilus* AC, heat-inactivated at an appropriate concentration, to the diet significantly improves the intestinal morphology, intestinal health, and metabolic profile of juvenile *L. crocea*. Adding 10¹⁰ CFU/g of heat-inactivated *L. acidophilus* AC produced the most favorable response, particularly in terms of CF, intestinal structure, gut health, and improved metabolism. When the dosage was increased to 10¹¹ CFU/g, no further beneficial effects were observed, and negative effects, such as reduced metabolic efficiency and enhanced inflammatory responses emerged, indicating that the dose-response relationship for this intervention has plateaued or even begun to decline. This finding provides scientific evidence for the precise application of heat-inactivated probiotics in aquaculture.

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CONFLICT OF INTEREST STATEMENT

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.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Writing – original draft: Linfeng Huang (Lead). Writing – review & editing: Linfeng Huang (Lead). Supervision: Jiahang Xu (Lead). Investigation: Zhiqiang Ran (Lead). Visualization: Shanshan Wang (Lead). Formal Analysis: Haoyu Qiu (Lead). Software: Chenzhi Tao (Lead). Methodology: Ping Wang (Lead). Funding acquisition: Ping Wang (Lead).

ATTESTATION STATEMENTS

Data related to any of the subjects in the study has not been published previously. All study and manuscript data will be made available to the journal editors upon request before and/or after manuscript publication for review or query. This study was designed and reported in accordance with the ARRIVE 2.0 guidelines for animal research, and the corresponding checklist was completed and submitted.

DATA SHARING STATEMENT

Data will be made available on request.

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REFERENCES

1. Fisheries F. *The State of World Fisheries and Aquaculture 2024 Blue Transformation in Action.*; 2024.
2. Cabello FC, Godfrey HP, Tomova A, et al. Antimicrobial use in aquaculture re-examined: its relevance to antimicrobial resistance and to animal and human health. *Environmental microbiology*. 2013;15(7):1917-1942. doi:[10.1111/1462-2920.12134](https://doi.org/10.1111/1462-2920.12134)
3. Newaj-Fyzul A, Al-Harbi A, Austin B. Developments in the use of probiotics for disease control in aquaculture. *Aquaculture*. 2014;431:1-11. doi:[10.1016/j.aquaculture.2013.08.026](https://doi.org/10.1016/j.aquaculture.2013.08.026)
4. Ringø E, Hoseinifar SH, Ghosh K, Doan HV, Beck BR, Song SK. Lactic acid bacteria in finfish—An update. *Frontiers in microbiology*. 2018;9:1818. doi:[10.3389/fmicb.2018.01818](https://doi.org/10.3389/fmicb.2018.01818). PMID:30147679
5. Hoseinifar SH, Sun YZ, Wang A, Zhou Z. Probiotics as means of diseases control in aquaculture, a review of current knowledge and future perspectives. *Frontiers in microbiology*. 2018;9:2429. doi:[10.3389/fmicb.2018.02429](https://doi.org/10.3389/fmicb.2018.02429). PMID:30369918
6. Peng M, Tabashsum Z, Patel P, Bernhardt C, Biswas D. Linoleic acids overproducing *Lactobacillus casei* limits growth, survival, and virulence of *Salmonella typhimurium* and enterohaemorrhagic *Escherichia coli*. *Frontiers in Microbiology*. 2018;9:2663. doi:[10.3389/fmicb.2018.02663](https://doi.org/10.3389/fmicb.2018.02663). PMID:30443248
7. Zhang D, Li A, Guo Y, Zhang Q, Chen X, Gong X. Molecular characterization of *Streptococcus agalactiae* in diseased farmed tilapia in China. *Aquaculture*. 2013;412:64-69. doi:[10.1016/j.aquaculture.2013.07.014](https://doi.org/10.1016/j.aquaculture.2013.07.014)
8. Ren X, Han Y, Zeng F, et al. Effects of dietary heat-killed *Lactobacillus plantarum* and compound probiotics on the growth performance, antioxidative capacity, intestinal morphology and microbiota of Pacific white shrimp *Litopenaeus vannamei*. *Aquaculture Research*. 2022;53(9):3516-3530. doi:[10.1111/are.15857](https://doi.org/10.1111/are.15857)
9. Qiu H, Huang L, Wang H, et al. Effects of *Lactobacillus acidophilus* AC on the growth, intestinal flora and metabolism of zebrafish (*Danio rerio*). *Fish & Shellfish Immunology*. 2024;149:109570. doi:[10.1016/j.fsi.2024.109570](https://doi.org/10.1016/j.fsi.2024.109570)
10. Baeverfjord G, Krogdahl Å. Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar* L., distal intestine: a comparison with the intestines of fasted fish. *Journal of Fish Diseases*. 1996;19(5):375-387. doi:[10.1111/j.1365-2761.1996.tb00376.x](https://doi.org/10.1111/j.1365-2761.1996.tb00376.x)
11. Gao Z, Wang X, Tan C, Zhou H, Mai K, He G. Effect of dietary methionine levels on growth performance, amino acid metabolism and intestinal homeostasis in turbot (*Scophthalmus maximus* L.). *Aquaculture*. 2019;498:335-342. doi:[10.1016/j.aquaculture.2018.08.053](https://doi.org/10.1016/j.aquaculture.2018.08.053)
12. Cain K, Swan C. Barrier function and immunology. In: *Fish Physiology*. Elsevier; 2010:111-134. doi:[10.1016/S1546-5098\(10\)03003-7](https://doi.org/10.1016/S1546-5098(10)03003-7)
13. Knudsen D, Urán P, Arnous A, Koppe W, Frøkiær H. Saponin-containing subfractions of soybean molasses induce enteritis in the distal intestine of Atlantic salmon. *Journal of agricultural and food chemistry*. 2007;55(6):2261-2267. doi:[10.1021/jf0626967](https://doi.org/10.1021/jf0626967)
14. Sparagon WJ, Gentry EC, Minich JJ, et al. Fine scale transitions of the microbiota and metabolome along the gastrointestinal tract of herbivorous fishes. *Animal Microbiome*. 2022;4(1):33. doi:[10.1186/s42523-022-00182-z](https://doi.org/10.1186/s42523-022-00182-z). PMID:35606844
15. Zhang W, Yu X, Ma R, et al. Research progress on feed nutrition regulation of cultured *Larimichthys crocea* quality. *Feed industry*. 2024;45(22):1-13.
16. Luan Y, Li M, Zhou W, et al. The fish microbiota: research progress and potential applications. *Engineering*. 2023;29:137-146. doi:[10.1016/j.eng.2022.12.011](https://doi.org/10.1016/j.eng.2022.12.011)
17. Kim PS, Shin NR, Lee JB, et al. Host habitat is the major determinant of the gut microbiome of fish. *Microbiome*. 2021;9(1):166. doi:[10.1186/s40168-021-01113-x](https://doi.org/10.1186/s40168-021-01113-x). PMID:34332628
18. Medina M, Sotil G, Flores V, Fernández C, Sandoval N. In vitro assessment of some probiotic properties and inhibitory activity against *Yersinia ruckeri* of bacteria isolated from rainbow trout *Oncorhynchus mykiss* (Walbaum). *Aquaculture Reports*. 2020;18:100447. doi:[10.1016/j.aqrep.2020.100447](https://doi.org/10.1016/j.aqrep.2020.100447)
19. Elworthy S, Rutherford HA, Prajsnar TK, et al. Activated PI3K delta syndrome 1 mutations cause neutrophilia in zebrafish larvae. *Disease Models & Mechanisms*. 2023;16(3):dmm049841. doi:[10.1242/dmm.049841](https://doi.org/10.1242/dmm.049841). PMID:36805642

20. Nie Z, Xu X, Shao N, et al. Integrative analysis of microbiome and metabolome reveals the linkage between gut microbiota and carp growth. *Environmental Research*. 2023;220:115133. doi:[10.1016/j.envres.2022.115133](https://doi.org/10.1016/j.envres.2022.115133)
21. Che M, Lu Z, Liu L, Li N, Ren L, Chi S. Dietary lysophospholipids improves growth performance and hepatic lipid metabolism of largemouth bass (*Micropterus salmoides*). *Animal Nutrition*. 2023;13:426-434. doi:[10.1016/j.aninu.2023.04.007](https://doi.org/10.1016/j.aninu.2023.04.007). PMID:37251277
22. Hwang DF, Yeh YH, Lai YS, Deng JF. Identification of cyprinol and cyprinol sulfate from grass carp bile and their toxic effects in rats. *Toxicol*. 2001;39(2-3):411-414. doi:[10.1016/S0041-0101\(00\)00134-3](https://doi.org/10.1016/S0041-0101(00)00134-3)
23. Jiang Y, Liu Z, Zhang L, Liu W, Li H, Li X. Phosphatidylserine Counteracts the High Stocking Density-Induced Stress Response, Redox Imbalance and Immunosuppression in Fish *Megalobrama amblysephala*. *Antioxidants*. 2024;13(6):644. doi:[10.3390/antiox13060644](https://doi.org/10.3390/antiox13060644). PMID:38929083
24. Li M, Wang X, Qi C, et al. Metabolic response of Nile tilapia (*Oreochromis niloticus*) to acute and chronic hypoxia stress. *Aquaculture*. 2018;495:187-195. doi:[10.1016/j.aquaculture.2018.05.031](https://doi.org/10.1016/j.aquaculture.2018.05.031)
25. Hofmann AF, Hagey L. Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. *Cellular and Molecular Life Sciences*. 2008;65:2461-2483. doi:[10.1007/s00018-008-7568-6](https://doi.org/10.1007/s00018-008-7568-6). PMID:18488143
26. Hagey LR, Møller PR, Hofmann AF, Krasowski MD. Diversity of bile salts in fish and amphibians: evolution of a complex biochemical pathway. *Physiological and Biochemical Zoology*. 2010;83(2):308-321. doi:[10.1086/649966](https://doi.org/10.1086/649966). PMID:20113173
27. Tocher DR. Metabolism and functions of lipids and fatty acids in teleost fish. *Reviews in fisheries science*. 2003;11(2):107-184. doi:[10.1080/713610925](https://doi.org/10.1080/713610925)
28. Xu H, Ai Q, Mai K, et al. Effects of dietary arachidonic acid on growth performance, survival, immune response and tissue fatty acid composition of juvenile Japanese seabass, *Lateolabrax japonicus*. *Aquaculture*. 2010;307(1-2):75-82. doi:[10.1016/j.aquaculture.2010.07.001](https://doi.org/10.1016/j.aquaculture.2010.07.001)
29. Lushchak VI. Environmentally induced oxidative stress in aquatic animals. *Aquatic toxicology*. 2011;101(1):13-30. doi:[10.1016/j.aquatox.2010.10.006](https://doi.org/10.1016/j.aquatox.2010.10.006)