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Research Article

Effects of Probiotics and Its Extracellular Products on the Growth Performance, Immune Response, and *Aeromonas hydrophila* Resistance of Grass Carp

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The goal of this study was to research the effects of *Lactobacillus buchneri* L3-9 and its extracellular products on growth, immunity, intestinal microorganisms, and disease resistance to *Aeromonas hydrophila* in grass carp. A total of 120 fish (80–90 g) were distributed into 12 aquariums (10 fish/aquarium) in three replicates and fed 1% of their body weight for 21 days. The grass carp were fed four different diets: a control (group C), a diet supplemented with 1×10^6 cell g^{-1} of *L. buchneri* L3-9 (group B), a diet supplemented with extracellular products of *L. buchneri* L3-9 (group E), and a diet supplemented with *L. buchneri* L3-9 and the extracellular products (group BE). Compared with the control diet, the B, E, and BE diets increased the weight gain rate by 4.49%, 2.59%, and 4.38%, respectively ($p < 0.05$), and significantly decreased the feed conversion ratio by 0.72, 0.57, and 0.76, respectively ($p < 0.05$). Groups B, BE, and E showed significant decreases in mortality, with values of 26.67%, 16.67%, and 23.33%, respectively ($p < 0.05$). Group B showed a significant increase in superoxide dismutase (SOD) activity and total antioxidant capacity (T-AOC) and decrease in malondialdehyde (MDA) content compared to the control group ($p < 0.05$). The highest catalase (CAT) activity was found in group E ($p < 0.05$). In group B, the expression of the gut proinflammatory gene *TNF-α* was downregulated ($p < 0.05$), and the expression of anti-inflammatory genes, including *TGF-β1*, *IL-10*, and *Tlr-8*, was upregulated compared to that in the control group ($p < 0.05$). Groups BE and E showed an increased abundance of *Lactobacillaceae*. Additionally, a drastic decrease in the abundance of pathogenic bacteria such as *Aeromonadaceae* and *Enterobacteriaceae* was in these groups compared to the control group. In conclusion, *L. buchneri* L3-9 and its extracellular products could improve the growth performance, immune responses, and resistance to *A. hydrophila* in grass carp. This study provides insights for the development and application of microecological preparations.

1. Introduction

Grass carp are widely farmed worldwide and have the largest annual output of any fish; grass carp account for more than 10% of the total global aquaculture output, and the amount of farmed grass carp continues to increase [1]. However, high-density carries farming creates the risk of the outbreaks of a wide range of diseases, which has severely affected the development of grass carp aquaculture. *Aeromonas hydrophila* is a common pathogen in aquaculture, readily causing hemorrhagic septicemia in aquatic animals and severely affects the development of the aquaculture industry [2]. *A. hydrophila* is the most harmful pathogen in grass carp aquaculture because of its high mortality rate. Antibiotics are the most commonly used tools for disease prevention and control diseases, followed by vaccines, antibacterial peptides, and other biological agents [3–5]. The massive use of antibiotics in aquaculture has led to many problems, such as increased bacterial resistance, antibiotic residues, and

pollution of the water environment, which is not conducive to the health of aquatic livestock [6–8]. In addition, antibiotics added to the feed kill the beneficial bacteria in the gut, resulting in disturbance of the intestinal flora, which affects fish health [9]. Bacterial resistance caused by antibiotics poses a serious threat to human health [10]. Studies have shown that global antibiotic resistance will worsen in the coming years as more antibiotics use increases [11]. The extensive use of antibiotics and disinfectants has a great impact on the breeding environment, and antibiotic residues in fish affect food health [12]. Because of the problems caused by the extensive use of antibiotics, new safe and alternative methods are urgently needed to address bacterial diseases in aquatic products.

Probiotics are widely used to replace antibiotics in feed. Probiotics are widely used as feed additives to promote the growth and enhance the disease resistance of fish, and they are widely accepted as potential antiviral products [13]. Probiotics are a safe feed additive in aquaculture that can provide nutrients, regulate intestinal microorganisms, improve digestive enzyme activity, promote digestion and absorption, improve immunity, promote growth, and enhance the health of cultured animals [14]. Many studies have shown that probiotics can regulate the intestinal flora, promote fish intestinal health, and improve fish body health to enhance disease resistance [15, 16]. Some studies have shown that adding *Bacillus* to diets can enhance the disease resistance of red sea bream and reduce infection by *Edwardsiella tarda* [17]. Probiotics can change the health of fish when administered orally, especially to control bacterial disease in fish [18, 19]. *Bacillus licheniformis* and *Bacillus pumilus* can enhance the resistance of rohu to *A. hydrophila*. Adding *B. pumilus* to diets can enhance the resistance of Nile tilapia and reduce the incidence of disease [20]. In addition to the large number of probiotics used to replace antibiotics, prebiotics and synbiotics are also being increasingly used. Prebiotics improve the health of animals by promoting the propagation of beneficial bacteria in their gut [21]. Synbiotics bear the functions and characteristics of both probiotics and prebiotics [22]. There are many studies on probiotic use in aquaculture. However, there are few studies on probiotic metabolites and the combined effects of probiotics and their metabolites [23]. The metabolites of some probiotics also play an important role in the intestinal tract. Given this lack of relevant research, this study examined the effects of *L. buchneri* L3-9 and its extracellular products on the growth and immunity of grass carp. In our laboratory, a strain of *L. buchneri* L3-9 was previously isolated, and its extracellular product was shown to have a strong antibacterial effect against *A. hydrophila*. Therefore, it can be used to control intestinal pathogen abundance and can be combined with probiotics to regulate the intestinal microbial flora and address bacterial diseases in fish. Therefore, our present research objective was to study the effects of supplementation with *L. buchneri* L3-9 and its extracellular metabolites on the growth, immunity and resistance to *A. hydrophila* challenge of grass carp during a 21-day feeding trial.

Table 1: Ingredients of grass carp basic feed.

Ingredient	Percentage
Crude protein	28.0
Crude ash	15.0
Crude fat	4.0
Crude fiber	12.0
Moisture	12.5
Total phosphorus	1.4

2. Materials and Methods

2.1. Fish. Grass carp weighing 80–90 g and with an average body length of 19–20 cm were obtained from the Hubei fish seed field in Ezhou, Hubei, China. Before the experiment, they were fed in a plastic aquarium (150 l of water) at 22 −25°C for 21 days. A total of 300 grass carp were fed for the experiment. The fish were fed twice a day (9 : 00 and 16 : 00) at a rate of 1% of their body weight. The grass carp feed was obtained from Tongwei Biotechnology Co., Ltd. The nutrient composition of the feed is shown in Table 1. During this period, one-third of the aerated tap water was replaced every 2 days.

2.2. Lactobacillus buchneri L3-9 and Its Safety. L. buchneri L3-9 was obtained from the College of Life Science and Technology (Huazhong Agricultural University, Wuhan, China). *L. buchneri* L3-9 is stored in the China Center for Type Culture Collection (CCTCC), and the deposit number is CCTCCNO: M2018511. In vitro bacteriostatic experiments showed that *L. buchneri* L3-9 fermentation broth had a good inhibitory effect on *A. hydrophila*. Therefore, it has good potential resistance to *A. hydrophila*.

To evaluate the safety of *L. brucei* L3-9, grass carp were intraperitoneally injected with 0.1 ml of saline containing 10^8 cells ml^{-1} *L. buchneri* L3-9. The control group was injected with 0.1 ml of sterile saline (0.85% NaCl) [24]. All grass carp were injected intraperitoneally using a 1 ml sterile syringe. There were 10 grass carp per tank and three replicates per treatment. The grass carp were fed in a plastic aquarium (150 L water) at 22–25°C. Grass carp death was recorded every day for 2 weeks. There were no dead fish in the control and experimental groups during this period.

2.3. L. buchneri L3-9 and Extracellular Product Preparation. L. buchneri L3-9 was inoculated into liquid MRS medium at pH 6, consistent with the intestinal pH value of grass carp [25], and cultured for 48 h in a culture shaker at 30°C to measure the number of viable bacteria and the antibacterial effect. Then, the fermentation solution was centrifuged at 3,000x *g* at 4°C for 15 min, the supernatant was collected, and the bacteria were diluted with sterile water and frozen at −20°C before being added to the diet.

2.4. Diet Preparation. The basal diet was used as the control diet (C). The three experimental diet groups were the live bacteria-fed group (B), extracellular product-fed group (E), and live bacteria and extracellular product-fed group (BE). The basal diet (C-1, C-2, C-3) contained sterile water added

Table 2: Sequences of primers used for RT-qPCR in this study.

Primer	Primer sequence (5′–3′)	Accession no.	Product size (bp)	Amplification efficiency (%)
TNF-α	F: TGATGGTGTCGAGGAGGAAGGC R: TTGAGCGTGAAGCAGACAGCAG	HQ696609	112	100
IL-6	F: CAGCAGAATGGGGGAGTTATC R: CTCGCAGAGTCTTGACATCCTT	KC535507	134	97.35
IL-10	F: GCAACAGAACATCAATAGTCCTT R: CACCCTTTTCCTTCATCTTTTCA	HQ388294	251	91.36
Nrf2	F: CTGGACGAGGAGACTGGA R: ATCTGTGGTAGGTGGAAC	KF733814	234	94.69
TLR8	F: TCACATCGCTTCCAGGTCTC R: ACGGTGAAATAATGGGGGTT	HQ638214	133	98.74
TGF-β1	F: CCACTGTAGAACTAAACCAGGAG R: CTGTGATGTTGAACCATATGTGC	EU099588.1	156	99.11
β-Actin	F: GGCTGTGCTGTCCCTGTA R: GGGCATAACCCTCGTAGAT	XM_051886219	101	99.95

at 1% of the diet weight; the bacterial suspension (B-1, B-2, and B-3) was added at a final dose of 1×10^6 cell g^{-1} diet, and the extracellular products (E-1, E-2, E-3) were added at 1% of the diet weight; and in the BE group, the live bacteria and extracellular products (BE-1, BE-2, BE-3) were administered at a final dose of 1×10^6 cell g^{-1} diet and at 1% of the diet weight, respectively [26]. For different diet preparation processes, the appropriate amount of live bacteria and extracellular product suspension was evenly sprayed into the feed, the amount was 15% of the weight of the feed. Keep turning the feed while spraying, so that the feed absorbs evenly. Then, the feed dried on the sterile operating table until moisture content was 10%, the feed was then stored at −20°C for later use. To monitor the change in the effective viable bacterial count of *L. buchneri* L3-9 in the diet, the viable bacterial counts in the live bacteria-fed group were determined every 7 days. The results showed that the levels of *L. buchneri* L3-9 decreased over 14 days of storage. Therefore, fresh diets were prepared once a week to ensure sufficient levels of viable bacteria. The feed used is extruded feed with good absorbability.

2.5. Fish Feeding and Sampling. The fish were randomly divided into the control group (group C) and groups B, E, and BE (each group contained 30 fish) with three replicates. The control group fish were fed the basal diet, while group B was fed the *L. buchneri* L3-9-containing diet, group E was fed the extracellular product-containing diet, and group BE was fed both the *L. buchneri* L3-9 and extracellular product-containing diets. The fish were fed twice a day (9 : 00 and 16 : 00) at a rate of 1% of their body weight and weighed weekly, and the amount of bait in each tank was adjusted weekly.

2.6. Growth Performance. At the end of the 21-day feeding experiment, the fish were starved for 24 h, and the total weight of the fish in each tank was determined. The weight gain rate (WGR), feed conversion ratio (FCR), condition factor (CF), and visceral body rate (VSI) were determined as follows:

$$\text{WGR} = (\text{Final weight} - \text{initial weight})/\text{initial weight} \times 100\%, \quad (1)$$

$$\text{FCR} = \text{Dry feed intake}/\text{wet weight gain}, \quad (2)$$

$$\text{CF} = 100 \times \text{fish weight} \times \text{fish length}^3, \quad (3)$$

$$\text{VSI} = 100 \times (\text{visceral weight}/\text{fish weight}), \quad (4)$$

$$\text{SR} = 100\% \times (\text{final number}/\text{initial number}). \quad (5)$$

2.7. Analysis of Gut Intestinal Section of Grass Carp. Two fish were taken randomly from each plastic aquarium after 21 days for dissection and analysis. Thus, a total of six fish were collected from every treatment for gut-intestinal section analysis. A 2 cm midgut sample was placed into 3 ml of 4% paraformaldehyde and stored at 4°C until analysis.

2.8. Blood Sample for Immunological Measurement. Blood was sampled from the caudal vasculature using a 2.5 ml syringe after the fish were euthanized by MS222 (75 mg/l). Then, plasma samples were placed at 4°C for 12 hr. Plasma samples were centrifuged at 3,000x *g* for 10 min at 4°C to collect serum and stored at −80°C for analysis of superoxide dismutase (SOD) activity, catalase (CAT) activity, malondialdehyde (MDA) content, and total antioxidant capacity (T-AOC). These biochemical indices were determined by a commercial kit from Jiancheng Bioengineering Institute of Nanjing. All analyses were performed in three replicates. The detection limit and catalog number of the kits used were as follows: total superoxide dismutase assay kit (5.0–122.1 U/ml) (A001-1), catalase assay kit (0.2–24.8 U/ml) (A007-1), malondialdehyde assay kit (0–113.0 nmol/ml) (A003-1), and total antioxidant capacity assay kit (0.2–55.2 U/ml) (A015-1).

2.9. Analysis of the Intestinal Microbiota. The intestine was removed under sterile conditions and cleaned with phosphate-buffered saline (PBS). The midgut contents were carefully

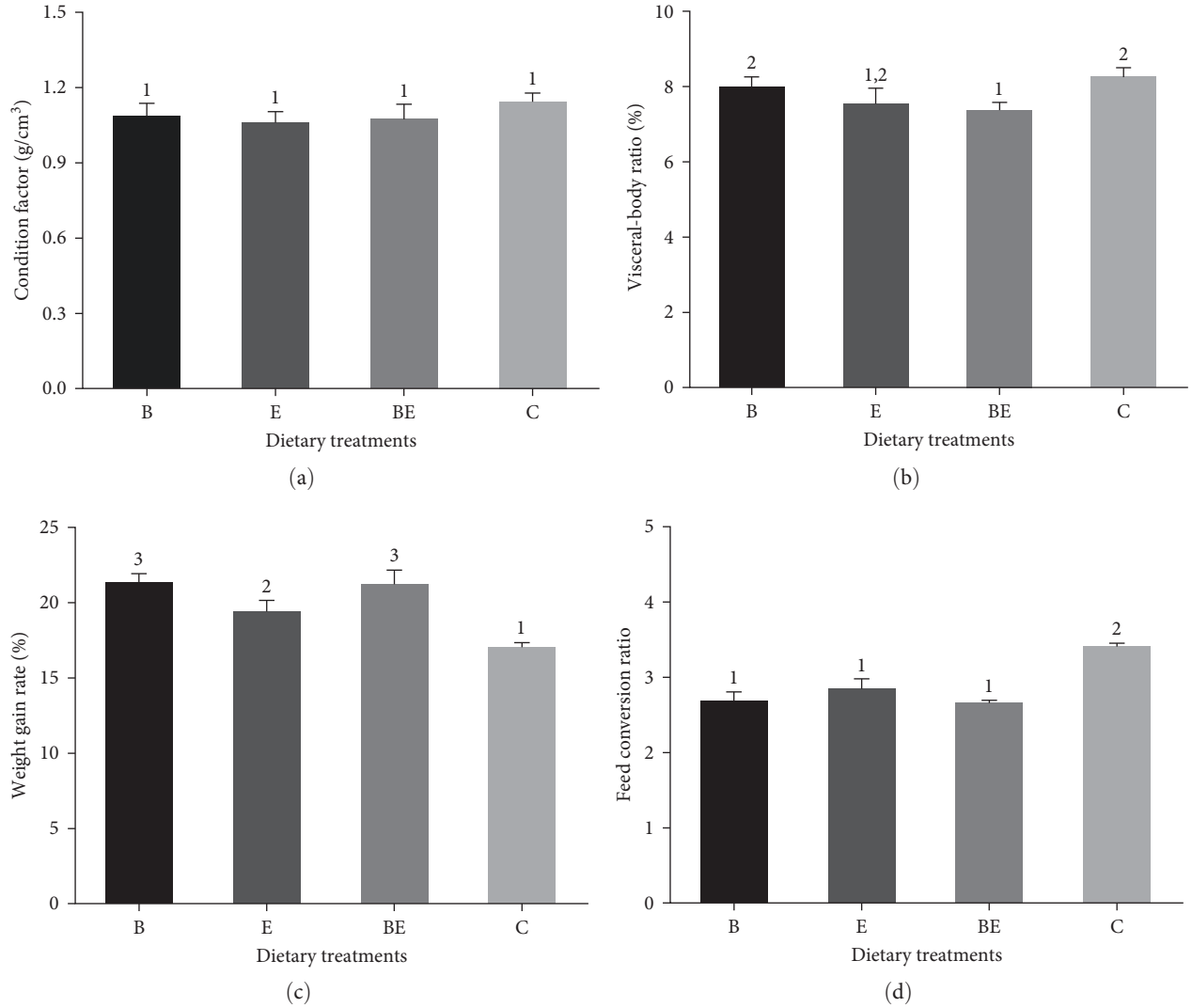

Figure 1: Growth performance and feed utilization of grass carp fed the different diets for 3 weeks. Condition factor (CF) (a), visceral body rate (VSI) (b), weight gain rate (WGR) (c), and feed conversion ratio (FCR) (d). The data are the average of three replicates. Different numbers indicate significant differences between different treatment groups ($p < 0.05$).

collected and put into tubes, which were quickly stored at −80°C for analysis of the intestinal microbial structure. High-throughput sequencing technology was used to analyze the intestinal flora of grass carp in each group. The gut microbiota of six fish from each group was analyzed. The samples were then sent to Beijing Qingke Biotechnology Co., Ltd., to determine the intestinal microbial structure.

2.10. Gene Expression Analysis. The intestinal tract of grass carp in each group was collected, and the mRNA expression levels of the tumor necrosis factor α (*TNF-α*), interleukin 6 (*IL6*), interleukin 10 (*IL10*), transforming growth factor β1 (*TGF-β1*), toll-like receptor 8 (*TLR8*), and NF-E2-related factor 2 (*Nrf2*) genes were measured. The TB Green Premix Ex TaqTM II kit was used in the Bio-Rad CFX96 real-time quantitative fluorescent PCR assay system (Bio-Rad, USA). Accurate biology was employed to determine the mRNA levels of the *TNF-α*, *IL6*, *IL10*, *TGF-β1*, *TLR8*, and *Nrf2* genes. The PCR procedure was as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The results were analyzed by the $2^{-\Delta\Delta CT}$ method [27]. The PCR-specific primers were designed according to the sequences in Table 2.

2.11. Challenge Study. After 21 days of rearing, protection against the pathogen in grass carp in the experimental groups was tested by challenging fish with *A. hydrophila* obtained from the College of Life Science and Technology (Huazhong Agricultural University, Wuhan, China). A total of 24 fish with three replicates in each group were used for the challenge experiment. For grass carp challenge, 0.2 ml of *A. hydrophila* suspension (1×10^7 cfu ml^{-1}) was intraperitoneally injected in the fish using a 1 ml sterile syringe [28]. The dose was decided in a previous experiment based on the calculation of lethal dose 50 (LD50). The death of grass carp in each group was recorded every day for 10 days. The dead grass carp showed symptoms of *A. hydrophila*.

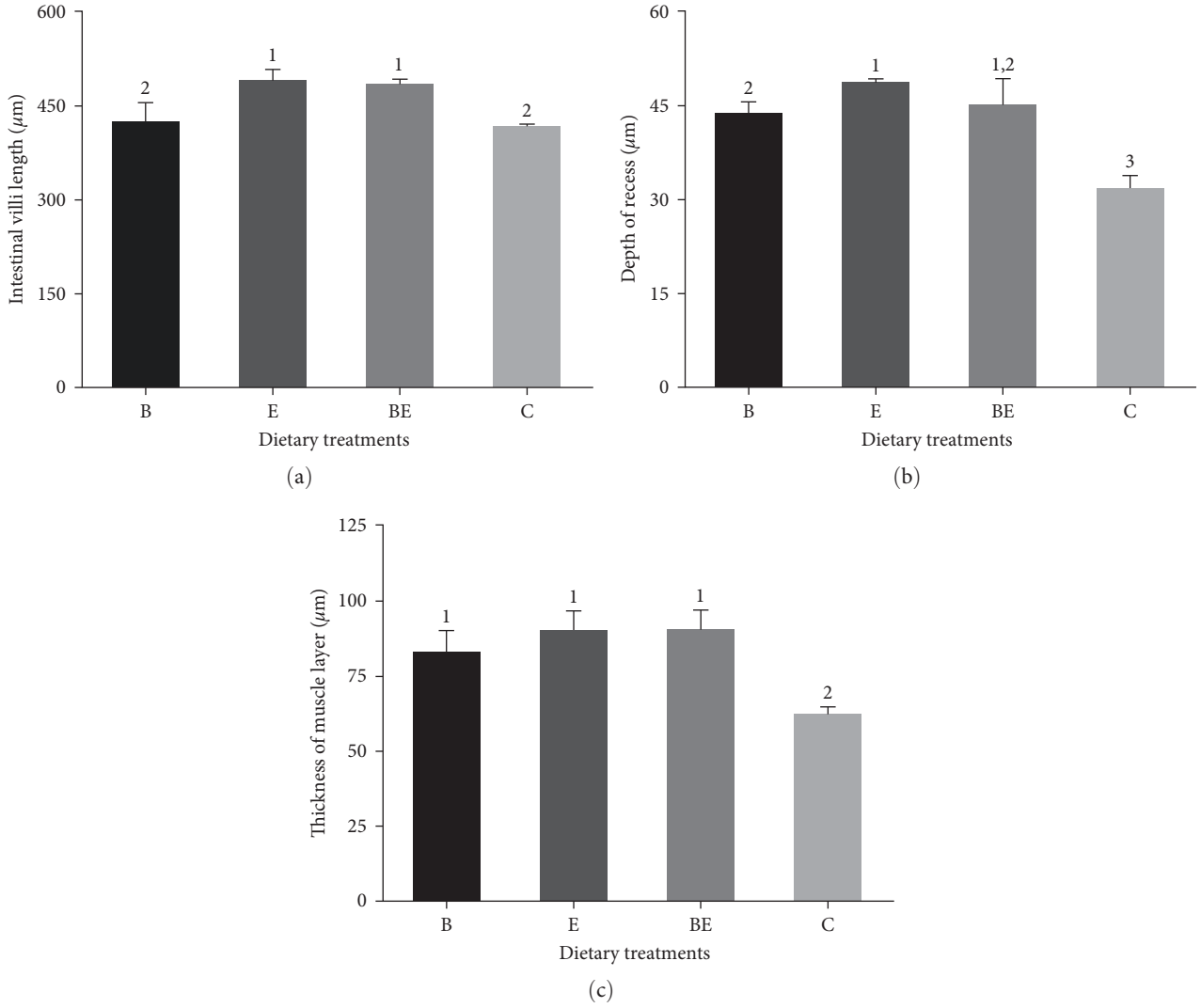

Figure 2: Effect on the intestinal tract of grass carp fed the different diets for 3 weeks. Intestinal villus length (a), depth of the recess (b), and thickness of the muscle layer (c). The data are the average of three replicates. Different numbers indicate significant differences between different treatment groups ($p < 0.05$).

2.12. Statistical Analysis. IBM SPSS Statistics 20 software was used to conduct one-way analysis of variance (ANOVA) and Duncan's multiple comparisons. All data are presented as the mean $\pm$ SD (standard deviation), with $p < 0.05$ being statistically significant.

3. Results

3.1. Growth Indicators. The growth performance and FCR of grass carp fed the different diets for 3 weeks are shown in Figure 1. Compared with the control diet, the B, E, and BE diets increased the WGR by 4.49%, 2.59%, and 4.38%, respectively, and decreased the FCR by 0.72, 0.57, and 0.76, respectively, after 3 weeks of feeding, and the differences were significant ($p < 0.05$). However, there were no significant differences among the different experimental groups ($p > 0.05$). The fish fed the B and BE diets had a significantly higher WGR and lower FCR than the fish fed the E diets ($p < 0.05$). The VSR of the fish fed the BE diets was lower than that of the fish fed other diets ($p < 0.05$). The survival rate and CF were not significantly different among the different dietary treatments ($p > 0.05$).

3.2. Intestinal Tract Indicators. Fish fed the B, E, and BE diets had a significantly increased intestinal villus length, depth of recess, and thickness of muscle layer compared with those fed the control diet ($p < 0.05$), as shown in Figure 2. Fish fed the E and BE diets had a significantly higher intestinal villus length and depth of recess compared with those fed the B diet, and the difference was significant ($p < 0.05$). However, there were no significant differences in the muscle layer thickness among the experimental dietary treatments ($p > 0.05$). The intestinal tract of fish fed the B, E, and BE diets was in a significantly better state than that of fish fed the control diet, as shown in Figure 3 ($p < 0.05$).

3.3. Disease Resistance. Mortality decreased significantly in groups B, BE, and E, by 26.67%, 16.67%, and 23.33%,

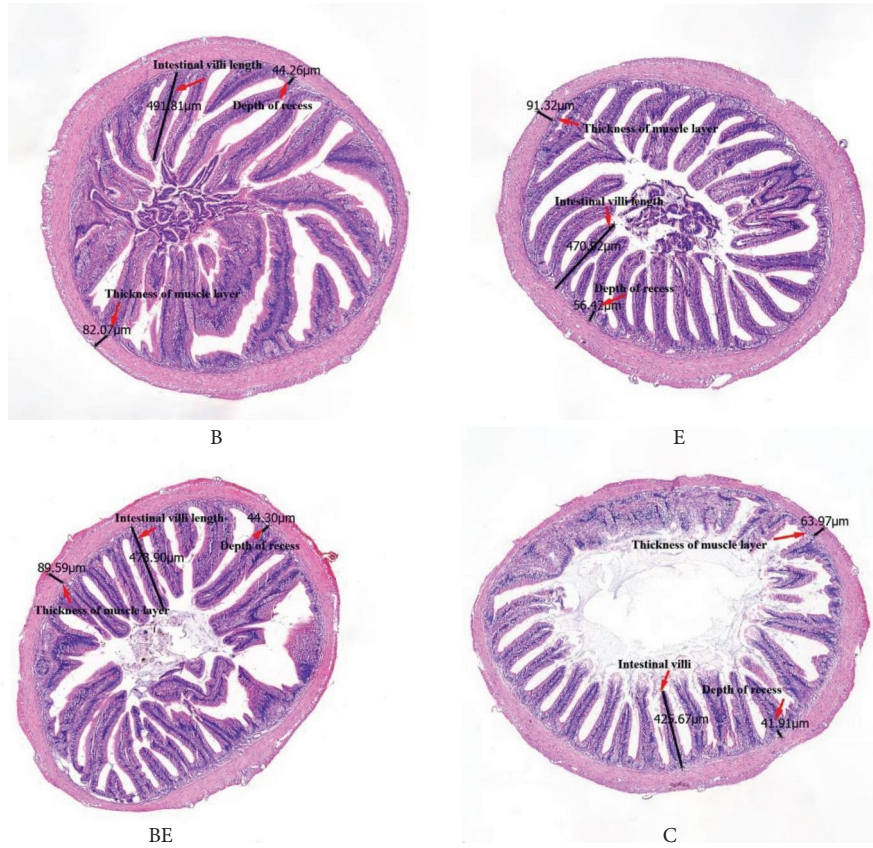

FIGURE 3: Comparison of intestinal sections of grass carp fed different diets for 3 weeks.

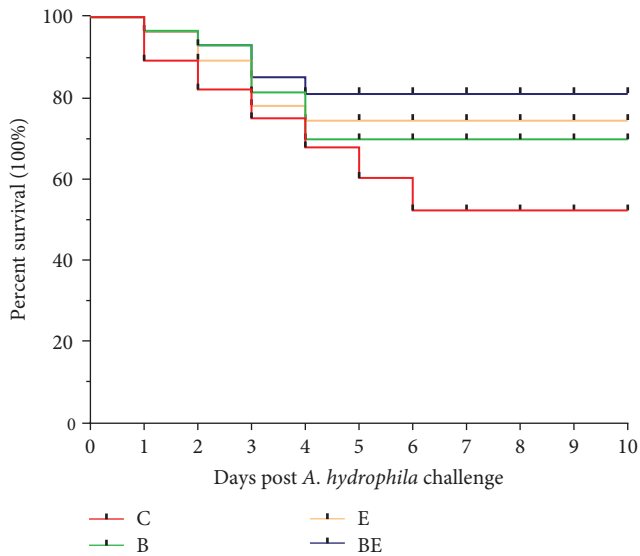

FIGURE 4: Survival rate of grass carp fed different supplemental diets after artificial challenge with *A. hydrophila* via intraperitoneal injection. The data are the average of three replicates.

respectively, when the fish were challenged with live *A. hydrophila* compared with the control group ($p<0.05$). The fish in the control group exhibited the highest mortality rate of 56.67% ($p<0.05$). All the dead fish showed bleeding from the anus and accumulation of yellow fluid in the intestine, which are characteristics of *A. hydrophila* infection. After 10 days, there was no further disease incidence, and the survival rate of the BE group was the highest (83.33%) (Figure 4) ($p<0.05$).

3.4. Immune Responses. Group B exhibited in a significant increase in SOD activity, T-AOC and CAT activity ($p<0.05$). However, this group exhibited a significant decrease ($p<0.05$) in MDA content compared to the control group after 21 days of feeding (Figure 5). Group BE showed no significant differences in SOD activity, T-AOC, and MDA content compared to the control. However, this group showed a significant increase ($p<0.05$) in CAT activity after 21 days of feeding. Group E showed obviously higher CAT activity ($p<0.05$) than the other treatment groups. In addition, group E exhibited significantly improved MDA content ($p<0.05$) compared to the control. However, this group showed no significant differences in SOD activity and T-AOC compared to the control ($p>0.05$).

3.5. Relative Expression of Immune-Related Genes. Compared with the control group, group B showed increased relative mRNA expression of *TGF-β1*, *IL-10*, and *Tlr-8*, as shown in Figure 6 ($p<0.05$). However, the relative mRNA expression of *TNF-α* was decreased, as shown in Figure 7 ($p<0.05$). Compared with the control group, group E showed significantly increased relative mRNA expression of *Tlr-8* ($p<0.05$). However, there were no significant differences in the relative mRNA

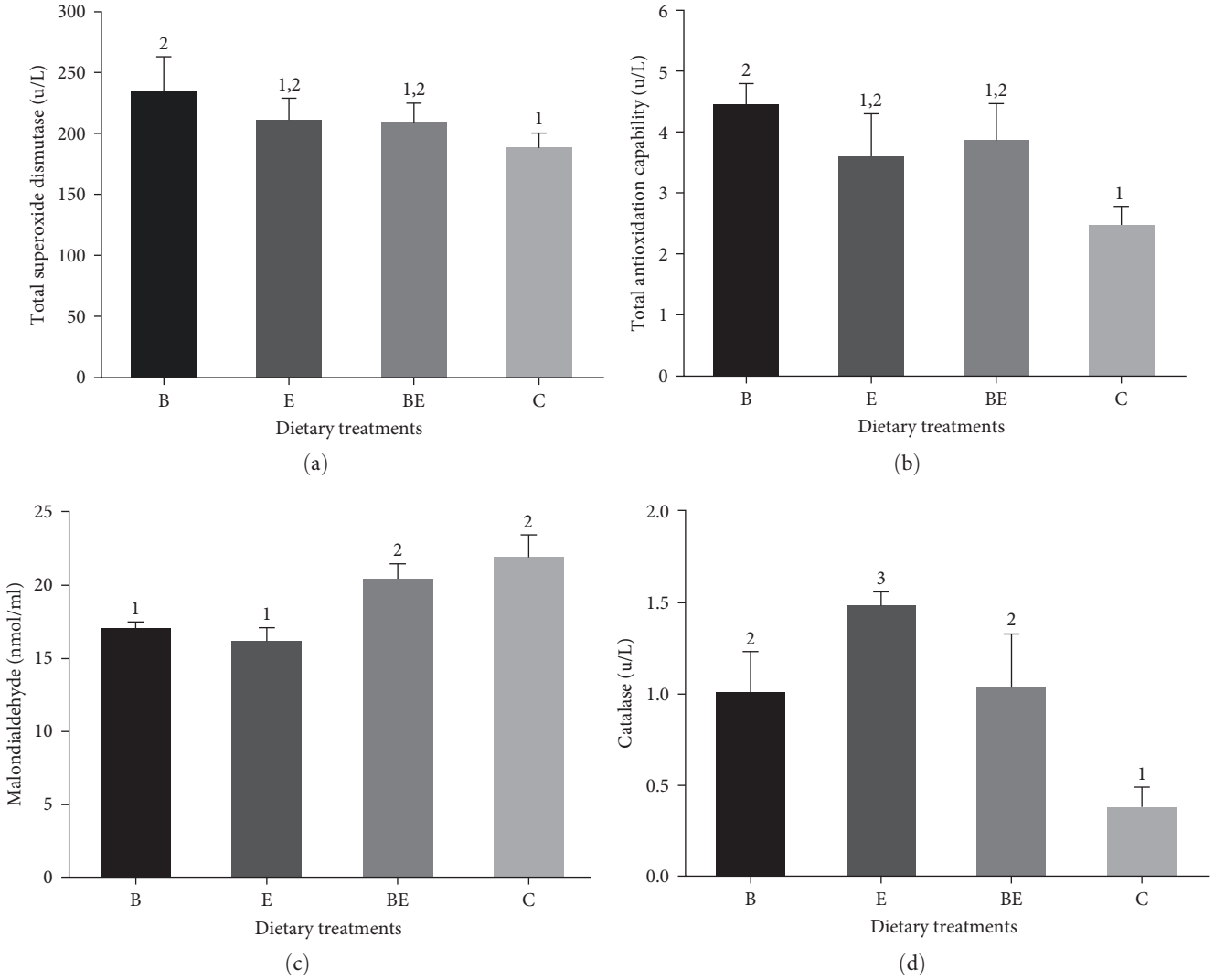

Figure 5: Effect on the total superoxide dismutase (SOD) activity (a), total antioxidant capacity (T-AOC) (b), malondialdehyde (MDA) content (c), and catalase (CAT) activity (d) in grass carp after feeding with different diets for 21 days. The data are the average of three replicates. Different numbers indicate significant differences between different treatment groups ($p < 0.05$).

expression of other genes ($p > 0.05$). Compared with the control group, group BE showed increased relative mRNA expression of *Tlr-8* and *TGF-β1* ($p > 0.05$). However, the relative mRNA expression of *TNF-α* was decreased. Group B showed the most obvious effect on the expression of intestinal immune-related genes in grass carp, followed by group BE.

3.6. Gut Microbiota Analysis of Grass Carp. To examine the effects of different diets on the grass carp intestinal microflora, samples from groups B, E, BE, and C were analyzed. The numbers of unique OTUs for the B, E, BE, and C groups were 2,361, 9,103, 8,265, and 4,886, respectively (Figure 8). Alpha diversity analysis showed that the community richness (Chao, Ace) and diversity (Shannon) of the E and BE groups were higher than those of the control group (Table 3). However, the community richness (Chao, Ace) and diversity (Shannon) of the B group were lower than those of the control group. The most abundant bacterial phyla were *Comamonadaceae*, *Enterobacteriaceae*, *Xanthomonadaceae*, *Aeromonadaceae*, and *Lachnospiraceae*. Groups BE and E exhibited an increased abundance of *Lactobacillaceae* (Figure 9). Additionally, a drastic decrease in the abundance of pathogenic bacteria such as *Aeromonadaceae* and *Enterobacteriaceae* was detected compared to the control. The addition of *L. buchneri* L3-9 to the diet resulted in an increased abundance of *Lactobacillaceae*.

4. Discussion

Lactic acid bacteria, as a safe potential antibiotic replacement product, are being increasingly used in aquaculture. Many studies have shown that the application of lactic acid bacteria in aquaculture can improve the intestinal microbiota, promote fish growth, enhance immunity, and improve fish disease resistance [29–32]. Many studies have shown that adding probiotics to feed can promote fish growth, which is consistent with the results of this paper [33]. The present study confirmed that supplementation with *L. buchneri* L3-9 and its extracellular products could increase the growth rate

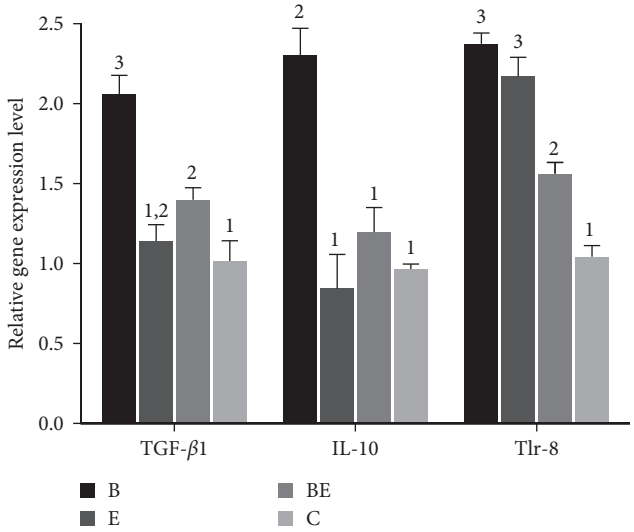

FIGURE 6: The expression of the *TGF-β1*, *IL-10*, and *Tlr-8* genes was observed after feeding grass carp supplemented diets for 21 days. The data are the average of three replicates. Different numbers indicate significant differences between different treatment groups ($p < 0.05$).

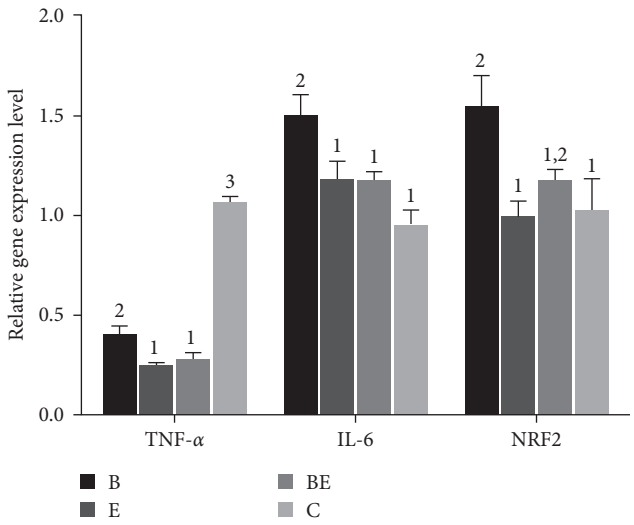

FIGURE 7: The expression of the *TNF-α*, *IL-6*, and *Nrf2* genes was observed after feeding grass carp supplemented diets for 21 days. The data are the average of three replicates. Different numbers indicate significant differences between different treatment groups ($p < 0.05$).

of grass carp. The results indicate that the weight gain rates in the B, E, and BE groups were significantly increased ($p < 0.05$) compared with that in the control group. The feed conversion ratios in the B, E, and BE groups were significantly decreased ($p < 0.05$) at the end of the study period (21 days). Previous studies have shown that after supplementation with 1.0×10^8 cfu/g *Lactobacillus plantarum* for 28 days, the SGR was significantly higher than that of the control group

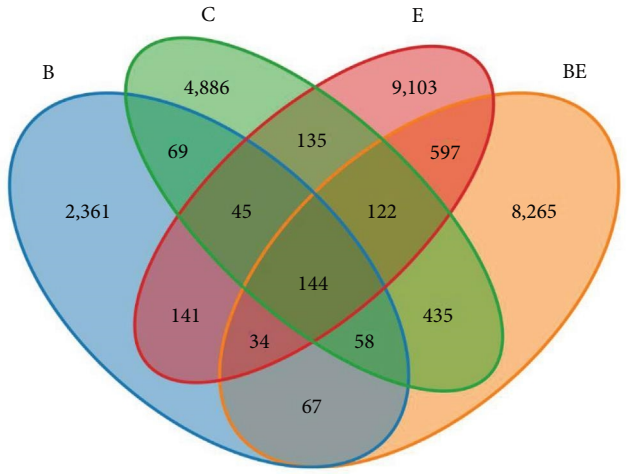

FIGURE 8: Venn diagram showing the unique and shared OTUs in grass carp after feeding with different diets for 21 days.

[34]. Similarly, after feeding with rhamnose for 56 days, the WG and SGR of red snapper increased significantly, which was similar to the results of this study [35]. To our knowledge, the current study is the first to examine the effects of *L. buchneri* L3-9 and its extracellular products on the growth, immunity, intestinal microbes, and disease resistance of grass carp, and the results indicate the potential of the development of *L. buchneri* L3-9 as a microecological agent.

The intestinal villus length and depth of the recess affect the absorption capacity for intestinal nutrients, and the thickness of the muscular layer affects intestinal health. Probiotics may improve fish growth by increasing the length and density of intestinal villi and thereby increasing the intestinal absorption area [36]. Our culture results showed that the intestinal villus lengths in the E and BE groups were significantly increased compared with those in the control group. However, in the B group, the value was significantly lower. In addition, the intestinal villus length in group E was the highest among all groups. The crypt depth and thickness of the muscle layer increased significantly in groups B, BE, and E compared with the control group. However, there was no significant difference among groups B, BE, and E. This indicates that adding extracellular metabolites of *L. buchneri* L3-9 can improve the intestinal health status of grass carp and promote digestion and absorption.

Previous studies have shown that adding plant extracts, probiotics, and antimicrobial peptides to feed can boost immune levels in fish [37–39]. SOD and T-AOC are important indices to measure the antioxidant capacity of the body. SOD can regulate the level of superoxide and hydrogen peroxide in the body to maintain the health of fish [40]. CAT scavenges free radicals from the body, reducing harmful effects and supporting proper immune function [41]. Malondialdehyde (MDA) is negatively correlated with antioxidant capacity, so it is useful to evaluate antioxidant capacity in fish [42]. Studies have shown that SOD and CAT in intestine of crucian carp increased significantly after feeding complex probiotics ($10^6 - 10^8$ cfu/ml) [43]. In this study, SOD and T-AOC levels were higher in group B than in the other

Table 3: Alpha diversity analysis of grass carp for 21 days ($n = 3$).

Group	ACE	Chao1	Simpson	Shannon	Coverage
B	537.85 ± 44.68^{a}	538.25 ± 44.89^{a}	0.83 ± 0.02^{a}	4.84 ± 0.19^{a}	1.00 ± 0.00
E	$1{,}468.75 \pm 79.74^{b}$	$1{,}465.79 \pm 81.99^{b}$	0.92 ± 0.04^{a}	6.88 ± 0.79^{b}	1.00 ± 0.00
BE	$1{,}900.79 \pm 63.79^{c}$	$1{,}896.52 \pm 63.57^{c}$	0.89 ± 0.04^{a}	6.58 ± 0.69^{ab}	1.00 ± 0.00
C	680.49 ± 36.34^{a}	678.06 ± 37.42^{a}	0.90 ± 0.02^{a}	5.37 ± 0.36^{ab}	1.00 ± 0.00

Values are means of triplicate groups and presented as mean ± SE. Values in the same column having different superscript letters are significantly different ($p < 0.05$).

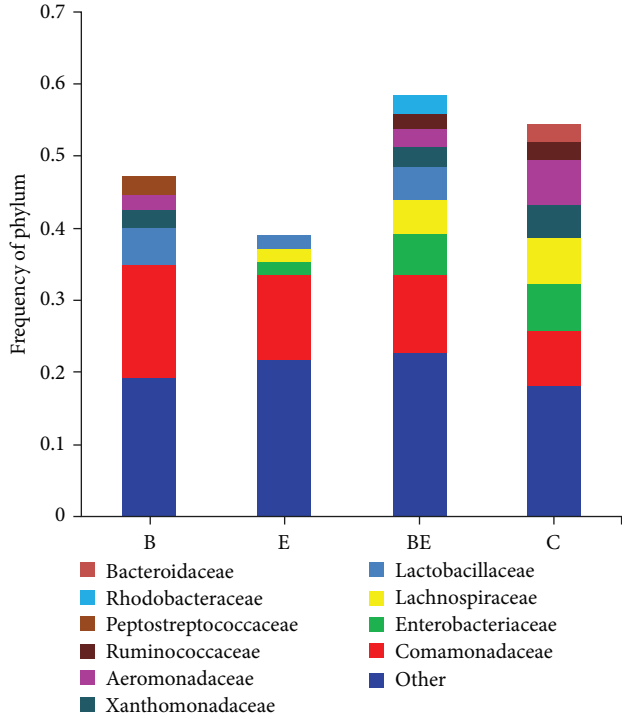

Figure 9: Distribution bar plot of different bacterial phyla observed after feeding with different diets for 21 days in grass carp.

groups, and groups BE and E also increased but not significantly compared to the control group. The CAT levels in the B, BE, and E groups were higher than those in the control group, and the levels of CAT in group E were the highest. The levels of MDA in the B, BE, and E groups were lower than those in the control group, but group BE was not significantly different from the control group. According to the analysis of serum immune indices, the immune level of grass carp in group B was enhanced most obviously, followed by that in group E. These results demonstrated that *L. buchneri* L3-9 and its extracellular products have some effect on the antioxidant capacity of fish. The single addition of *L. buchneri* L3-9 had the most obvious effect on the serum immune indices of grass carp. Compared with the control group, the mixed group supplemented with *L. buchneri* L3-9 and its metabolites had the least significant effect on serum immune indices. Further study is needed on the interaction mechanism between *L. buchneri* L3-9 and its metabolites.

The intestinal tract is the digestive and absorption organ of fish, and it is also the immune organ. Intestinal tract health affects the disease resistance of fish [44]. There are many cytokines related to fish immunity, including pro-inflammatory factors (*TNF-α*, *IL-8*, and *Nrf2*) and anti-inflammatory factors (*TGF-β1*, *IL-10*, and *Tlr-8*). The level of the *TNF-α* gene indicates the response of fish to invasion by bacteria and viruses. *IL-10* and *TGF-β1* can inhibit the release of inflammatory cytokines, thus reducing inflammation in fish. When 5×10^{8} cfu/g *Lactobacillus lactis* was added to the carp diet, the expression of the immune-related cytokines *IL-10* and *TGF-β1* was significantly upregulated, and the expression of the pro-inflammatory cytokines *TNF-α*, *IL-6*, and *IL-12* was significantly downregulated, which enhanced the immune ability of carp [45]. Some studies have also shown that a hybrid tilapia diet supplemented with 1.0×10^{9} cfu/g *Lactobacillus brevis* significantly increased the gene expression of the anti-inflammatory cytokine *TGF-β1* [46]. Studies have revealed that probiotic mixtures can promote the expression of the anti-inflammatory cytokine *IL-10*, enhancing the body's resistance to disease [47]. In our study, the mRNA levels of *IL10*, *TGF-β1*, and *Tlr-8* in group B were significantly upregulated, while *TNF-α* showed downregulation compared with the control group. The mRNA levels of *Tlr-8* in groups E and BE were significantly upregulated, while there were no significant differences in the expression of *IL10* and *TGF-β1* compared with the control group. These results indicated that a diet with *L. buchneri* L3-9 and its extracellular products could affect the expression of intestinal immune-related genes. These findings suggested that dietary *L. buchneri* L3-9 and its extracellular products can induce nonspecific immunity and promote the fish immune system to resist pathogen invasion. Among the treatments, the addition of *L. buchneri* L3-9 had the most obvious effect on the expression of intestinal immune-related genes. Although the extracellular products of *L. buchneri* L3-9 enhanced the expression of intestinal immune-related genes, some of the genetic differences were less significant than those in the control group. More research needs to be done to identify the exact active ingredient or the appropriate concentration and optimal time of addition.

Intestinal tract is the main nutrient absorption and immune organ of fish and plays a decisive role in determining the health of fish by regulating the interaction between the body and food, the environment, and pathogenic microorganisms [48]. The intestinal flora composition is a key factor affecting intestinal health, affecting digestion, absorption, immunity, and metabolism of fish [49, 50]. We studied the effects of *L. buchneri* L3-9 and its metabolites on the intestinal microbial community distribution of grass carp. It was found that the abundance of pathogenic bacteria such as *Aeromonadaceae*, *Enterobacteriaceae*, and *Lachnospiraceae* in groups E and BE was low, while the abundance of beneficial bacteria such as *Lactobacillaceae* was significantly

increased. Previous research has shown that many probiotics secrete antibacterial substances that kill disease-causing bacteria [51, 52]. This study shows that *L. buchneri* L3-9 can produce antibacterial substances to kill *A. hydrophila*, which may be the reason for the decrease of intestinal pathogenic bacteria in grass carp in this study. Alpha diversity refers to the species diversity of intestinal microorganisms, which is mainly used to reflect the richness and evenness of microorganisms [53]. The alpha diversity (Chao1 & Shannon) of the group fed *L. buchneri* L3-9 in the present study was lower than that of the control group but not significantly different, suggesting that *L. buchneri* L3-9 had little effect on the diversity of grass carp intestinal microbiota. Previous studies have shown that probiotics can significantly increase the intestinal microbial community composition of Nile tilapia [54], which is inconsistent with the results of this study. In this study, the alpha diversity (Chao1) of the BE and E groups was higher than that of the control group, which showed a significant difference, indicating that extracellular metabolites of *L. buchneri* L3-9 increased the intestinal flora diversity of grass carp.

In this study, we found that *L. buchneri* L3-9 and its extracellular metabolites can improve the immunity and resistance of grass carp to *A. hydrophila*. The feeding in different groups was treated for 21 days, and *A. hydrophila* was challenged. The survival rate of each group at 10 days was recorded. Studies have shown that the survival rate of the BE group was the highest. The results indicate that the groups B, BE, and E can enhance the disease resistance of fish, and the group BE has the best effect. This may be closely related to the extracellular products of *L. buchneri* L3-9 protecting the fish intestinal tract from attack by *A. hydrophila* by regulating microbial diversity, regulating the intestinal microorganism innate immune response, increasing the expression of immune-related genes, and increasing the number of probiotic bacteria. This result is consistent with previous studies, adding probiotics or probiotic extracellular products to the diet can significantly improve the disease resistance of fish.

5. Conclusion

In conclusion, this study reported for the first time that supplementation of *L. buchneri* L3-9 and its extracellular metabolites in diets can promote growth performance, enhance immunity, regulate immune-related gene expression, and enhance anti-*A. hydrophila* characteristics of grass carp. This study preliminarily revealed the mechanism of *L. buchneri* L3-9 in the prevention and control of *A. hydrophila*. *L. buchneri* L3-9 kills pathogenic bacteria such as *A. hydrophila* by producing antibacterial substances, increases the number of probiotics such as *lactic acid* bacteria, changes the structure of intestinal flora, thus regulating intestinal digestion and absorption, and enhances the disease resistance of grass carp. Enhance the expression of anti-inflammatory related cytokines, reduce the expression of pro-inflammatory related cytokines, and regulate the immunity of grass carp. Therefore, *L. buchneri* L3-9 and its extracellular metabolites are promising probiotics, which provide a new idea for the development of pathogenic bacteria products and the prevention and control of bacterial diseases. The mechanism of prevention and treatment of *A. hydrophila* needs to be further studied.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

This experiment has passed the animal experiment ethics review of the Experimental Animal Center of Huazhong Agricultural University. The Animal Experimental Ethics Number (ID number): HZAUFI-2023-0050.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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