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Effect of Different Fermented Feed on the Growth, Immunity, and Intestinal Glora of *Litopenaeus Vannamei*

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Abstract: In this research, 8 strains with the capability to produce digestive enzymes or against pathogens were isolated from the shrimps' gut. To obtain fermented feed with low pH, high content of acid-soluble protein, or high number of viable bacteria, the combination of strain, fermentation conditions was investigated. Then, a six-week feeding experiment in shrimps was conducted using 4 diets (F0: feed without fermentation; F1: fermented feed with low pH; F2: fermented feed with high content of viable bacteria; F3: fermented feed containing acid-soluble protein). At 21 days, T-AOC and SOD in F2 and F3 were significantly higher ($P < 0.05$) than those in the control group; GSH-Px activity in the F1 group and F3 group increased significantly; there are no significant differences ($P > 0.05$) among the four groups in GOT and GPT. The bacterial groups of the 4 experimental groups in the Phylum are composed mainly of Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes, and the F1 group had the highest relative proportion of Firmicutes.

Keyword: Fermented feed; Fermented condition; Immune parameters; Intestinal flora

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1. Introduction

Recently, fermented feed as a trend of feed development has shown advantages over ordinary feed. Fermentation is a dynamic process involving microorganisms, substrates, and environmental conditions, transforming complex substrates into simpler compounds [1]. There are many types of bacteria used in fermentation, which can basically be divided into several categories: *Bacillus*, Yeast, *Lactobacillus*, Actinomycetes, and molds. During fermentation, enzyme-producing microorganisms can promote biosynthesis due to adaptability and growth of internal microbial mechanisms, leading to accumulation or accumulation of biologically active compounds. In terms of feed fermentation technology, the compatibility of bacteria strains, substrates, the activation of strains, and the degree of drying also need further study. Research on the pre-digestion of raw material, improvement of feed utilization, improvement of carcass intestinal health, and improvement of the quality of livestock products are becoming a

development trend of fermented feed. Studies have shown that in terms of promoting growth, using fermented feed can improve the feed utilization, growth, feed utilization, immune response, and disease resistance of white shrimp [2]. However, the evaluation of the effects of fermented feed on cultured subjects is not comprehensive.

Litopenaeus vannamei is the most farmed shrimp species in the world [3]. However, there are limited studies on the use of compound strains for shrimp feed fermentation. At present, most of the probiotics added to the feed of *L. vannamei* are non-indigenous strains. Bacillus and photosynthetic bacteria account for a relatively large proportion [4]. In this study, the authors selected bacteria with digestive enzyme production and antibacterial activity from the intestine of *L. vannamei* and explored the optimal conditions for the fermentation of compound bacteria and fed them with fermented feed. Shrimps were fed with optimized fermented feed, and the effects of fermented feed on prawns' intestinal physiological functions from the aspects of shrimp growth and body composition, intestinal digestive enzymes, antioxidant factors, immune indicators, antimicrobial peptide gene expression, and improvement of the composition of the intestinal flora were systematically evaluated.

2. Materials and methods

2.1. Screening and identification of bacterial strains

2.1.1. Isolation and identification of isolated strains

The bacterial strains were isolated from the gut of 80 Pacific White Shrimp that came from Shandong Yantai. Every 10 guts producing homogenate by a homogenizer was regarded as one sample. Bacterial genomic DNA was extracted according to the method of Medici et al. [5]. Amplifying partial fragments of 16S rDNA and 18S rRNA by PCR. The PCR products were sent to Bioengineering (Shanghai) Co., Ltd. for sequencing. The sequencing results are compared and analyzed on the BLAST module of the National Center for Biotechnology Information (NCBI), and the tested strains are classified according to the homology sequence.

2.1.2. Screening of Bacillus, Lactobacillus, and Saccharomyces and determination of antimicrobial sensitivity and hemolytic activity

The protease, lipase, and amylase activities of strains were measured by the spot-on-the-lawn method [6]. The antibacterial activities of *Lactobacillus* were determined by the Oxford Cup antibacterial method [7]. Antimicrobial susceptibilities were determined by a standard microbiological method [3]. 100 μL of bacterial suspension was evenly coated on MHA agar plates. Subsequently, 22 different antibiotic disks, including penicillin, cefotaxime, cephradine, kanamycin, gentamicin, doxycycline, tetracycline, enrofloxacin, ciprofloxacin, florfenicol, erythromycin, and ampicillin were placed on the plates, then incubated at 30 ℃ for 24 h. The diameter of the transparent circle was measured. 20 μL of candidate strains were spotted and inoculated on blood agar plates and incubated at 37 °C for 48 h [8]. Hemolysis activities were determined by observing a transparent circle in the plates.

2.2. Feed fermentation and experiment design

The details of the diet composition are given in **Table 1**. To evaluate the effect of combination of strains on fermented feed, 6 strains named SLVGB3(B1), SLVGB7(B2), SLVGL4(L1), SLVGL12(L2), SLVGY2(Y1), and SLVGY9(Y2) from 3 genera composed a total of 8 combinations (B1+L1+Y1, B1+L1+Y2, B1+L2+Y1, B1+L2+Y2, B2+L1+Y1, B2+L1+Y2, B2+L2+Y1, B2+L2+Y2). The complete pellets of commercial feed were fermented by each of 8 combinations of strains at 10^8 CFU g^{-1} concentration for 48 h, and the ratio of material to water was 1:0.5 g/mL, and the temperature was 35 °C. In addition, the polyethylene bag with a one-way air valve

was perpetually used during the fermenting process. Each fermented sample was performed in triplicate.

Table 1. Ingredients and nutritional composition of the basal diet.

Ingredients	Composition (%)
Fish meal/%	35
Peanut meal/%	5
Soybean meal/%	17
wheatmeal/%	30
Squid meal/%	2
Shrimp meal/%	1
Shrimp shell meal/%	0.5
Fish oil/%	2
phosphatide oil/%	4
Calcium dihydrogen phosphate/%	1.5
Mineral premix/%	1.5
Vitamin premix/%	0.5
Total	100
Nutrient Index	
Crude protein/%	42
Crude fat/%	5
Crude fiber/%	5
Crude ash/%	16
Moisture/%	12

2.3. Optimum conditions of the feed fermented by strains combination

In order to explore the effects of different fermentation conditions on fermented feeds, three bacterial combinations were selected as fermentation strains, and orthogonal experiments were carried out with three levels of four factors (inoculation amount, ratio of material to water, fermentation time, and fermentation temperature). The conditions of each group of the orthogonal experiment are shown in **Table 2** below.

Table 2. The conditions of each group of the orthogonal experiment

	A. Inoculum amount (%)	B. Material to water ratio (g:ml)	C. Fermentation time (h)	D. Fermentation temperature (℃)
1	3	1:0.4	36	28
2	6	1:0.6	48	32
3	12	1:0.8	60	36

2.4. Rearing animals

L. vannamei used in this experiment comes from farms in Shandong Yantai. Every 3 tanks are regarded as a group, composing 4 groups (control, I, II, III) that receive different diet. 720 shrimps were divided into 12 plastic water tanks (66×51×36cm). Maintaining in appropriate temperature (23–25°C), sanity (31‰), and pH (7.8–8.2), shrimp were fed their respective diets 3 times (8:00, 14:00and 20:00) with 5% body weight per day for 6 weeks during the experiment.

Half of the water in each tank was replaced daily, and the water used in the rearing experiment was taken from the natural sea.

2.5. Survival and growth

Shrimp were weighed on 0 d and 42 d, the survival rate (%), weight gain (%), and specific growth rate (SGR) were calculated as follows:

Weight gain (WG) (%) = $100*(W_f - W_i)/ W_i$

Specific growth rate (SGR) (% day^{-1}) = $100*(\ln W_f - \ln W_i)/t_i$

Feed conversion rate (%) = $W_d/ (W_f - W_i)$

Survival (%) = $100*N_t/N_0$

Composition analysis of shrimp body, including moisture, crude protein, ash, and crude lipid, was measured according to AOAC (1995) procedures [9].

2.6. Digestive enzyme assay

Fifteen shrimps were selected randomly from each tank for dissection. The hepatopancreas and intestines were respectively sampled, and then the protease activity was measure by the method of Oliver and Lowry et al. [10]; the lipase activity was measure by the method of Duncombe, W.G. [11]; the amylase activity was measure by the method of Pan et al. [12].

2.7. Immune response parameters and gene expression assay

After collecting hemolymph, we measured total haemocyte counts (THC) by the method of Pan et al. [12]. Phagocytic activity was evaluated by the method of Yue et al. [13]. L-dihydroxyphenylalanine (L-DOPA) produced dopachrome, according to which phenoloxidase (PO) activity of plasma was determined spectrophotometrically by the method of Yeh [14]. Basing on the autooxidation of pyrogallol, SOD activity was measure as the method of Marklund and Marklund [15]. Total amounts of GSH were determined with the method of Anderson [16]. Commercial kits (provided by Nanjing Jiancheng Biological Engineering Institute, China) were used to determine T-AOC. According to methods described by Reitman and Frankel [17], GOT and GPT activities in serum were determined. Real-time quantitative PCR (qPCR) was used to detect the expression of immune-related genes, including Crustin, Penaiedin, and ALF. The primers used in qPCR are shown in **Table 3**.

Table 3. Primer sequences used in this study

Primer name	Primer sequences (5'–3')	GenBank accession number
***β-actin*-**F	CCACGAGACCACCTACAAC	AF300705
***β-actin*-**R	AGCGAGGGCAGTGATTTC	AF300705
Crustin -F	ATTCTGTGCGGCCTCTTTAC	AY488496
Crustin -R	ATCGGTCGTTCTTCAGATGG	AY488496
Penaiedin -F	CACCCTTCGTGAGACCTTTG	Y14926
Penaiedin -R	AATATCCCTTTCCCACGTGAC	Y14926
ALF-F	GCAGGTCGACGATTAGCTTTC	KU213608.1
ALF-R	GTTCCTCCACAGCCCAACAATC	KU213608.1

2.8. Intestinal microbiota analysis

The study extracted and assessed the DNA of intestinal microbiota, and all DNA samples were sent to Novogene Biological Information Technology Co. (Tianjin, China) for analysis.

3. Result

3.1. Isolation and screening of bacterial strains

A total of 41 strains were identified. As shown in **Figure 1**, there are 4 strains of *Lactobacillus* (SLVGL4, SLVGL5, SLVGL7, SLVGL12) with the capabilities to inhibit 3 indicator pathogens. SLVGL4 and SLVGL12 showed higher antibacterial activity (**Figure 1**, A). Strains (SLVGB3, SLVGB4, SLVGB7, SLVGB9, SLVGY2, SLVGY6, SLVGY7, SLVGY12) secreting three major digestive enzymes were detected. Higher protease activities of the strains SLVGB3 and SLVGB7 were observed among the four Bacillus. And the protease activities of strains SLVGY7 and SLVGY12 were higher in the 4 strains of yeast. Moreover, no obvious transparent hydrolysis circle was observed around the colony in the haemolytic test.

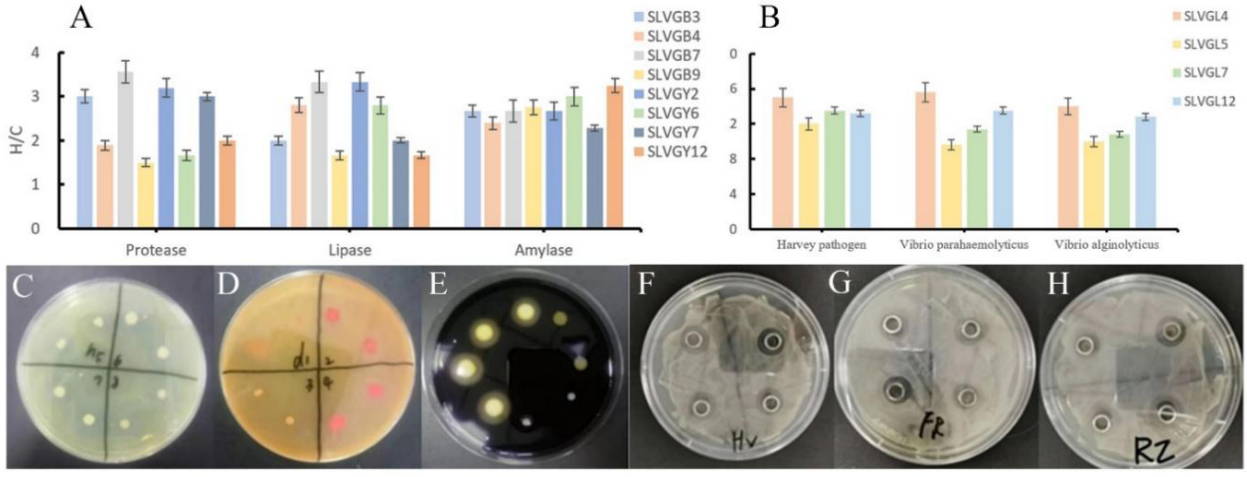

Figure 1. (A) Enzymatic activity of isolated *Bacillus* and Yeast. (B) Antibacterial activity of *Lactobacillus plantarum* (measured by the diameter of the inhibition zone (unit: mm). Representative graph of strains producing digestive enzymes (C) Protease, (D) Lipase, (E) Amylase and *Lactobacillus plantarum* inhibition (F) *Harvey pathogen*, (G) *Vibrio parahaemolyticus*, (H) *Vibrio alginolyticus*

3.2. Evaluation of feed fermented by strains combination

The quality evaluation of the fermented feed by 8 strain combinations is shown in **Table 4**. It can be inferred that at the 48 h time point of fermentation, the pH of each combination was significantly lower than that of the control group ($P < 0.05$), and compatibility 1 showed the lowest pH. The acid soluble protein in compatibility 8 was as high as 46.77±0.37% while the rest combinations were below 40, but still significantly higher than the control group ($P < 0.05$). As for the number of viable bacteria in the colony, the combination 6 is the most one. So, compatibility 1 (SLVGB3+SLVGL4+SLVGY2), compatibility 8 (SLVGB7+SLVGL12+SLVGY9), and compatibility 6 (SLVGB7+SLVGL4+SLVGY9) were selected as feed combinations for further feeding experiments.

Table 4. pH, acid-soluble protein(%), and number of viable bacteria/(×108 CFU·g-1) of fermented feed. SLVGB3(B1), SLVGB7(B2), SLVGL4(L1), SLVGL12(L2), SLVGY2(Y1), SLVGY9(Y2)

Conbination	pH	Acid soluble protein (%)	Number of viable bacteria (×10 8 CFU·g^{-1})
Control	6.00±0.07	9.90±0.02	0.00
B1+L1+Y1	5.33±0.02	12.28±0.23	18.00±1.0
B1+L1+Y2	5.38±0.03	15.74±0.24	19.00±1.5
B1+L2+Y1	5.68±0.01	16.40±0.17	2.00±0.01
B1+L2+Y2	5.82±0.02	11.90±0.26	5.00±0.1
B2+L1+Y1	5.39±0.01	13.50±0.12	21.00±2
B2+L1+Y2	5.36±0.03	14.29±0.34	30.00±3.0
B2+L2+Y1	5.68±0.05	14.57±0.08	8.00±0.5
B2+L2+Y2	5.79±0.04	16.77±0.37	3.00±0.3

3.3. Shrimp survival and growth

After 42 d experiment, the growth of shrimps is shown in **Table 5**. Group F3 showed the highest weight gain rate, reaching 264.5%, significantly higher than F1 and F2 groups ($P < 0.05$). The weight gain rate of F1 and F2 was significantly higher than that of the control group ($P < 0.05$). Feed conversion rates of F1 were significantly lower than other groups ($P < 0.05$), while there was no significant difference among F1, F3, and the control group. The survival rate of group F1 was significantly higher than that of other groups, reaching 96.6% while no significant difference was observed in F2, F3, and the control group. The body composition of shrimps after 42 d experiment was shown in **Table 6**. The highest composition of crude protein was observed in F3, reaching 74.58%, with the lowest composition of moisture, whihe crude protein in F2 was lower than the group F3, while the content of moisture in F2 was higher than F3; F1 showed the lowest content of crude protein, and content of water.

Table 5. Effects of fermented feed on the growth performance of *L.vannamei*

Index	F0	F1	F2	F3
Initial weight (g)	2.0±0.0	2.0±0.0	2.0±0.0	2.0±0.0
Final weight (g)	$6.62±0.04^{a}$	$6.84±0.02^{b}$	$7.16±0.01^{c}$	$7.29±0.03^{c}$
Weight gain rate(%)	$230.59±4.95^{a}$	$242.05±2.59^{b}$	$248.22±2.39^{c}$	$264.5±3.67^{d}$
Feed conversion rate (%)	$1.46±0.01^{c}$	$1.38±0.03^{b}$	$1.27±0.04^{a}$	$1.28±0.02^{a}$
Survival rate (%)	$86.6±1.02^{a}$	$96.6±0.97^{b}$	$93.3±1.41^{ab}$	$90.0±1.35^{ab}$

Note: F0, control; F1, group fed with fermented feed (B1+L1+Y1); F2, group fed with fermented feed (B2+L1+Y2) , F1, group fed with fermented feed (B2+L2+Y2). Data are means ±SD (n =3)

Table 6. The body composition of *L.vannamei*

Body composition (%)	F0	F1	F2	F3
Crude protein	$16.36±0.09^{a}$	$16.5±0.48^{a}$	$17.06±0.18^{b}$	$17.58±0.35^{b}$
Crude fat	$1.61±0.30^{a}$	$1.3±0.05^{a}$	$1.54±0.50^{a}$	$1.77±0.63^{a}$
Crude ash	$2.44±0.02^{a}$	$2.32±0.04^{a}$	$2.52±0.03^{a}$	$2.33±0.05^{a}$
Moisture	$78.42±0.31^{a}$	$77.01±0.17^{a}$	$78.81±0.41^{a}$	$76.8±0.83^{a}$

Note: F0, control; F1, group fed with fermented feed (B1+L1+Y1); F2, group fed with fermented feed (B2+L1+Y2) , F3, group fed with fermented feed (B2+L2+Y2). Data are means ±SD (n =3)

3.4. Evaluation of digestive enzyme activities of shrimp

The results of protease, amylase, and lipase activities on 21 d and 42 d were shown in **Figure 2**. The activity of protease, lipase, and amylase in both hepatopancreas and gut in the test groups was higher than that in the control group. In hepatopancreas, after 21 and 42 days of culturing, the activity of protease in test groups increased significantly ($P < 0.05$), and group F3 showed the highest activities of protease, lipase, and amylase. The lipase activities of the F2 groups show no significant difference compared with F1 group ($P > 0.05$), while F3 was significantly higher than F2 and F1 ($P < 0.05$).

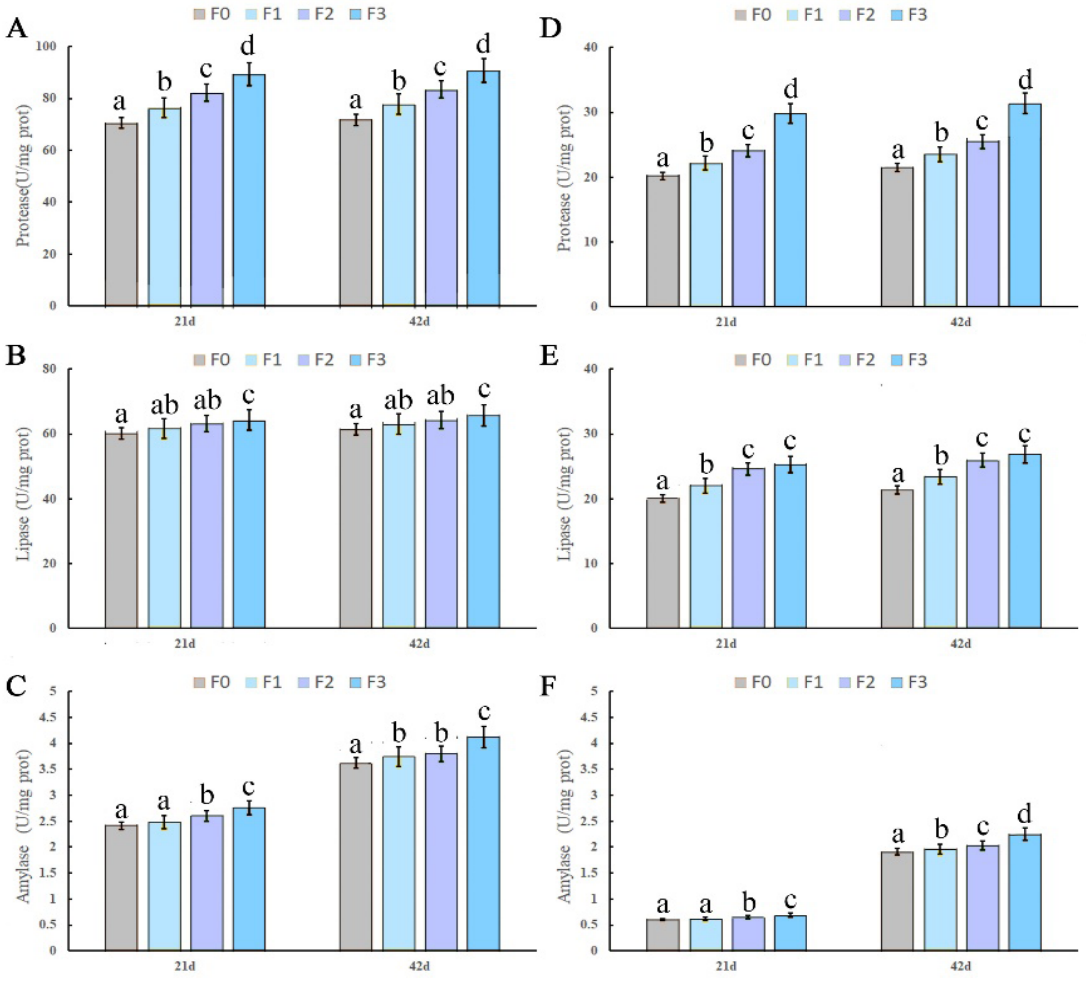

Figure 2. Effect of different feeds on protease (A), lipase (B), and amylase (C) activity in hepatopancreas and protease (D), lipase(E), and amylase (F) activity in gut. Data are means ± SD. (n =3)

3.5. Effects of fermentation diet on biochemical parameters of shrimp

Effects of fermentation diet on biochemical parameters of *L. vannamei* are shown in **Figures 3** and **4**. In **Figure 3**, the activity of GOT and GPT was observed to have higher values in the test group, but not significantly among the four groups ($P < 0.05$).

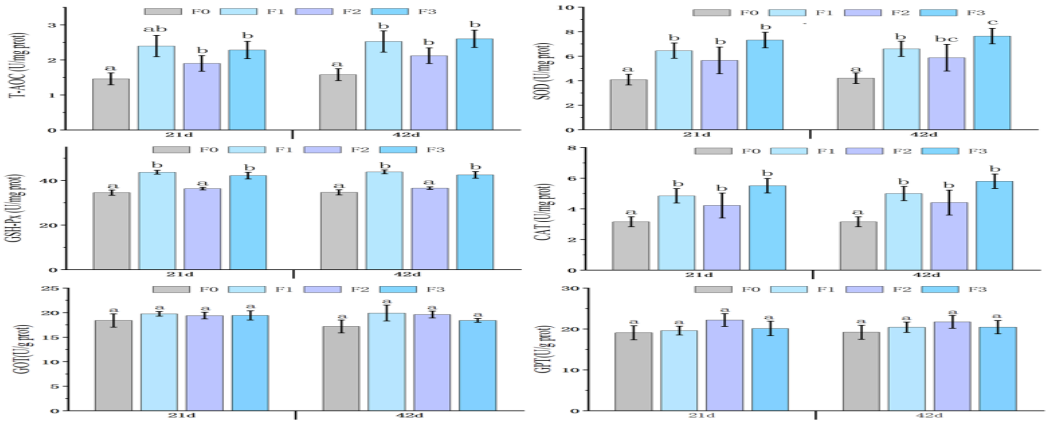

Figure 3. (A) glutamic oxalacetic transaminase (GOT) and (B) glutamic-pyruvic transaminase (GPT) activity in hemolymph of *L. vannamei* fed fermentation diet. Data are means ± SD. (n =3)

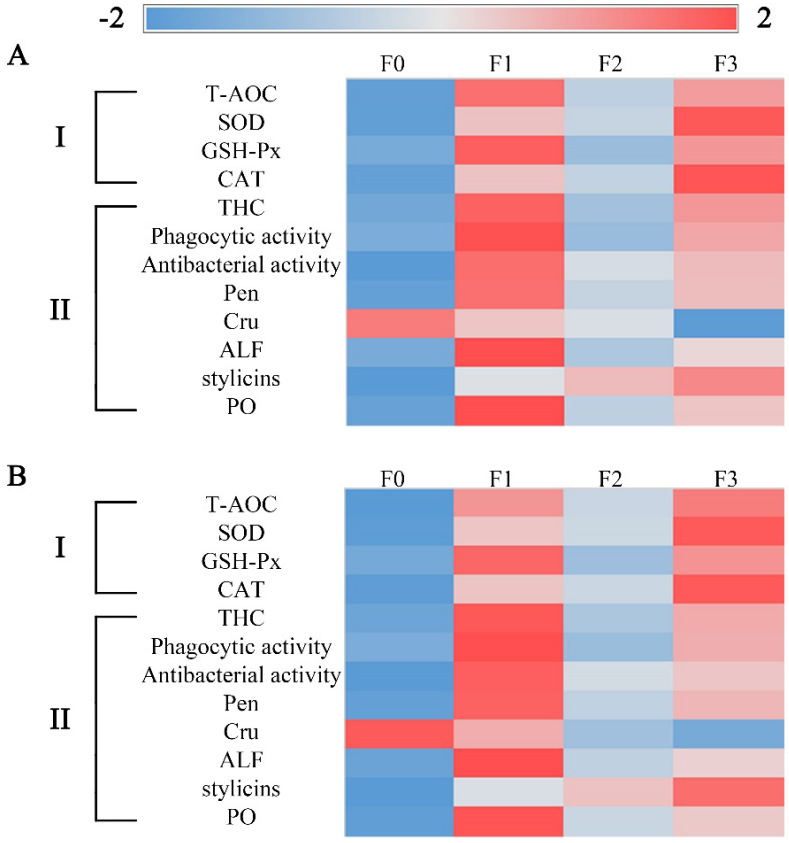

Figure 4. The anti-oxidation factors, immune parameters, and immune-related genes expression in the hemocytes of *L. vannamei* for (A) 21 and (B) 42 days

In **Figure 4**, at 21 days, T-AOC and SOD in F2 and F3 were significantly higher ($P < 0.05$) than those in the control group, while T-AOC in F1 showed no significantly difference compared with the control group. At 42 days, T-AOC and SOD in treatment groups increased significantly ($P < 0.05$). GSH-Px activity in the F1 group and F3 group increased significantly, while the GSH-Px activity in the F2 group showed no significant increase.

At 21 and 42 days of culture, the THC, phagocytic activity, antibacterial activity, and PO activity of F1 groups were increased significantly ($P < 0.05$). PO activity of the test groups was significantly higher than that of the control group ($P < 0.05$). There is no significant increase between the F2 and control groups in THC, phagocytic activity, and antibacterial activity. In addition, the expression of Pen in F0 was significantly lower ($P < 0.05$) than other groups at 21 d and 42 d, while the expression of Cru showed no significant difference among the 4 groups. In general, all detected genes in shrimps in groups fed with fermented feed were observed to have significantly higher expression than those in the control group, except for Cru.

3.6. Intestinal microbiota analysis of shrimp

As shown in **Figure 5**, there are a total of 204 OTUs in the intestinal flora of all groups, accounting for only 20.69% of the control group. In the group of fermented feed, the percentage of OTUs accounted for 33.71%, 63.95%, and 20.24%. The bacterial groups of the 4 experimental groups in the Phylum are mainly composed of Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes. The relative proportion of Proteobacteria accounts for more than 70% of the intestinal flora in the control group and the F1 group, while accounting for about 40% in the F3 group. In groups F2 and F3, the relative proportion of the Bacteroides phylum was significantly higher than that of the control group ($P < 0.05$). In F3 groups, Bacteroides account for nearly 40% of the intestinal flora. At the same time, the relative proportion of actinomycetes was significantly lower than the control group ($P < 0.05$), and it is also the lowest among the 4 experimental groups.

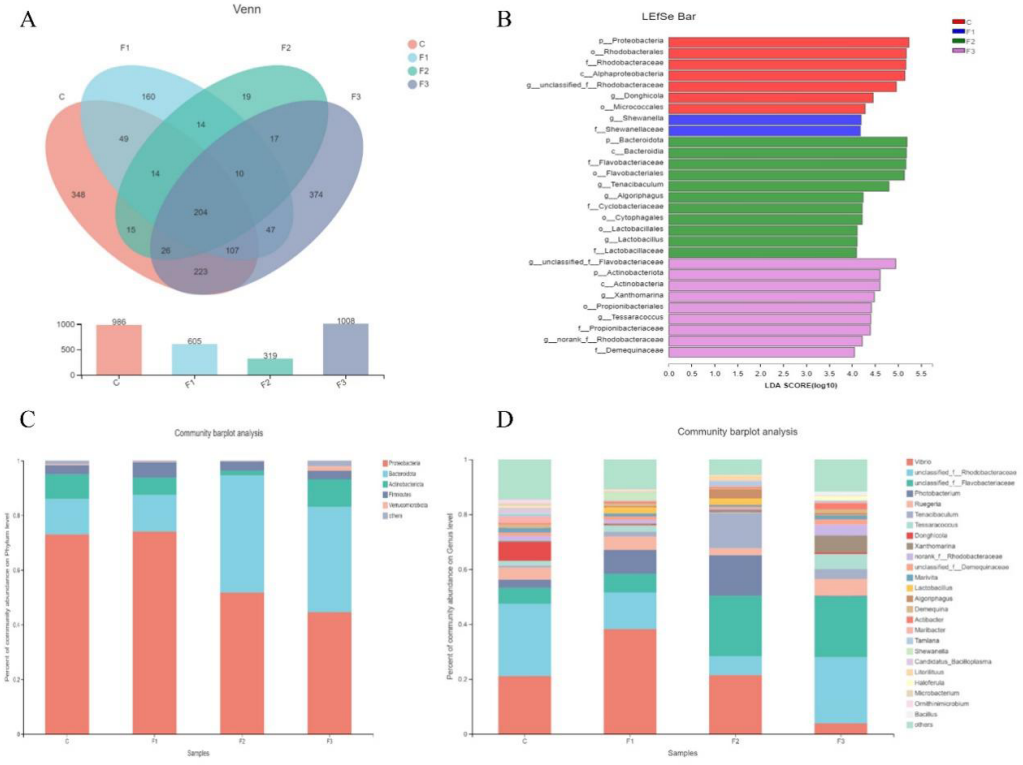

Figure 5. (A) Relative abundance of intestinal flora of *L. vannamei*; (B) The KEGG pathway annotation of *L. vannamei* fed diets fermented with different diets; (C) Relative abundance of intestinal flora of *L. vannamei* at "Phylum" level; (D) Relative abundance of intestinal flora of *L. vannamei* at "Genus" level

4. Discussion

Adding enzyme preparations or probiotics to aquatic animal feed has become the main way to improve the utilization of aquatic animal feed. In this study, well-performing Bacillus, lactic acid bacteria, and yeast were isolated from the intestines of *L. vannamei. Lactobacillus* colonizes the digestive tract, then forms a dominant flora through nutritional competition and secretion of some bacteriostatic substances, inhibiting the growth and reproduction of pathogenic bacteria. It is reported that dietary incorporation of *Lactobacillus plantarum* improves the health and immune performance [18]. Moreover, parts of *Bacilli* are hemolytic, which usually endangers the health of the host [19]. In this study, the hemolysis of the strains was evaluated, and the results showed that the candidate strains did not exhibit hemolysis, which makes them suitable to be used in aquaculture.

In addition, temperature, time, solid-liquid ratio, and bacterial inoculum are the main factors that affect solid-state fermentation. In this study, the screening indicators (the acid-soluble protein content, pH, and the number of viable bacteria in fermented feed) of the compatibility of the complex bacteria were set respectively in order to evaluate the effects of fermented feed to promote the growth of prawns, inhibit pathogenic bacteria, and improve the intestinal microecology. It has practical and guiding significance. In this research, the authors observed in F3 a significant increase in weight gain rate and a significant decrease in feed conversion rate. At the same time, the intestinal protease and lipase activity of the F3 was significantly higher than that of the control group. Hemocytes and humoral immune factors are important components of the shrimp immune system: SOD, PO, and T-AOC are important enzymes in body fluids as important indicators of the immunity of marine animals. The immune-related indicators in F1 were up-regulated, including the total number of blood cells, phagocytosis rate, and antimicrobial peptide gene expression. The pH value of the feed in F1 group was lower, indicating that the immune upregulation may be caused by the secretion of short-chain fatty acids by probiotics. Hepatopancreas is an important organ responsible for detoxification, metabolism and excretion in the body. The results of this experiment showed that the GOT and GPT of the experimental feed group were not significantly higher than those of the control group ($P > 0.05$), indicating that the fermented feed did not damage the hepatopancreas and was not toxic to the shrimp. It also proves the safety of the strains [20]. In terms of flora composition, *Proteobacteria* is the most abundant phylum. It was consistent with previous reports [21]. Firmicutes contains many well-known beneficial bacteria, such as *Bacillus*, Clostridium, *Lactobacillus*, etc. It is consistent with the research that fermenting the feed with commercial strains [22]. At the genus level, compared to the control group, the number of *Vibrio* increased first and then decreased in the treatment group, and significantly decreased in the F3 group. Hence, the results indicated that the F3 group feed has a certain regulatory effect on the intestinal flora.

5. Conclusion

In summary, the fermented feed group (F3 group) contains high acid-soluble protein content promotes the growth of *L. vannamei*, improves its immunity, and improves the diversity of intestinal flora. Considering the importance of the growth of shrimp culture, the authors conclude that the fermented feed group (F3 group) performs better than the other groups.

Disclosure statement

The authors declare no conflict of interest.

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