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Research Article

Effects of Dietary *Bacillus licheniformis* and Combined Herbs Extracts Supplementation on Physiological and Immune Characteristics, Microbial Community, and Vibriosis Resistance of *Apostichopus japonicus*

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This study was conducted to evaluate the effects of *Bacillus licheniformis* and combined herbs extracts on the physiological and immune characteristics, intestinal microbiota, and vibriosis resistance of sea cucumber *Apostichopus japonicus*. The sea cucumbers were fed with basal diets supplemented with *B. licheniformis* (B group), combined herbs extracts (C group), or both (BC group). The BC group exhibited the better growth performance and higher digestive and immune enzyme activities, whereas the lowest parameters appeared in the control group. This revealed that dietary *B. licheniformis* and combined herbs extracts in combination could improve digestion, food intake, phosphatase-responsiveness, and oxidation resistance more efficiently compared with the other groups. The BC group was also more capable of regulating intestinal flora balance of sea cucumbers by increasing the beneficial bacteria related to energy synthesis and metabolic conversion, and inhibiting the potential pathogens associated with organic damage and metabolic disorders. Furthermore, the BC group showed greater ability of improving disease resistance by reducing the cumulative mortality rates and the counts of *Vibrio splendidus*. These results collectively demonstrated that dietary *B. licheniformis* and combined herbs extracts could improve the physiological and immune parameters, optimize the microbial community, and enhance the resistance to vibriosis of sea cucumbers, and the health-promoting effects were more obvious by supplying them in combination.

1. Introduction

Sea cucumber *Apostichopus japonicus* has been highly prized as an economically important farmed echinoderm species in China, both as a delicacy and medicine [1]. However, the inappropriate factors of management, the degeneration of sea cucumber germplasm, and the extreme farming conditions have seriously hindered the healthy development of the sea cucumber breeding industry in recent years [2]. Previous studies indicated that the implementation of health management in *A. japonicus* aquaculture depended on the formulation of the diet from the nutritional and immunological perspectives to a large extent [3]. Therefore, it is of great significance for searching natural and environment-friendly dietary additives to acquire better health of farmed *A. japonicus* [4].

Generally, the improvement of the health status of animals could be accomplished by applying probiotics or herbal medicines as the dietary supplements [5]. Probiotics are known to contain many active microorganisms, which

might confer health benefits to the hosts [6]. Numerous studies have found the genus *Bacillus* could be effective in improving health and reducing the disease risk in aquaculture [7]. Among them, *Bacillus licheniformis* possesses the significant advantages of confronting pathogenic microorganisms, producing lots of extra cellular enzymes or polypeptides, and improving the immunity of the host, which make it a very appropriate probiotic candidate [8–10].

In addition to probiotics, the herbal medicine extracts have been utilized in aquaculture due to the features of cheaper cost, low toxicity, and less side effects [11–13]. The typical herbal medicines, such as *Astragalus membranaceus*, *Angelica sinensis*, *Lonicera japonica*, *Rehmannia glutinosa*, *Paeonia lactiflora*, *Scutellaria baicalensis*, *Radix bupleuri*, and *Glycyrrhizae*, possess many medicinal properties and biological effects that could be due to polysaccharides, organic acids, alkaloids, steroids, phenols, glycosides, terpenoids, flavonoids, and different types of active components presented in the extracts [14–16]. The herbal supplements have been proved to be effective in improving the growth, immune response, microbial communities, and disease resistance in rainbow trout [17], Nile tilapia [18], common carp [19], shrimp [20], and sea cucumber [21]. Generally, probiotics and herbal supplements are usually studied individually, while some studies have demonstrated that dietary supplementation jointly might bring better results than the administration of them separately. The compounds of *B. licheniformis* and combined herbs extracts could provide abundant probiotic bacteria and bioactive components, and the investigation of the interactions of them would also be of great significance.

Up to date, the combined applications of *B. licheniformis* and herbal medicines in sea cucumber aquaculture have been still studied barely. Thus, the positive effects of *B. licheniformis* and combined herbs extracts on the physiological and immune parameters, intestinal microbiota, and vibriosis resistance of *A. japonicus* were assessed in this study. The results would offer original insights into the healthy and scientific breeding in *A. japonicus*, and the other species shared the similar feeding habits.

2. Materials and Methods

2.1. Experimental Animals and Diet Preparation. Healthy sea cucumber individuals were provided by the Breeding Center of Liaoning Ocean and Fisheries Science Research Institute. The basal diet was formulated with marine mud and sargasso (1 : 1). The authors confirmed that the ethics, as mentioned on the author guidelines, had been fully adhered to. Sea cucumbers used in this research were obtained from commercial sea cucumber catches; therefore, approval from any ethics committee or institutional review board was not necessary. The *B. licheniformis* was isolated from *A. japonicus* and stored at −80°C by us previously. This strain was cultured for 24 h at 28°C in the shaken bottles containing the liquid trypticase soy broth (TSB) medium. After incubation, the fresh cells were collected by centrifugation at 3000 rpm for 10 min, washed three times in sterile normal saline, and then resuspended and added into the basal diet. The raw medicinal herbs including *Astragalus membranaceus*, *Scutellaria baicalensis*, *Angelica sinensis*, and *Lonicera japonica* were purchased from a local Chinese pharmacy and mixed at an optimum ratio of 2 : 1 : 1 : 1, which was designed in accordance with the previous study and modified from the preliminary screening results [22]. The four herbs were pulverized in a commercial grinder, and decocted with deionized water (1 : 5, w/v) three times for 2.5 h each. Thereafter, the extracts were filtered through a sterile muslin cloth and the filtrates were concentrated in a rotary vacuum evaporator to obtain the residue. The resulting extracts were freeze-dried and stored at −20°C. The *B. licheniformis* and combined herb extracts were supplemented to the basal diet at doses of 10^7 cfu·g^{-1} and 2.0% (w/w), configured on the basis of the pre-experimental results and our published study [23].

2.2. Feeding Experiment. After they acclimated the basal diet and breeding conditions for 15 days, the sea cucumbers with the similar sizes were classified into four groups randomly with 50 animals in each tank (100 cm × 80 cm × 80 cm) in triplicate. The control group was fed with the basic diet, and the other groups were fed with the basic diet added with *B. licheniformis* (B group), combined herbs extracts (C group), or both (BC group). During the 30-day feeding trial, all individuals were fed the designated diets once daily at a feeding rate of 5% of the body weight. The residual food and feces were cleared by siphoning, and 50% seawater was replaced with fresh seawater daily. Water temperature was kept at 16 ± 2°C, salinity 30 ± 1, pH 8.0 ± 0.5, and the dissolved oxygen was no less than 6 mg L^{-1} that was maintained by continuous aeration.

2.3. Sampling Procedures and Enzyme Assays. During the feeding trial, three sea cucumbers from each treatment were selected randomly for measuring the digestive and immune enzyme activities every ten days. The intestines were gathered from the dissected animals and homogenized with phosphate-buffered saline (PBS) using a manual glass homogenizer. The homogenates were then centrifuged at 4000 rpm for 15 min at 4°C, and the supernatants were collected for digestive enzyme analysis. Meanwhile, the coelomic fluid was collected and pooled and then mixed with anticoagulant with equal volume proportion. The coelomic fluid sample was centrifuged at 4000 rpm for 15 min and the supernatants were then collected for the immunological analyses.

The levels of the digestive enzyme activities (amylase, trypsin, lipase, and cellulose) and the immune enzyme activities, including acid phosphatase (ACP), alkaline phosphatase (AKP), lysozyme (LZM), and superoxide dismutase (SOD), were assessed by the reagent kits (Nanjing Jiancheng Bioengineering Institute, China) following the operating instructions, and each sample was measured in triplicate.

2.4. Growth Performance. The growth performance parameters of *A. japonicus* were measured before and after the feeding trial. Specific growth rate (SGR) and feed efficiency ratio (FER) were calculated as follows:

$$\text{SGR} = \frac{(\ln W_t - \ln W_0) \times 100}{t},$$
$$\text{FER} = \frac{(W_t - W_0)}{f}, \quad (1)$$

where W_t and W_0 are the final and initial body weight of the *A. japonicus*, t is the test days, and f is the feed intake.

2.5. Analysis of Intestinal Microbiota. The intestinal contents of the sea cucumbers were collected for the analysis of community compositions. Genomic DNA was extracted using a DNA extraction kit according to the operation instruction. The successful extraction of DNA was obtained by agarose gel electrophoresis. The V3-V4 regions of bacterial 16S rRNA gene were achieved by PCR amplification and sequenced by Illumina MiSeq. The paired-end reads were assembled on the basis of the specific barcode and shortened by eliminating the redundant portions. Reads were picked to the amplicon sequence variants (ASVs) by the DADA2 plug-in unit. Every ASV was attributed to the taxonomy on the basis of Greengenes database, and then the hierarchical abundance tables were established [24]. Singletons were then excluded to perfect the efficacy of the database. In the end, the ASV abundance data were standardized based on the sample with the minimum tag number.

2.6. Vibrio Challenge Trials. The disease resistance test was performed after the feeding trial. The cryopreserved *Vibrio splendidus* strain, which had been selected and identified in our laboratory previously [25], was activated in the 2216E medium at 180 rpm at 28°C for 24 h. The viable cells were then harvested by centrifuging at 6000 rpm for 10 min, and resuspended with sterilized seawater. Every individual was then challenged with an intraperitoneal injection of 100 μL at a suitable dose of 10^8 cfu·mL^{-1}. The dose was defined on the basis of the mean lethal dose (DL50) measurement and our published study [26]. The challenge experiment lasted for 15 days, during which the disease symptoms and cumulative mortality were monitored daily, and the sea cucumbers were routinely managed as the feeding trial.

The intestinal pathogenic vibrio count was assessed according to the spread plate count method [27]. At days 0, 1, 3, 6, 10, and 15 after injection, 1 g of the intestinal tissue from each individual was homogenized in 9 mL sterile seawater, and 100 μL of the homogenate was spread onto the thiosulfate citrate bile salts sucrose (TCBS) medium uniformly of three replicates. The numbers of pathogenic vibrios were checked after growing at 28°C for 48 h. The green colonies were appraised as the *V. splendidus* based on the defined protocol of the phenotypic identification [28].

2.7. Bioinformatics and Statistical Analysis. Alpha diversity analysis was assessed by the PAST v3.22 software. Boxplots were established to exhibit the bacterial diversities using the "ggplot2" package in the *R* v3.6.1. The abundance of major bacteria at different taxonomic levels was visualized with the "ggplot2" package in *R* v4.0.2. Duncan's multiple range tests were conducted to compare the mean differences of the growth performance parameters, enzyme activities, and cumulative mortalities within groups. Statistical analyses were conducted by the SPSS program version 21.0, and the statistical significance threshold was set at $P < 0.05$.

3. Results

3.1. Growth Performance. The SGR and FER of *A. japonicus* in the four treatments are presented in Table 1. All dietary treatments showed an improvement in growth during the feeding period. The greatest SGR and FER were both displayed in the BC group, while the control group produced the lowest values. In addition, the SGR did not differ between the B and C groups, while the FER exhibited by sea cucumbers in the C group appeared higher than the B group.

Values are given as the means and standard errors of three replicates. Data in the same column with different superscript letters indicate significant differences among treatments ($P < 0.05$). The letter CK, B, C, and BC represent the control group, *B. licheniformis* treatment group, combined herbs extracts treatment group, and combination treatment group.

3.2. Digestive Enzyme Activities Assay. The effects of basal diet supplemented with *B. licheniformis* and combined herbs extracts on the digestive enzyme levels of sea cucumbers are displayed in Figure 1. The levels of amylase and trypsin in all groups increased obviously except the control group. The BC group displayed the greater values, followed by the C and B groups (Figures 1(a) and 1(b)). The lipase activities with groups tended to increase sustainably and peaked on day 20, and then decrease slowly at the later period. The BC group exhibited slightly higher values than the B group, and no significant changes were detected between the C and control groups (Figure 1(c)). During the feeding entire trial, the cellulase activities changed gently and no clear differences were detected with groups (Figure 1(d)).

3.3. Immune Enzyme Activities Assay. The effects of basal diet supplemented with *B. licheniformis* and combined herb extracts on the levels of the immune enzymes of sea cucumbers are displayed in Figure 2. It can be seen that the activities of ACP and AKP increased significantly during the feeding period, and the maximum values appeared in the BC group followed by the B, C, and control groups (Figures 2(a) and 2(b)). Considerable increases in LZM levels were observed from day 10 to day 20 with groups, and then less amplitude changes were detected during the later period. The BC group displayed the maximum values, while the minimums occurred in the control group (Figure 2(c)). With regard to the SOD activities, gradual increases were observed among the treatments except the control group, and the values reached at the original levels on day 30. As expected, the BC group displayed higher values than the others. In addition, no obvious changes in SOD activities were detected between the B and C groups (Figure 2(d)).

Table 1: Effects of basal diet supplemented with *B. licheniformis* and combined herbs extracts on the growth performance of *A. japonicus*.

Diet groups	Initial (g)	Final (g)	SGR	FER
CK	6.64 ± 0.83^{a}	6.98 ± 0.64^{b}	0.17 ± 0.02^{c}	0.033 ± 0.005^{c}
B	6.75 ± 0.71^{a}	7.51 ± 0.49^{ab}	0.36 ± 0.03^{b}	0.048 ± 0.007^{bc}
C	6.54 ± 0.43^{a}	7.29 ± 0.81^{ab}	0.37 ± 0.11^{b}	0.071 ± 0.016^{b}
BC	6.57 ± 0.76^{a}	8.03 ± 0.92^{a}	0.67 ± 0.08^{a}	0.139 ± 0.012^{a}

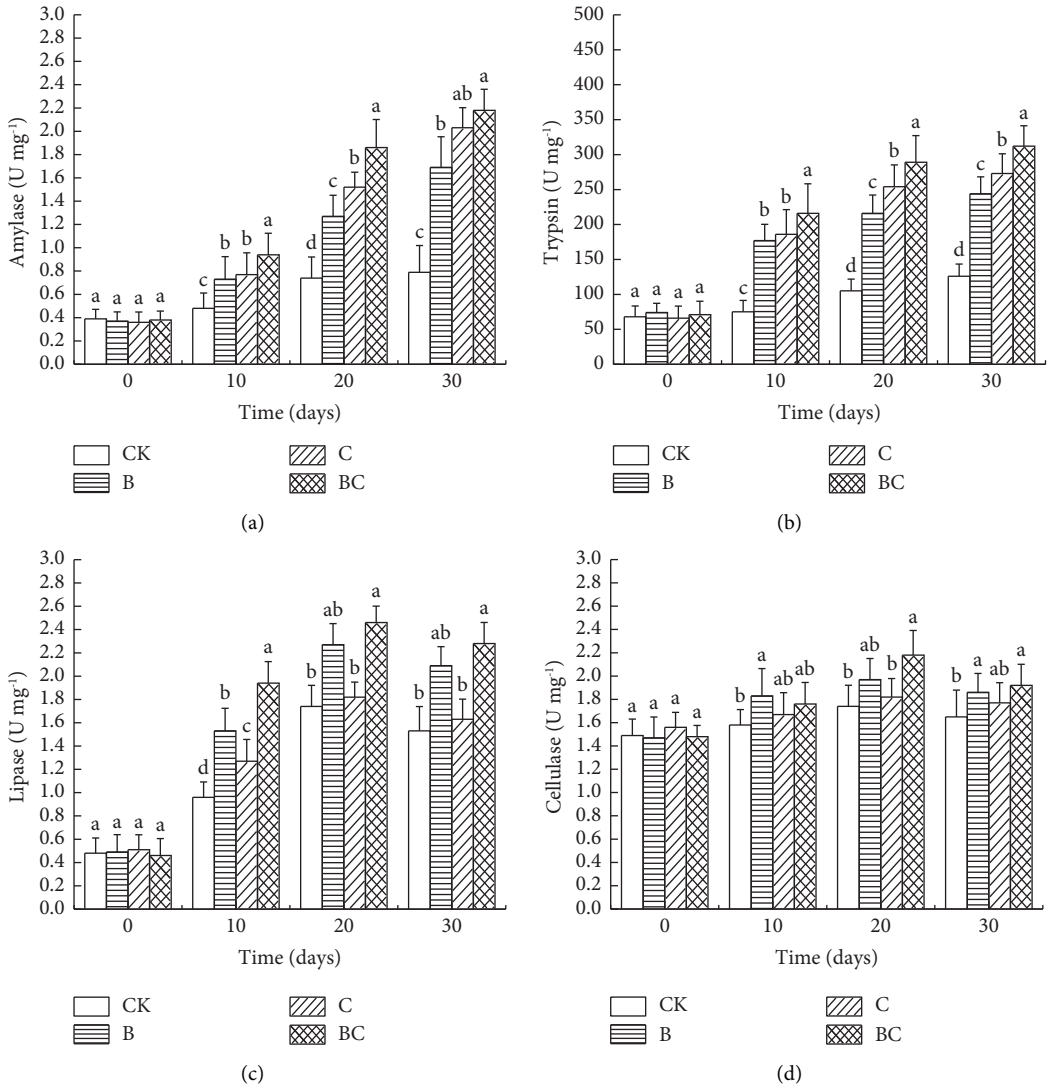

Figure 1: Effects of *B. licheniformis* and combined herb extracts' supplementation on the digestive enzyme levels of *A. japonicus*: (a) amylase, (b) trypsin, (c) lipase, and (d) cellulase. Data are the means and standard errors of three sea cucumbers at the same sampling time, with different letters indicating significant differences among treatments ($p < 0.05$).

3.4. Analysis of Intestinal Microbiota Composition. The alpha diversity indices of the gut microbiota in *A. japonicus* are displayed in Figure 3. For richness, the maximum values of Chao1 and observed species were detected in the BC group followed by the C group, and no obvious changes were detected between the B and control groups (Figures 3(a) and 3(b)). For diversity, obvious increases in Shannon and Simpson indices were detected in the BC group than others, and the control group produced the lowest values. In addition, no obvious changes were detected between the B and C groups (Figures 3(c) and 3(d)).

The intestinal microbiota composition of *A. japonicus* hierarchically before applying *B. licheniformis* and combined herb extracts is displayed in Figure 4. The *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* were the predominant phyla in the *A. japonicus*. The classes *Alphaproteobacteria*, *Gammaproteobacteria*, *Flavobacteria*, and *Clostridia*, and the families *Rhodobacteraceae*, *Flavobacteriaceae*, *Clostridiaceae*, *Colwelliaceae*, and

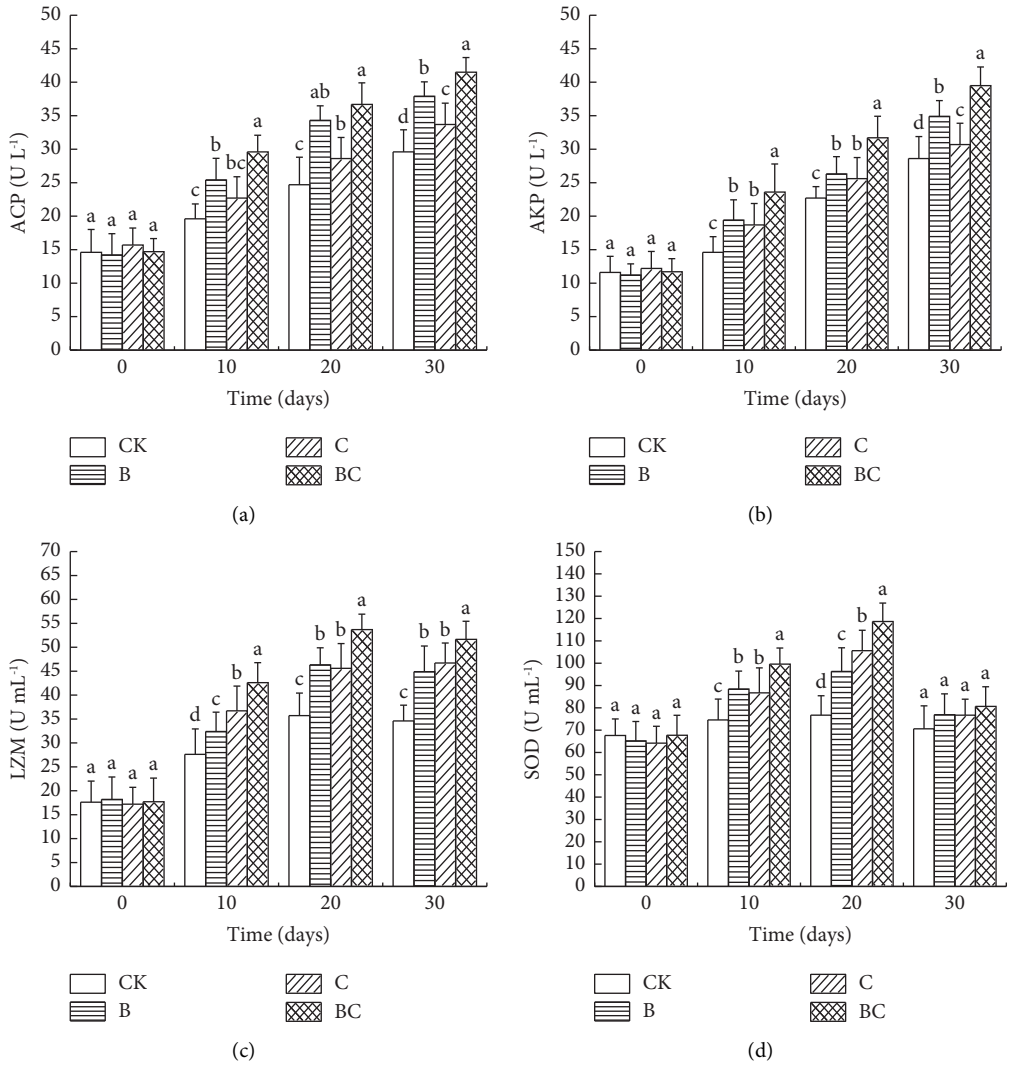

Figure 2: Effects of *B. licheniformis* and combined herb extracts' supplementation on the immune-related enzyme levels of *A. japonicus*: (a) ACP, (b) AKP, (c) LZM, and (d) SOD. Data are the means and standard errors of three sea cucumbers at the same sampling time, with different letters indicating significant differences among treatments ($p < 0.05$).

Verrucomicrobiaceae made up the majority of the microbial community. At the genus level, the higher abundance of *Colwellia*, *Rhodobacter*, *Phaeobacter*, and *Flavobacterium* was observed in the samples. Moreover, *Lutibacter*, *Roseovarius*, *Ruegeria*, *Rubritalea*, *Roseburia*, and *Vibrio* were also detectable.

After the feeding period, the highest abundance of *Proteobacteria* and the lowest abundance of *Bacteroidetes* were observed in the control group. Conversely, the abundances of *Proteobacteria* and *Bacteroidetes* in the BC group were contrary to the control group. The maximum *Firmicutes* was detected in the B group followed by the control, C, and BC groups. In addition, the highest abundances of *Verrucomicrobia* and *Tenericutes* were detected in the BC group, whereas the minimums were found in the control group (Figure 5(a)). At the genus level, the high-proportioned*Rhodobacter* were detected in the BC and B groups. The maximum abundance of *Colwellia* was presented in the BC group followed by the C, B, and control groups. The highest proportions of *Lutibacter*, *Arcobacter*, and *Vibrio* were presented in the control group, while the lower values occurred in the BC group. Moreover, significant increase in the number of *Bacillus* was detected in the BC group followed by the B group, whereas there was no detectable *Bacillus* in the C and control groups. In addition, some new genera, such as *Neptunomonas*, *Psychrobacter*, *Halomonas*, and *Cobetia*, emerged in the samples to some extent (Figure 5(b)).

3.5. Resistance against V. splendidus. Cumulative mortality rates of *A. japonicus* with groups during the bacterial challenge are displayed in Figure 6. The typical symptoms of infection in the tested sea cucumbers were observed in succession after injection, whereas no sea cucumber was confirmed dead until day 5 in the control group. After that, the first dead individuals were detected on days 6, 7, and 9 in the B, C, and BC groups. During the challenge

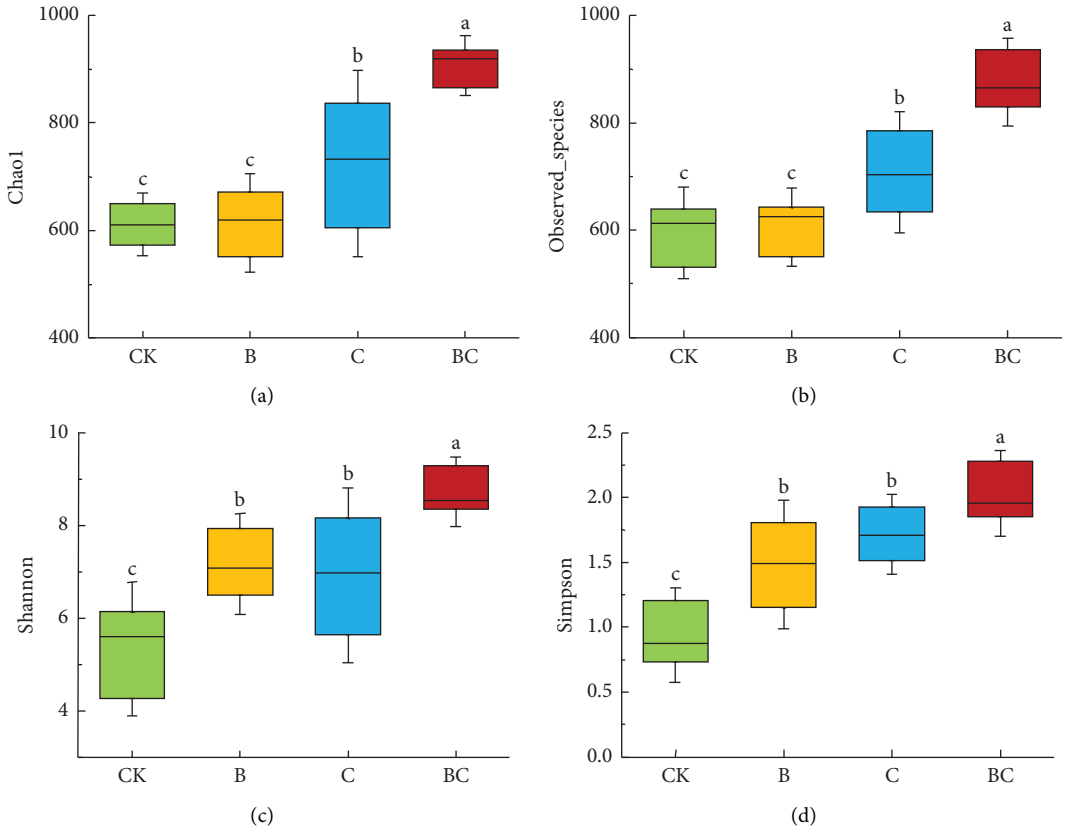

FIGURE 3: Boxplots for microbial alpha diversity of sea cucumbers with groups: (a) Chao1, (b) observed species, (c) Shannon index, and (d) Simpson index. Box charts with different letters represent significantly different differences ($p < 0.05$).

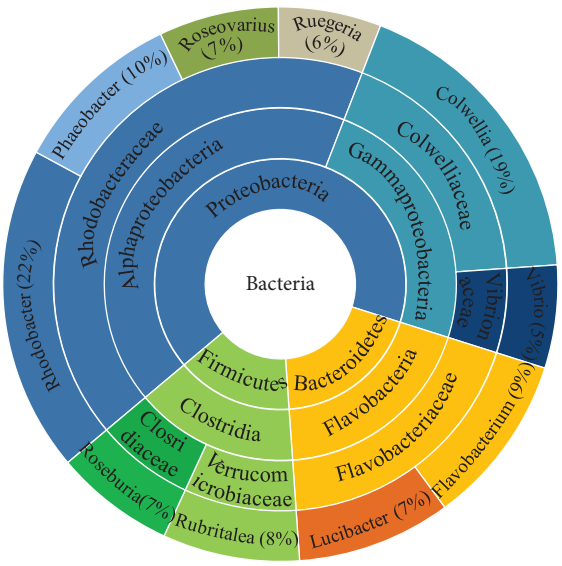

FIGURE 4: Intestinal microbiota composition of *A. japonicus* before applying *B. licheniformis* and combined herb extracts at four taxonomic levels.

test, the sea cucumbers in the control group produced the higher cumulative mortality rate, followed by the B and C groups. Unsurprisingly, the minimums appeared in the BC group.

The antimicrobial effects of *B. licheniformis* and combined herb extracts on *V. splendidus* of *A. japonicus* are displayed in Figure 7. Before the injection of *V. splendidus*, no vibrios were identified among the groups with the exception of a few emerging in the control group. As time went on, counts of *V. splendidus* increased remarkably from days 1 to 3 after injection with groups. After that, the increased amounts of *V. splendidus* were not distinct. At various times of the test, the control group exhibited the maximum values of vibrios, while the BC group produced the minimums. In addition, the B group displayed the higher counts of vibrios than the C group.

4. Discussion

4.1. Enhancement of Growth Performance. Growth performance was an essential index to assess the effects of dietary supplement on the aquatic animals. Generally, the relatively better growth performance could be associated with an increase in digestive ability and nutrient absorption [29]. Extensive studies have endorsed the positive effects on growth performance of the probiotics and herbal medicines in aquaculture [7, 30]. Consistently, the results in this study showed that *B. licheniformis* and combined herb extracts could effectively enhance the growth performance parameters of sea cucumbers, and the beneficial effect was more obvious when they were administered in combination. This

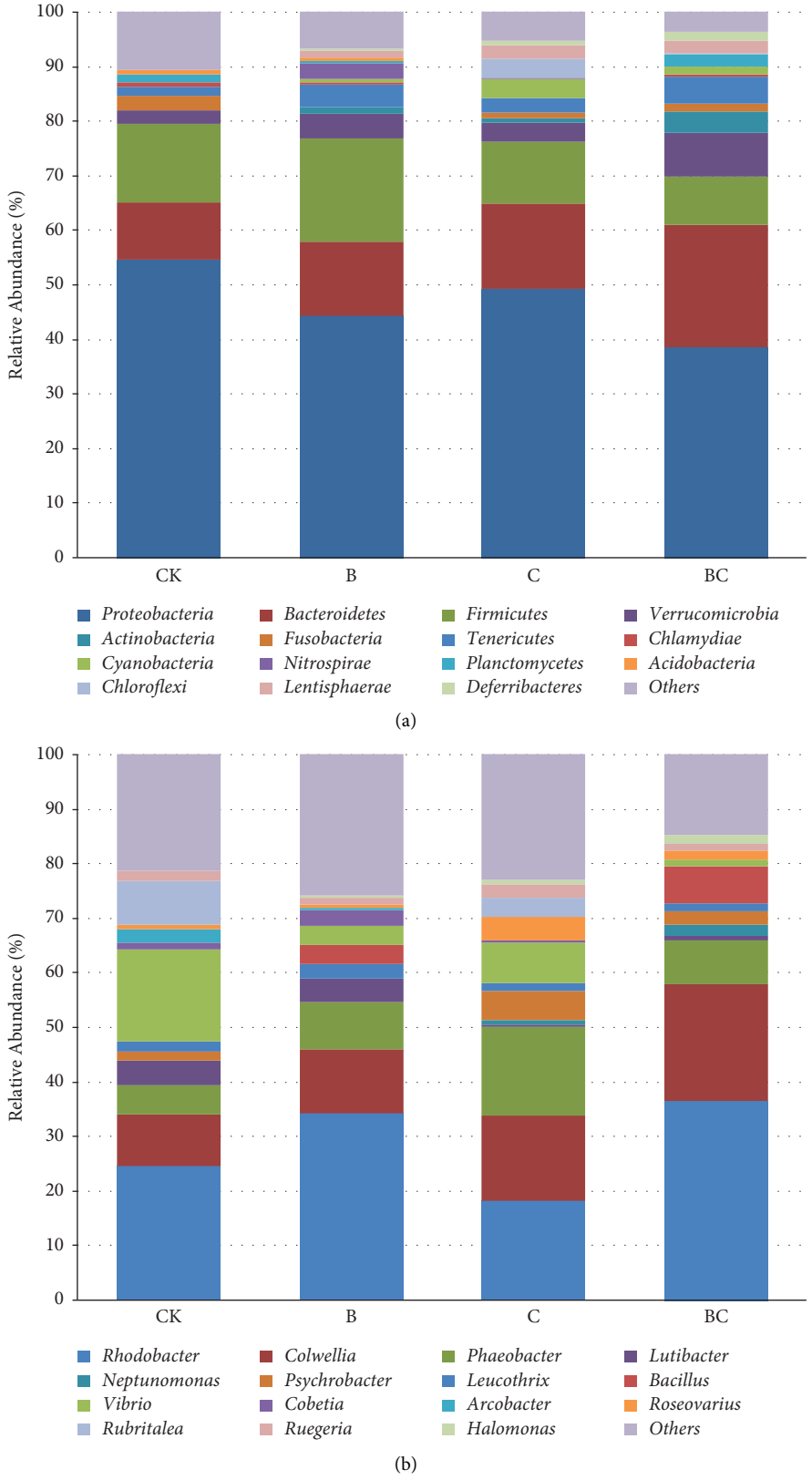

Figure 5: Effects of *B. licheniformis* and combined herb extracts on distribution of gut microbiota of sea cucumbers at phylum (a) and genus (b) levels after feeding trial.

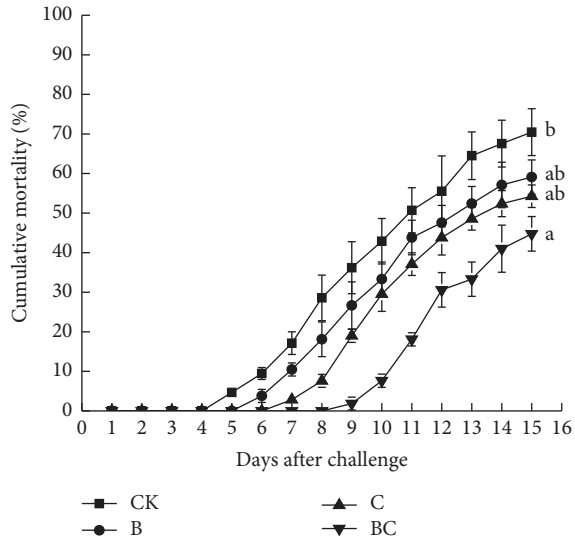

Figure 6: Cumulative mortality rates after a challenge with *V. splendidus* for 15 days of *A. japonicus* dietary supplementation with *B. licheniformis* and combined herb extracts. Values were shown as means of three replicates. Different superscript letters denote significant differences among treatments ($p < 0.05$).

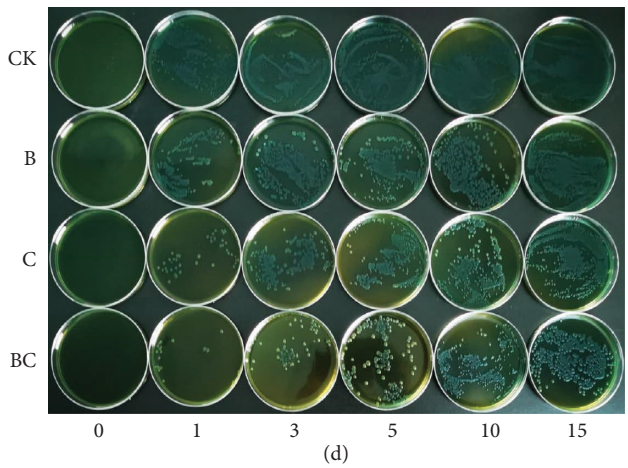

Figure 7: Counts of vibrios in the intestines of sea cucumbers dietary supplementation with *B. licheniformis* and combined herb extracts after *V. splendidus* challenge.

may be due to that new active substances can be obtained through the interaction between *B. licheniformis* and combined herb extracts, which can transform the anti-nutritional contents of the herbal medicines, reduce the toxic side effects, and even improve the appetite and nutrition condition of sea cucumbers [31]. The value of FER in the B group did not reach the statistical significance compared with the control group. It could be explained by the administration of *B. licheniformis* alone may restrain the proliferation of the indigenous microorganisms to a certain extent through the competition for nutrition, which would exert a negative effect on the feed conversion [2]. Although not being remarkable, the FER in the B group remained slightly higher than the control group.

4.2. Enhancement of Digestion. The levels of digestive enzymes, such as amylase, trypsin, lipase, and cellulose, are reliable indicators of digestibility and food uptake of the sea cucumbers [32]. In the present study, obvious increases in amylase and trypsin activities were found with groups except the control group. The higher amylase and trypsin activities are considered to be able to stimulate the decompositions of carbohydrate and protein, which may play a functional role in digestion and absorption [33]. Unsurprisingly, the highest values occurred in the BC group, suggesting that the dietary supplements of *B. licheniformis* and combined herb extracts in combination could stimulate sea cucumber to produce more digestive enzymes for metabolic regulation and proliferation of beneficial microorganisms [34]. The higher lipase activities were observed in the BC and B groups, and no remarkable changes were detected between the C and control groups. The phenomenon might be due to that the mode of action of the combined herb extracts in this study seemed ineffective on the lipases, and the exogenous lipases produced by the extracts may not offer much contribution to the total activity of the sea cucumbers [35]. As for the cellulose activities, no significant changes were identified with groups, and the BC group did not show a distinct advantage over other groups. It would be that the amylase, trypsin, and lipase activities were more susceptible indicators than the cellulose activities for *A. japonicus* in response to *B. licheniformis* and combined herb extracts during the feeding trial [36]. Nevertheless, this remains hypothetical and needs to be confirmed in follow-up studies. These results showed that the supplementation of *B. licheniformis* and combined herb extracts jointly had been more profitable to the digestive ability for *A. japonicus* than the application of them individually by means of the nutrition supply and enzyme synthesis.

4.3. Enhancement of Immune Response. Regulation of the immune response is one of the key benefits of functional dietary supplementation. ACP and AKP are typical lysosomal enzymes, which could digest the invading organisms and provide the basic immunity [37, 38]. In the current study, sea cucumbers in the BC group displayed the optimal activities of ACP and AKP, suggesting that the phosphatase-response capability of the BC group was clearly superior to the others. It also could be inferred that dietary *B. licheniformis* and combined herb extracts in combination might endow the coelomocytes of sea cucumbers with the greater ability to phagocytize and eliminate the invading pathogens [39]. LZM is speculated to play a major role in bacterial lysis and fibrin hydrolysis [40]. The LZM activities grew remarkably and then held a steady level in this study. This may be explained that more LZM enzymes were secreted by accessing the serum to release multiple hydrolases, while a processing time was needed to take part in the immune response. As the test proceeds, the animals were adapted to the stimulation, and then the stable values were triggered by a self-regulation mechanism [41]. Sure enough, the BC group exhibited the optimal LZM activities with groups. SOD is a vital component of the antioxidant

response system of the host [42]. The activities of SOD exhibited the inverted V-shapes trends among the treatments and the maximums emerged in the BC group. It could be reasonably speculated that dietary supplements of *B. licheniformis* and combined herb extracts could stimulate sea cucumbers to produce more SOD enzymes to suppress the oxidative stress and enhance the stability of the lysosomal membrane at the initial stages of the test [43]. As time went on, the oversaturated enzymes have caused immunosuppression and induced immune fatigue, which presented significant time response and reduced the antioxidant ability of sea cucumbers [44]. In general, dietary *B. licheniformis* and combined herb extracts in combination provided a better immunoreactivity for sea cucumbers than the separate supplementation.

4.4. Variation in Intestinal Microbiota. The intestinal microbiota composition plays a major part in the health of *A. japonicus* [45]. Bacterial alpha diversity is applied in analyzing the community richness and the complexity of species diversity [46]. The increases in diversity and richness would enhance the integrity and stability of the intestinal microbiota profile, while the lower values may be connected with higher susceptibility to invasion [5]. In the present study, the BC group produced the highest Chao1 and observed species parameters, as well as the indices of Shannon and Simpson, suggesting that the species' richness and diversity of the microbial community had increased dramatically in the BC group. In contrast, the minimums of Chao1 and observed species were detected in the B and control groups, while the competitive advantage of increasing richness indices had not yet shown in the B group. This may be due to that administration of *B. licheniformis* individually would result in growth depression of the indigenous bacteria through the competition for nutrients and have changed the richness of some bacteria.

In this study, the intestinal microbiota composition of *A. japonicus* before applying *B. licheniformis* and combined herb extracts was similar to the previous studies, and the detectable bacteria were considered to be the indigenous bacteria in the gut of *A. japonicus* [47]. The results suggested that the intestinal microbiota profile held relatively steady at hierarchical levels of the phylum, class, family, and genus.

After the feeding period, an alteration was induced in dominant microbial phyla of sea cucumbers with the treatments of *B. licheniformis* and combined herb extracts either alone or in combination. The higher proportion of *Bacteroidetes* and the lower proportion of *Proteobacteria* were observed in the BC group. Generally, *Bacteroidetes* could promote nutrient absorption and produce new metabolites that were correlated with lipid and protein conversion [48], while some bacteria of *Proteobacteria* were related to chronic phlogosis and metabolic diseases, which were actually harmful to the sea cucumbers [49]. Moreover, the abundances of *Verrucomicrobia*, *Actinobacteria*, and *Tenericutes* were found to increase more dramatically in the BC group compared to the other three groups. The phylum *Verrucomicrobia* was metabolically diverse and could serve as a suitable model for investigating methane oxidation, phosphorus availability, and polysaccharide degradation [50]. The phylum *Actinobacteria* was regarded as an important source to develop marine drugs and bioactive substances, and the phylum *Tenericutes* possessed the productive versatility and metabolic adaptability, which could maintain the bacterial community homeostasis and resist the invading organisms [51].

At the genus level, the higher abundances of *Rhodobacter*, *Colwellia*, *Neptunomonas*, and *Halomonas* were detected in the BC, B, and C groups, suggesting that administration of *B. licheniformis* and combined herb extracts had produced the benefits of regulating the distribution of microflora during the feeding period. Relatively, the BC group was better than the advantage of the other two. This can be explained reasonably that *Rhodobacter* may help to degrade the intake organic matter and toxic substances, and serve as an energy source of the host [52]. The *Colwellia* could secrete extracellular cold-active enzyme products, which would maintain high metabolic rates in cold conditions [53]. The *Neptunomonas* and *Halomonas* could break down carcinogens and fatty acid residues, and play a crucial role in the digestion of ingested detritus, which might be potentially useful for biotechnological processes [54]. Most noteworthy, the higher proportion of *Bacillus* was detected in the BC group followed by the B group, indicating that the *B. licheniformis* was able to colonize the gut of *A. japonicus* when it was added to the basic feed. In addition, the higher abundance of opportunistic pathogens, such as *Vibrio*, *Lutibacter*, and *Arcobacter*, was found in the control group, which were lower in the BC group. These typical genera were associated with organic damage and metabolic disorders, and may increase the risk of infections [55]. The results confirmed that the *B. licheniformis* and combined herb extracts could modulate the microbial composition of *A. japonicus* by increasing the beneficial microorganisms and restraining potential pathogens.

4.5. Resistance to Pathogenic Bacteria. As the causative pathogen of skin ulcerative syndrome in *A. japonicus*, the *V. splendidus* could secret a variety of extracellular products, which are toxic to hosts [56]. After injection with *V. splendidus*, the first detected dead individual appeared in the control group, followed by the B, C, and BC groups in this study. This implied that *B. licheniformis* and combined herb extracts would defer the occurrence of disease in sea cucumber. In addition, the values of cumulative mortality showed by sea cucumbers in the BC group were observably lower than the other groups. This indicated that the application of *B. licheniformis* and combined herb extracts in combination could produce a synergistic benefit in inhibiting pathogenic bacteria. Similarly, the minimum values of *V. splendidus* on the TCBS agar appeared in the BC group, while the control group exhibited the maximum counts during the challenge test. The results suggested that the BC group could eliminate *V. splendidus* in intestines of sea cucumbers more effectively.

Previous studies have proven that the genus *Bacillus* could exclude other bacteria through the production of antimicrobial agents [7]. The combined herb extracts used in this study had been already documented for stimulating bacteriostasis. Among them, *Astragalus membranaceus* and *Angelica sinensis* could effectively regulate the functions of the immune cells by means of enhancing respiratory burst, improving phagocytic activity and plasma lysozyme activity [57, 58]. The *Scutellaria baicalensis* possessed pharmacological activities, including antibacterium, anti-inflammation, and antihypoxia responses, as well as immunostimulating properties to combat against fish diseases [12]. *Lonicera japonica* extract contained various active components, which are responsible for potential bioactivities [59]. It was speculated that the combined herb extracts would provide the specificity substrates for the *B. licheniformis* to enhance the reproductive capacity in the intestine, and the *B. licheniformis* would in turn stimulate the combined herbs extracts to express antimicrobial effects on the elimination of pathogenic bacteria on the basis of a synergistic mechanism [5, 10]. Therefore, the application of *B. licheniformis* and combined herb extracts in combination could counteract the adverse effects of pathogenic bacteria more efficiently.

5. Conclusions

To conclude, the present study confirmed that *B. licheniformis* and combined herb extracts could effectively enhance the physiological and immune characteristics, optimize the composition of the intestinal microbiota, and improve the vibriosis resistance of sea cucumbers. In addition, dietary supplementation of *B. licheniformis* and combined herb extracts in combination displayed better positive effects than the separate supplementation. These results indicated that *B. licheniformis* and combined herb extracts would be functional as food supplements to produce the health-promoting effects in *A. japonicus*, and the findings may be particularly significant to the development of green and environment-friendly feed products in aquaculture.

Data Availability

The data used to support the findings of this study are available from the corresponding author on request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Xuda Wang and Zunchun Zhou conceptualized the study; Xuda Wang, Shilei Li, Ying Dong, Rui Mi, and Jingwei Jiang contributed to methodology and performed formal analysis; Zunchun Zhou, Shilei Li, and Ying Dong were responsible for funding acquisition; Xiaoyan Guan, Bo Ye, Guiying Liu, Zhenjun Zhao, Danni Liu, Zhong Chen, Xuewen Gao, and Chenyu Zhang investigated the data; Zunchun Zhou was involved in study supervision; Xuda Wang prepared the original draft; and Zunchun Zhou reviewed and edited the manuscript.

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Supplementary Materials

The graphical abstract had been provided as the supplementary materials and uploaded under the supplementary section. (*Supplementary Materials*)

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