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Natural Feed Supplements Improve Growth, Non-Specific Immune Responses and Resistance against *Vibrio alginolyticus* in *Lates calcarifer*

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Abstract: This study aimed to investigate the effects of dietary natural feed supplement on the growth performance, non-specific responses, and disease resistance in *Lates calcarifer*. Three commercial products (A, B, and C) containing a basal probiotic mixture were tested. Product A contained a basal mixture of *Paenibacillus* sp., *Bacillus subtilis*, *Bacillus amyloliquefaciens,* and *Lactobacillus rhamnosus* (10^7 cfu/g for each probiotic); product B contained additional *Lactobacillus plantarum* (10^{10} cfu/g); and product C contained additional soybean peptides (500 g/kg) and garlic powder (1 g/kg). Each product was supplemented into subject diets at dosages of 1 or 2 g/kg (designated as the A1, A2, B1, B2, C1, and C2 groups, respectively). Following an eight-week trial, growth parameters (specific growth rate and feed conversion ratio), non-specific immune responses (O_2^- production, phagocytic rate, and phagocytic index), and the results of a challenge test against *Vibrio alginolyticus* were evaluated. The results show that all probiotic supplement groups exhibited an improvement in growth performance compared to the control group (non-probiotic diet). In terms of non-specific immunity parameters, a significant improvement in O_2^- production was found in the C2 group, whereas significant improvements in phagocytic activity were found in all the B and C groups. The C2 group displayed optimal O_2^- production, phagocytic rate, and phagocytic index results. For the challenge test, the C groups showed higher *Vibrio* resistance than the other experimental groups and the control group. These results suggest that product C, given at dosages of 2 g/kg, may serve as a growth-promoting and immunostimulatory additive for the cultivation of Asian seabass.

Keywords: *Lates calcarifer*; probiotics; growth; non-specific immune response; *Vibrio alginolyticus*

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1. Introduction

Disease outbreaks in fish and other economic aquatic species can cause huge losses and hinder aquaculture development. For example, vibriosis, one of the most prevalent bacterial diseases, affects *Epinephelus* sp. groupers at every stage of development and causes death in up to 50% of infected fish [1]. Traditionally, the control of vibriosis is achieved through antibiotics and disinfectants such as chloramphenicol and erythromycin, which are used against *Vibrio harveyi* infections in giant tiger prawns, and hypochlorites for the treatment of vibrio-contaminated water [1]. However, such practices are usually accompanied by a negative impact on aquatic animal health and environment [2]. Single and mixed non-pathogenic probiotic products have been proven to be positive promoters of aquatic fish growth, survival, and health. Previous studies have reported that incorporating probiotics into fish diets could improve intestinal microbiota, absorptive ability, digestive enzyme activity, and expression levels of immune-related genes [3]. The administration of

probiotics is therefore considered an alternative method for disease control and prevention in various aquatic animal species [4].

Currently, various microorganisms such as *Saccharomyces, Bacillus, Lactococcus, Micrococcus, Enterococcus, Lactobacillus,* and *Photorhodobacterium* are considered to be probiotics in aquaculture [5]. *Bacillus* and lactic acid bacteria (LAB) are the most commonly used probiotics for aquaculture functional feed formulations. *B. subtilis* produces a wide range of peptide antibiotics that are effective in inhibiting the growth of pathogens. This bacterium can also produce secondary metabolites, fine chemicals, and heterologous proteins for immune modulation [6]. LAB can produce molecules with bactericidal activity against intestinal pathogens or trigger immune responses in the host [7]. Different administration modes for probiotics have been proposed. Several studies have found that the co-administration of probiotics with prebiotics or plant products is beneficial. Garlic (*Allium sativum*) has many bioactive compounds. The various properties of garlic such as antibacterial, antioxidant and immunostimulant activity have made it suitable for combination with probiotics in livestock farming [8,9].

Lates calcarifer, also known as barramundi or Asian seabass, is an important aquaculture fish species commonly found in Australia and southern Taiwan [10]. In Taiwan, *L. calcarifer* are often cultivated under high-density conditions to increase profitability. However, this farming process and environment expose the fish to multiple stressors, increasing their susceptibility to disease. The present study aimed to develop a practical framework for probiotic and plant product utilization in *L. calcarifer*. Using three novel commercial probiotic products, respectively, containing a basal mixture of *Paenibacillus* sp., *Bacillus subtilis*, *Bacillus amyloliquefaciens,* and *Lactobacillus rhamnosus* (10^7 cfu/g for each probiotic) (product A), additional *Lactobacillus plantarum* (10^{10} cfu/g) (product B), and additional soybean peptides (500 g/kg) and garlic powder (1 g/kg) (product C), the effects of dietary supplementation on the growth performance, non-specific immune responses, and disease resistance of Asian seabass were evaluated.

2. Materials and Methods

2.1. Experimental Fish Rearing

Healthy *Lates calcarifer* were transported from a commercial fish farm to Aquatic Animal Center, National Taiwan Ocean University (NTOU). Fish from freshwater were then acclimatized in seawater for two months prior to the experiment. Fish were kept in outdoor 8-ton fiberglass-reinforced plastic (FRP) tanks, and approximately 30% of the tank's water is exchanged every day with fresh seawater. Fish received pelleted bass feed (Tairoun Products Company Ltd., Taipei, Taiwan; crude protein > 43%, crude lipid > 6%, ash < 16%, fiber < 3% and moisture < 11%) two times daily at amounts equivalent to 3% of fish body weight.

2.2. Experimental Diets Preparation

A control diet (CT) obtained from Tairoun Products Co., Ltd. (Taipei, Taiwan) was crushed for basal ingredients. Three commercial probiotic products (A, B, and C from Green Wonder Biotech Co., Ltd. (Taichung, Taiwan)) were, respectively, added into the CT feed at dosages of 1 or 2 g/kg of feed (referred to the A1, A2, B1, B2, C1, and C2 groups). The feed powders were thoroughly mixed with α-starch (1 g/kg of feed) and reformed using a pelletizer. The diets were oven-dried at 40 °C for one day and then stored at 4 °C until use. Product A contained a basal mixture of *Paenibacillus* sp., *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Lactobacillus rhamnosus* (10^7 cfu/g for each probiotic). In addition to the basal probiotic mixture, *Lactobacillus plantarum* (10^{10} cfu/g) was included in product B, and soybean peptides (500 g/kg) and garlic powder (1 g/kg) were included in product C.

2.3. Feeding Trial

A total of 350 fish were randomly assigned to 7 groups placed in 14 outdoor 1-ton FRP tanks (25 fish/tank) with a seawater flow-through system. Fish were individually marked in tail. After acclimation of 1 week, the feeding trail started and lasted 8 weeks. Fish (average weight 50.79 ± 4.86 g) were fed with experimental diets twice daily at 09:00 and 16:00. Growth and death were observed and recorded every two weeks. Fish from each group were counted to calculate the survival rate (SR) and weighed individually to determine the weight gain (WG), specific growth rate (SGR), and feed conversion ratio (FCR). The growth parameters were calculated using the formula in our previous study [11]: WG = [(Final weight (g) − Initial weight (g))/Initial weight (g)] × 100%; SGR = [ln (Final weight (g)) − ln (Initial weight (g))/Time duration (days)] × 100%; FCR = Feed intake (g)/(Final weight (g) − Initial weight (g)). During the experimental period, the water quality parameters were monitored as follows: water temperature (25.5–30.5 °C), pH (6.9–8.1), dissolved oxygen > 6.1 mg/L. NTOU's Institutional Animal Care and Use Committee approved the experiments (Approval number: 109012).

2.4. Immunity Tests

Three fish from each group were randomly sampled and dissected on ice. Isolation of head kidney leukocytes and following immune tests were performed using a previously published method [12]. In brief, the head kidneys of the experimental fish were exsected, minced, and washed in Hanks balanced salt solution (HBSS; Gibco, Waltham, MA, USA). Cell lysates were passed through a 100 μm nylon mesh and the flow-through cell suspension was transferred to Percoll solution (Pharmacia, Stockholm, Sweden) with 30% and 50% density gradients. After being centrifuged at 400× *g* at 4 °C for 40 min, the leucocytes were obtained at the interface. The leucocytes were then washed three times with HBSS, followed by adjustments of 5×10^6 cells/mL.

The measurement of O_2^- production was performed to evaluate the respiratory burst activity. Moreover, 100 μL of leukocytes was loaded to a 96-well microplate. A centrifugation of 800× *g* at 4 °C for 20 min was used to remove the non-adherent cells and 100 μL of zymosan solution (0.1% in HBSS; InvivoGen, San Diego, CA, USA) was added to each well for 30 min at room temperature. After this step, the reaction mixture was discarded before adding 100 μL of 0.3% nitro tetrazolium blue chloride solution (Bio Basic Inc., Markham, Canada). After 30 min of incubation, the reaction was terminated by adding 100 μL of 100% methanol, followed by washing the cells with 70% methanol and air drying them at room temperature. In the last step, 120 μL of KOH (2 M) and 140 μL of dimethyl sulfoxide (Gibco, Waltham, MA, USA) were added into each well and measured at 630 nm with a microplate reader (BioTek, Santa Clara, CA, USA).

In the measurement of phagocytic activity, 100 μL of leukocytes was dispensed on a glass coverslip. After one hour of seeding, 100 μL of latex bead solution (3×10^6 beads, 0.8 μm size; Sigma-Aldrich, St. Louis, MO, USA) in HBSS was added and incubated for one hour at room temperature. The cells were then washed three times with sterilized ddH_2O and fixed for 5 min using 300 μL of pure methanol. Finally, fixed cells were stained with 5% Giemsa and air dried. Cells were observed under a light microscope (Olympus, Tokyo, Japan). The phagocytic rate (PR) was determined by phagocytic cell counts per 100 cells. The phagocytic index (PI) was calculated as the average counts of beads in phagocytic cells.

2.5. Challenge Test

V. alginolyticus was isolated from diseased fish and identified using 16S rDNA sequencing. The bacterial stock was cultured overnight on tryptic soy agar (NuCel, Maisons-Alfort, France) with the supplement of 3% NaCl. Following that, the single colony was inoculated to tryptic soy broth (NuCel, Maisons-Alfort, France) supplemented with 3% NaCl. A shaker (100 rpm) was used to incubate the broth cultures for 4 h at 30 °C before centrifugation at 1000× *g* for 10 min at 4 °C. Eleven fish from each group were challenged with *V. alginolyticus* after the feeding trial. An additional 11 fish from the CT group were injected

with sterile phosphate-buffered saline (PBS) as a negative control (CT-PBS group). A dose of 10^7 cfu/fish was injected into the abdomens of the fish using resuspended bacterial pellets dissolved in sterile PBS. Observations were conducted on the survival numbers of each group during a period of seven days.

2.6. Statistical Analyses

The experiments were conducted at least in triplicate, and data from growth and immunity tests were presented as means ± standard deviations (SD). SAS software version 9.4 was used for statistical analysis. Shapiro–Wilk tests and Levene test were performed to verify the normality of data and homogeneity of variance, respectively. A one-way ANOVA with post hoc Tukey's honest significant difference test was performed with significance set at $\alpha = 0.05$. Survival data for the fish challenge test were analyzed by Kaplan–Meier methods using the SPSS software version 22.0. The Mantel–Cox test was used to evaluate the differences between groups. Statistical significance (Sig.) is indicated by p values < 0.05.

3. Results

3.1. Improvement of Growth by Probiotics and Plant Products

The growth performance after four weeks is shown in Table 1. Compared to the CT group, the WG, SGR, and FCR of all experimental groups were significantly improved ($p < 0.05$). Among the experimental groups, the A1 group showed the highest level of growth after four weeks (SGR: 2.46 ± 0.07, FCR: 0.91 ± 0.03), but there were no significant differences between the experimental groups ($p > 0.05$). The growth performance after eight weeks is shown in Table 2. Compared to the CT group, the growth data of all experimental groups were improved. The WG and SGR of all experimental groups were significantly improved ($p < 0.05$), except in the B2 group. However, there were no significant differences in FCR among the experimental groups ($p > 0.05$). Among the experimental groups, the highest level of growth at eight weeks was achieved by the A2 group (SGR: 1.77 ± 0.10, FCR: 1.20 ± 0.10). No significant differences were found among the growth parameters of the experimental groups ($p > 0.05$). Moreover, the SR remained above 98% in each group after eight weeks of feeding, indicating that the three probiotic products are safe to use.

Table 1. Growth performance and survival of *L. calcarifer* fed with different natural feed supplement for four weeks.

Groups	Initial Weight (g)	Final Weight (g)	WG (%)	SGR (%)	FCR	FI	SR (%)
CT	49.82 ± 4.88 ^{a}	84.42 ± 8.57 ^{b}	69.60 ± 1.91 ^{b}	1.89 ± 0.04 ^{b}	1.20 ± 0.03 ^{a}	41.55	100
A1	50.27 ± 4.89 ^{a}	100.04 ± 11.64 ^{a}	99.01 ± 3.93 ^{a}	2.46 ± 0.07 ^{a}	0.91 ± 0.03 ^{b}	45.49	100
A2	50.79 ± 4.59 ^{a}	96.26 ± 11.44 ^{a}	89.49 ± 3.08 ^{a}	2.28 ± 0.06 ^{a}	1.00 ± 0.04 ^{b}	45.54	100
B1	50.58 ± 5.00 ^{a}	98.63 ± 12.60 ^{a}	95.00 ± 1.71 ^{a}	2.39 ± 0.03 ^{a}	0.95 ± 0.02 ^{b}	45.56	100
B2	51.96 ± 4.26 ^{a}	98.31 ± 13.51 ^{a}	89.20 ± 2.04 ^{a}	2.28 ± 0.04 ^{a}	0.99 ± 0.03 ^{b}	45.82	100
C1	50.92 ± 5.06 ^{a}	98.27 ± 11.16 ^{a}	92.98 ± 1.93 ^{a}	2.35 ± 0.04 ^{a}	0.98 ± 0.04 ^{b}	46.25	100
C2	50.75 ± 5.34 ^{a}	97.25 ± 14.74 ^{a}	91.63 ± 4.01 ^{a}	2.32 ± 0.07 ^{a}	0.99 ± 0.03 ^{b}	45.95	100
$F_{6,7}$	4.08	27.43	22.42	24.62	16.23		
p	0.0441	0.0002	0.0003	0.0002	0.0009		

Note: Values are presented as mean ± SD ($n = 50$ for each group). One-way ANOVA and Tukey's test were used for analysis. Different superscripts indicate significant differences ($p < 0.05$) among means in the same column. Abbreviations: WG: weight gain; SGR: specific growth rate; FCR: feed conversion ratio; FI: feed intake; SR: survival rate; CT: control; A1: A product 1 g/kg feed; A2: A product 2 g/kg feed; B1: B product 1 g/kg feed; B2: B product 2 g/kg feed; C1: C product 1 g/kg feed; C2: C product 2 g/kg feed.

Table 2. Growth performance and survival of *L. calcarifer* fed with different natural feed supplement for eight weeks.

Groups	Initial Weight (g)	Final Weight (g)	WG (%)	SGR (%)	FCR	FI	SR (%)
CT	49.82 ± 4.88 ^{a}	117.53 ± 14.21 ^{b}	136.59 ± 8.10 ^{b}	1.54 ± 0.06 ^{b}	1.32 ± 0.08 ^{a}	89.56	100
A1	50.27 ± 4.89 ^{a}	134.51 ± 18.28 ^{a}	167.57 ± 7.93 ^{a}	1.76 ± 0.05 ^{a}	1.23 ± 0.06 ^{a}	103.11	100
A2	50.79 ± 4.59 ^{a}	136.96 ± 19.06 ^{a}	169.66 ± 14.51 ^{a}	1.77 ± 0.10 ^{a}	1.20 ± 0.10 ^{a}	103.16	100
B1	50.58 ± 5.00 ^{a}	133.61 ± 19.24 ^{a}	164.23 ± 5.51 ^{a}	1.73 ± 0.04 ^{a}	1.24 ± 0.04 ^{a}	103.17	100
B2	51.96 ± 4.26 ^{a}	130.47 ± 19.32 ^{ab}	150.97 ± 8.53 ^{ab}	1.64 ± 0.06 ^{ab}	1.32 ± 0.08 ^{a}	103.43	100
C1	50.92 ± 5.06 ^{a}	137.03 ± 17.48 ^{a}	169.10 ± 4.93 ^{a}	1.77 ± 0.03 ^{a}	1.21 ± 0.04 ^{a}	103.87	98
C2	50.75 ± 5.34 ^{a}	134.30 ± 25.70 ^{a}	164.64 ± 20.99 ^{a}	1.74 ± 0.14 ^{a}	1.27 ± 0.16 ^{a}	105.13	100
$F_{6,7}$	4.08	3.54	18.47	19.61	0.63		
p	0.0441	0.0021	<0.0001	<0.0001	0.7023		

Note: Values are presented as mean ± SD (n = 50 for each group except C1 where n = 49). One-way ANOVA and Tukey's test were used for analysis. Different superscripts indicate significant differences ($p < 0.05$) among means in the same column. Abbreviations: WG: weight gain; SGR: specific growth rate; FCR: feed conversion ratio; FI: feed intake; SR: survival rate; CT: control; A1: A product 1 g/kg feed; A2: A product 2 g/kg feed; B1: B product 1 g/kg feed; B2: B product 2 g/kg feed; C1: C product 1 g/kg feed; C2: C product 2 g/kg feed.

3.2. Improvement of Immunity by Probiotics and Plant Products

Figure 1 shows the effects of probiotic supplements on the O_2^- production of *L. calcarifer* leukocytes ($F_{6,35} = 7.74$, $p < 0.001$). The highest O_2^- production was found in the C2 group, which showed a significant improvement compared to the CT group ($p < 0.05$). The B2 and C1 groups showed higher O_2^- production levels than the CT group, but the differences were not significant ($p > 0.05$). No significant differences were found between the other experimental groups and the control group ($p > 0.05$). Figure 2 shows the effects of probiotic supplements on the PR of *L. calcarifer* leukocytes ($F_{6,14} = 199.30$, $p < 0.001$). Compared to the CT group, the PR of the B and C groups were significantly increased ($p < 0.05$), whereas the PR of the A groups were not ($p > 0.05$). The PR of the B1 group was significantly higher than that of the B2 group ($p < 0.05$). Moreover, the PR of the C groups were significantly higher than those of other groups ($p < 0.05$). The C2 group had the highest PR, but showed no significant difference compared with the C1 group ($p > 0.05$). Figure 3 shows the effects of probiotic supplements on the PI of *L. calcarifer* leukocytes ($F_{6,14} = 59.74$, $p < 0.001$). Compared to the CT group, the PI of the B and C groups were significantly increased ($p < 0.05$), but the PI of the A groups were not ($p > 0.05$). A higher PI was observed in the B1 group than in the B2 group ($p < 0.05$). In addition, the PI of the C groups were significantly higher than those of the B2 and A groups ($p < 0.05$). The PI of the C2 group was the highest, but showed no significant difference compared with the PI of the C1 group ($p > 0.05$).

3.3. Improvement of Disease Resistance by Probiotics and Plant Products

Disease resistance was evaluated using a *V. alginolyticus* injection challenge after the feeding trial. The survival curve is shown in Figure 4A. The lack of mortality in the CT-PBS group at seven days after the challenge indicated that the fish could survive the injection process. Surviving experimental groups C1, C2, and A1 showed respective survival rates of 45%, 36%, and 18%, whereas no fish survived in the other challenge groups. Pairwise comparison analysis of the survival between experimental groups is shown in Figure 4B. The survival probabilities of the C1, C2 and A1 groups were significantly higher than that of the CT group ($p = 0.001$, $p = 0.046$, $p = 0.027$, respectively). There was no significant difference between the three survival groups ($p > 0.05$).

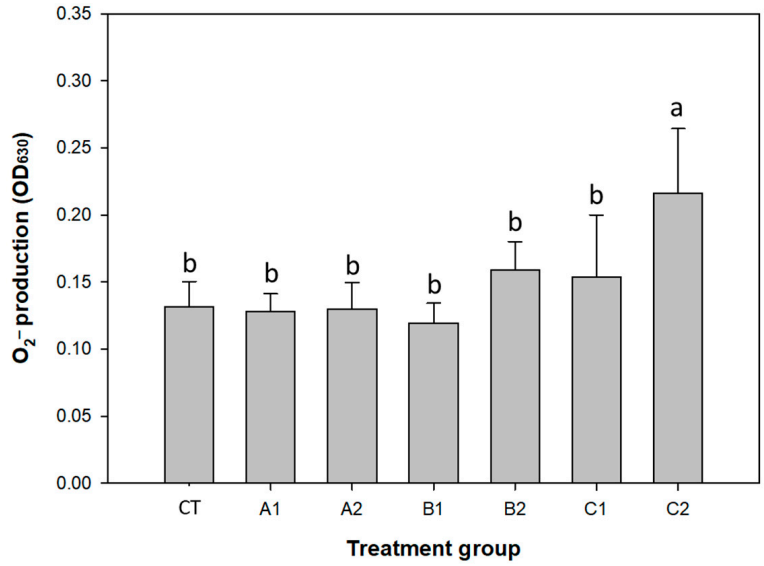


Figure 1. Superoxide anion (O_2^-) production of leukocytes taken from *L. calcarifer* fed with different commercial probiotics for 56 days. Values are shown as mean ± SD (n = 6). One-way ANOVA and Tukey's test were used for analysis. Treatment groups with significant differences ($p < 0.05$) are indicated above the bars by different letters. Each commercial product was supplemented into control diets (CT) at dosages of 1 or 2 g/kg and designated as the A1, A2, B1, B2, C1, and C2 groups.

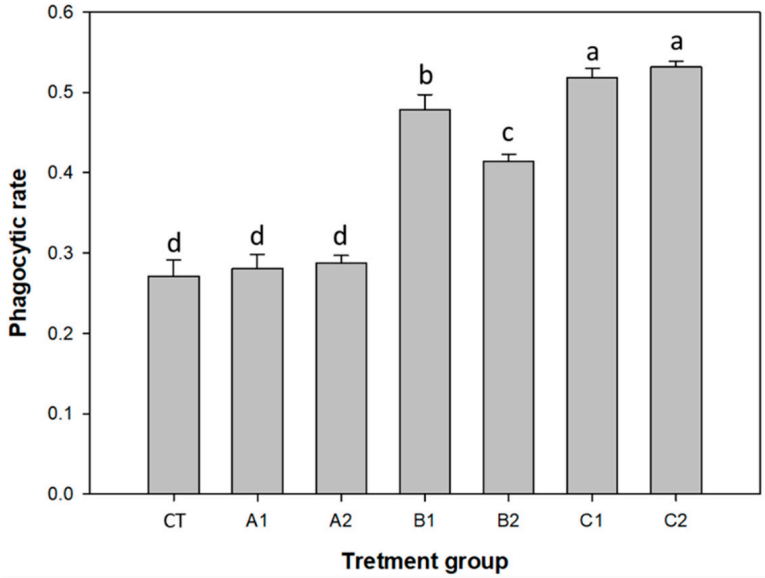


Figure 2. Phagocytic rate (PR) of leukocytes taken from *L. calcarifer* fed with different commercial probiotics for 56 days. Values are shown as mean ± SD (n = 3). One-way ANOVA and Tukey's test were used for analysis. Treatment groups with significant differences ($p < 0.05$) are indicated above the bars by different letters. Each commercial product was supplemented into control diets (CT) at dosages of 1 or 2 g/kg and designated as the A1, A2, B1, B2, C1, and C2 groups.

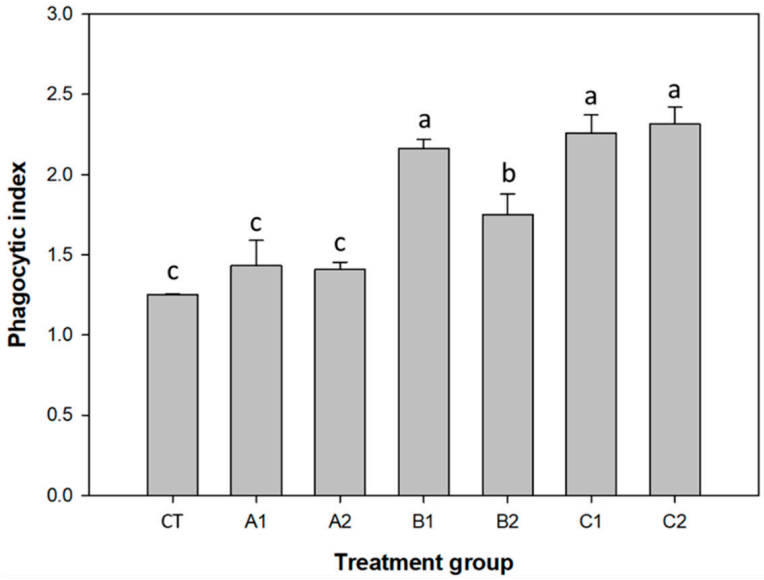
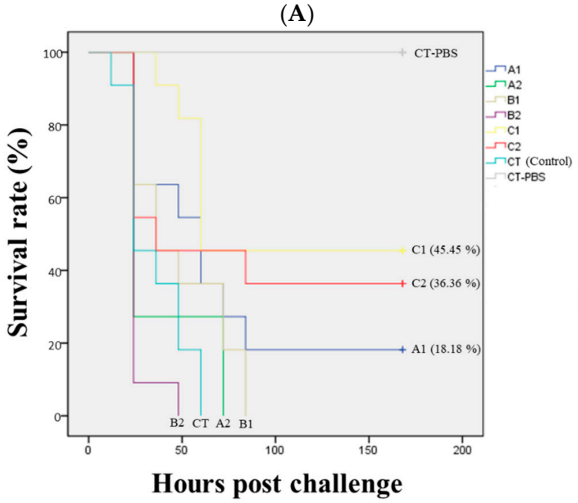


Figure 3. Phagocytic index (PI) of leukocytes taken from *L. calcarifer* fed with different commercial probiotics for 56 days. Values are shown as mean ± SD (n = 3). One-way ANOVA and Tukey's test were used for analysis. Treatment groups with significant differences ($p < 0.05$) are indicated above the bars by different letters. Each commercial product was supplemented into control diets (CT) at dosages of 1 or 2 g/kg and designated as the A1, A2, B1, B2, C1, and C2 groups.

(**A**)



(**B**)

Pairwise comparisons

		A1		A2		B1		B2		C1		C2		CT		CT-PBS	
	group	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	A1			2.136	.144	.780	.377	9.247	.002	2.231	.135	.401	.527	4.865	.027	15.593	.000
	A2	2.136	.144			1.449	.229	2.932	.087	7.197	.007	4.370	.037	1.190	.275	22.953	.000
	B1	.780	.377	1.449	.229			6.697	.010	6.051	.014	2.243	.134	2.830	.093	23.556	.000
	B2	9.247	.002	2.932	.087	6.697	.010			19.888	.000	6.735	.009	2.010	.156	23.261	.000
	C1	2.231	.135	7.197	.007	6.051	.014	19.888	.000			.896	.344	11.771	.001	7.796	.005
	C2	.401	.527	4.370	.037	2.243	.134	6.735	.009	.896	.344			3.995	.046	10.105	.001
	CT	4.865	.027	1.190	.275	2.830	.093	2.010	.156	11.771	.001	3.995	.046			23.502	.000
	CT-PBS	15.593	.000	22.953	.000	23.556	.000	23.261	.000	7.796	.005	10.105	.001	23.502	.000		

Figure 4. (**A**) Kaplan–Meier survivorship curves of *L. calcarifer* fed with different commercial probiotics for 56 days followed by *V. alginolyticus* challenge (n = 11 per group). Fish from the CT group were injected with sterile PBS as a negative control (CT-PBS group); (**B**) The pairwise comparisons between the groups were analyzed using the Mantel–Cox test.

4. Discussion

Possible role of probiotics as beneficial mediators in aquaculture include the improvement of nutrient utilization, microvilli surface area, gut microbial community, anti-stress enzymes activity, immune response, as well as the stimulation of decolonization of pathogen in host organisms [13]. The benefits of probiotic feed supplements on fish growth, specifically the components of the commercial products used in the present study, *Paenibacillus* sp. [14], *B. subtilis* [15], *B. amyloliquefaciens* [16], *L. rhamnosus* [17,18], and *L. plantarum* [19,20] were documented in previous literature. *B. subtilis* in particular has been proven to improve the growth performance of Asian seabass [21,22]. The dietary addition of *B. subtilis* (10^9 cfu/kg) has been shown to improve growth parameters, survival rates, body composition (increases proteins and decreases lipids), digestive enzyme activities (protease, amylase, and lipase) in the digestive tract, and hematological parameters (red blood cells, white blood cells, and hemoglobin levels). Moreover, soybean peptides, which were used in product C, were proven to benefit biological absorption and non-nutrition-related biological activities, displaying immunity, antibacterial, and antioxidant effects [23–25]. As for garlic powder, the other ingredient in product C, previous studies have shown that the inclusion of garlic (40 g/kg) in one's diet can significantly improve the growth and feed utilization of Asian seabass [26]. Therefore, improvements in the growth performance of *L. calcarifer* can be achieved with the dietary supplements of these functional and safe materials. Further investigations into the modulation of gut morphology, metabolism, and microbiota should be conducted to address the growth augmentation.

The present data show that non-specific immune responses of Asian seabass were improved in the B and C groups, but not in the A groups, indicating that the components of product B can enhance phagocytic activity, and the ingredients of product C can enhance both phagocytic and respiratory burst activities. Previous literature has shown that the dietary addition of *L. plantarum* (10^6–10^{10} cfu/kg) for two weeks can increase phagocytic activity in groupers (*Epinephelus coioides*) [20]. As no improvements in immunity were found in the A groups, we theorize that *L. plantarum* (1 and 2 $\times$ 10^7 cfu/kg), an ingredient of product B, may contribute to the improvement of phagocytic activity in Asian seabass. Garlic (*Allium sativum*) has been widely used as an aquaculture feed additive [26,27] and can be used as an immunostimulant to enhance resistance against bacteria, viruses, and parasites. Phagocytic activity and respiratory burst activity were improved in rainbow trout (*Oncorhynchus mykiss*) fed garlic powder (5 g/kg) for two weeks [28,29]. In addition, the dietary administration of raw garlic or garlic powder (10–80 g/kg) for eight days has been shown to enhance phagocytic and respiratory burst activity in *E. coioides* [30]. The present data show that trace amounts of garlic powder contained in product C may play an important role in improving the phagocytic and respiratory burst activity of Asian seabass.

The challenge test showed that the C groups had the best disease resistance against *V. alginolyticus*, which cause heavy mortalities in Asian seabass farming [31]. The C groups also had the highest levels of immunity improvement. A previous report showed that rainbow trout (*Oncorhynchus mykiss*) fed garlic powder (5 g/kg) for 14 days displayed improved resistance against *Aeromonas hydrophila* [29]. Increase in resistance against *Vibrio harveyi* was also found in *L. calcarifer* fed garlic powder (5 g/kg) for 14 days [32]. The antibacterial properties of garlic prevent the colonization of pathogenic bacteria in the intestine and influence immune responses by providing an ideal environment for the proliferation of beneficial probiotics [27]. It was noted that garlic extract can serve as prebiotics for LAB [33]. A 4% garlic extract is the optimal concentration that can effectively promote growth of *Lactobacillus acidophilus*. Moreover, dietary supplementation with soybean peptides replacing 50% of fish meal was shown to reduce mortality in yellow catfish (*Pelteobagrus fulvidraco*) after *A. hydrophila* infection [34]. Soybean peptides may improve immune responses and enhance the disease resistance of yellow catfish. This improvement of immune response was also reported in Japanese flounders, suggesting that the dietary supplement of 10% soybean peptide could enhance the survival of *Paralichthys olivaceus* under heat stress [35]. Product C contains 50% soybean peptides and 0.1% garlic powder. We therefore speculate

that the dietary additions of soybean peptides (0.5 and 1 g/kg) and garlic powder (0.001 and 0.002 g/kg) can effectively improve *Vibrio* resistance of Asian seabass.

Research on the use of probiotic mixtures and their effects on fish growth and immunity is presently limited. The immune efficiency and disease resistance of probiotics are associated with the strain, viability, dosage, and the duration of administration [36]. It has been reported that the dietary administration of a mixture of *B. subtilis* and *L. acidophilus* provided better disease protection for *Oreochromis niloticus* Nile tilapia than the use of a single probiotic [37]. However, the antagonistic interactions among probiotics should be carefully evaluated prior to the administration of probiotic mixtures for different aquatic animals. It has been reported that *L. plantarum* can inhibit the growth of *B. subtilis* and decrease probiotic efficiencies in *Scylla paramamosain* mud crabs [38], which could explain why the survival of the B groups was worse than that of the A groups in the present challenge test. In other words, *L. plantarum* may alleviate the beneficial effects provided by *B. subtilis*.

In sum, the present work indicates that the dietary administration of combined probiotics and plant products have positive effects on the growth performance, non-specific immune responses, and the pathogen resistance of the Asian seabass. The appropriate combination of multispecies probiotics and other bioactive substances may have complementary effects on fish aquaculture. This study provides a successful example of the use of natural feed supplements in sea bass mariculture.

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