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Multiple-strain probiotics appear to be more effective in improving the growth performance and health status of white shrimp, *Litopenaeus vannamei,* than single probiotic strains

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ABSTRACT

The probiotic efficiencies of the mixed probiotics containing *Lactobacillus pentosus* BD6, *Lac. fermentum* LW2, *Bacillus subtilis* E20, and *Saccharomyces cerevisiae* P13 for shrimp growth and health status improvement were better than those when using single probiotics. The probiotic mixture at a level of 10^8 colony-forming units (cfu) (kg diet)$^{-1}$ and the diets containing BD6 and E20 at 10^9 cfu (kg diet)$^{-1}$ significantly improved the growth and health status of shrimp, whereas the diets containing P13 or LW2 did not significantly affect the growth of shrimp. No significant difference in the carcass composition was recorded among the control and treatments. After 56 days of feeding, shrimp fed the diet containing the probiotic mixture ($10^7 \sim 10^9$ cfu (kg diet)$^{-1}$) had higher survival after injection with the *V. alginolyticus*, but 10^9 cfu (kg diet)$^{-1}$ of single probiotics (except for *S. cerevisiae* P13) had to be administered to improve shrimp survival. The better disease resistance of shrimp in groups fed the probiotic mixture might have been due to increased phenoloxidase activity, respiratory bursts, and lysozyme activity of hemocytes. Therefore, we considered that the probiotic mixture could adequately provide probiotic efficiency for white shrimp, and a diet containing 10^8 cfu (kg diet)$^{-1}$ probiotic mixture is recommended.

1. Introduction

Use of probiotics is considered an effective strategy for successful aquaculture because probiotics are one of the identified alternatives to antibiotics [1,2], and can inhibit pathogens [3], and improve animal growth and health status, and pond quality [4–6]. The word “probiotic” originates from the Greece words ‘pro’ and ‘bios’ which collectively mean ‘for life’, and hence they are widely regarded as beneficial microorganisms. 'Probiotic' was also defined by Fuller [7] as ‘a live microbial feed supplement which beneficially affects the host animal by improving microbial balance’ which is the most suitable definition for probiotics in cultured animals. Some microorganisms is now being used as probiotics for animal rising as well as aquaculture, such as *Saccharomyces*, *Debaryomyces*, *Bacillus*, *Lactococcus*, *Micrococcus*, *Enterococcus*, *Lactobacillus*, *Photorhodobacterium* and so on [8]. These probiotics are now garnering increasing scientific and commercial interest and are quite common in health-promoting functional feeds as well as therapeutic, prophylactic, and growth supplements [5,8–13].

Beneficial effects of the use of certain probiotics in humans, livestock, and poultry are well documented. The application of probiotics is also thought to bring positive effects in aquaculture. In marine fish aquaculture, probiotics were used for common snook, red drum, spotted sea trout, and striped mullet [14]. They observed that the application of probiotic bacteria to larval fish tanks (from egg through transformation) increased the survival, size uniformity, and growth rates, which might have been due to alterations of the microbial communities of both the environment and fish. Carnevali et al. [15] isolated *Lac. fructivorans* (AS17B) from the gut of seabass, *Sparus aurata*, and used live food, including *Brachinons plicatilis* and/or *Artemia salina,* and dry feed as vectors of probiotics for fish, and they found that the mortality of fish fry significantly improved. A *Bacillus* probiotic (BS11) used for tiger shrimp, *Penaeus monodon,* significantly increased its growth performance and survival rates [16]. In our previous works, the probiotic, *B. subtilis* [4,5] was able to increase the stress tolerance, growth

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Table 1
Ingredients of the experimental diets. C: Control; LW2: Lactobacillus fermentum; BD6: Lac. pentosus, P13: *Saccharomyces cerevisiae*; E20: Bacillus subtilis; MD7, MD8 and MD9 mean the diet contained the mixture of probiotics at levels of 10^7, 10^8 and 10^9 cfu (kg diet)$^{-1}$ diet, respectively.

Ingredients	Probiotic diets (g (kg diet)$^{-1}$)							
	CD	LW2D	BD6D	P13D	E20D	MD7	MD8	MD9
Fish meal (from Peru)	425	425	425	425	425	425	425	425
Soybean meal	200	200	200	200	200	200	200	200
Shrimp meal	50	50	50	50	50	50	50	50
α-starch	230	230	230	230	230	230	230	230
Fish oil	31	31	31	31	31	31	31	31
Vitamin premix	20	20	20	20	20	20	20	20
Mineral premix	40	40	40	40	40	40	40	40
Skim milk	4	3	3	3	3	3.96	3.6	0
LW2	0	1	0	0	0	0.01	0.1	1
BD6	0	0	1	0	0	0.01	0.1	1
P13	0	0	0	1	0	0.01	0.1	1
E20	0	0	0	0	1	0.01	0.1	1
Proximate composition (% dry matter)								
Crude protein	36.5 ± 0.21	36.5 ± 0.49	36.8 ± 0.04	36.6 ± 0.18	36.7 ± 0.36	36.9 ± 0.33	36.5 ± 0.07	36.5 ± 0.31
Crude lipid	7.1 ± 0.42	6.9 ± 0.12	7.2 ± 0.3	7.4 ± 0.31	7.5 ± 0.22	7.2 ± 0.34	7.3 ± 0.56	7.1 ± 0.36
Moisture	7.6 ± 0.03	6.8 ± 0.15	7.8 ± 0.34	7.5 ± 0.11	7.4 ± 0.2	6 ± 0.09	7.4 ± 0.06	5.6 ± 0.36
Ash	14.6 ± 0.03	14.7 ± 0.03	14.8 ± 0.02	14.8 ± 0.16	15.1 ± 0.04	14.5 ± 0.05	15 ± 0.03	14.6 ± 0.04

*Vitamin and mineral premix as given in Liu et al. (2009).

performance, and *Vibrio* resistance of white shrimp, *Litopenaeus vannamei*. A probiotic, *Lac. plantarum* improved the immune response and disease resistance of white shrimp [17]. However, the efficiency of a single strain of probiotic used for aquaculture animals sometimes exhibits great differences. In our previous studies, the improved growth performance and health status of white shrimp [4,5], and parrot fish, *Oplegnathus fasciatus* [18], were demonstrated when *B. subtilis* was used. However, the same probiotic used for mud crabs, *Scylla paramamosain,* can only enhance the health status but not the growth performance [19]. In addition, a dose difference also found in *B. subtilis* between parrot fish [18] and white shrimp [4,5].

Probiotic mixtures are considered to be more effective than a single probiotic strain in humans [20,21] as well as in aquaculture [22]. Evidence provided by a meta-analysis supported that multiple probiotics seemed to be the most effective way to prevent necrotizing enterocolitis (NEC) and reduced mortality in preterm infants of ≤34 weeks' gestation or those with a birth weight of ≤1500 g. In contrary to multiple probiotics, a single strain using *Lactobacillus* species had a border-line effect in reducing NEC, and single strains or *Bifidobacterium* species and *Saccharomyces boulardii* revealed no beneficial effects in terms of reducing NEC or mortality [21]. Aly et al. [3] reported that combined probiotics, including *B. subtilis* and *Lac. acidophilus,* for Nile tilapia, *Oreochromis niloticus*, produced superior growth performance and immune responses than did individual probiotics through oral administration. A study by Mohanty et al. [23] indicated that a mixture of bacteria and yeasts as probiotics resulted in better survival and higher body weight gain and nutrient utilization in catla, *Catla catla*, which was in accordance with results from Mohapatra et al. [24]. A synergistic effect of mixtures of probiotics containing *B. subtilis* E20, *Lac. pentosus* BD6, *S. cerevisiae* P13 and *Lac. fermentum* LW2 was able to improve both the growth performance, and immune response and disease resistance of Asian seabass, *Lates calcarifer,* against *Aeromonas hydrophila* [22].

As multiple strains of probiotics appear more effective than single strains of probiotics, we wanted to evaluate the effects of a probiotic mixture developed by Lin et al. [22] to see if it could also produce beneficial effects on the growth performance and health status of white shrimp. Also, comparisons of the probiotic efficiencies of mixed strains and single strains of probiotics were evaluated in this study.

2. Materials and methods

2.1. Animal and acclimation

White shrimp, *L. vannamei,* were obtained from a farm at the Department of Aquaculture, National Pingtung University of Science and Technology (Pingtung, Taiwan). Shrimp were acclimatized in a 10-ton cement tank with 5 tons of aerated brackish water (25‰) at 28 °C for 2 weeks before the trial. Shrimp were fed a commercial diet at 5% of their body weight twice a day.

2.2. Probiotic and diet preparation

Multiple strains of probiotics (*B. subtilis* E20, *Lac. pentosus* BD6, *Lac. fermentum* LW2, and *S. cerevisiae* P13) with synergistic effects on growth and health status improvement of Asian seabass were used in this study. *B. subtilis* E20 isolated from the human health food, natto, was considered as probiotic for shrimp aquaculture [5]. *Lac. pentosus* BD6, *Lac. fermentum* LW2, and *S. cerevisiae* P13 (gifts from Professor Chiu-Shia Chiu, the Department of Food Science of Technology, National Pingtung University of Science and Technology (NPUST)) were isolated respectively from pigeon feces, water of the Jingsi Lake located at NPUST, and fermented peaches. Baker's yeast, lactic acid bacteria, and *B. subtilis* E20 were respectively cultured in yeast malt (YM), de Man, Rogosa, and Sharpe (MRS), and nutrient broth (NB), according to Lin et al. [22], and then separated by centrifugation at 3000*g* for 10 min at 4 °C. The pellet was collected, mixed evenly with 20% skim milk, and stored at −80 °C. Frozen probiotic samples were dried in a freeze-dryer, and then probiotic powder was used to prepare the experimental diets.

Eight experimental diets were formulated in this study, including a control diet (CD) without probiotics, four experimental diets that contained a single strain of either *B. subtilis* E20, *Lac. pentosus* BD6, *Lac. fermentum* LW2, or *S. cerevisiae* P13, respectively denoted E20D, BD6D, LW2D, and P13D, and three diets with a mixture of the above probiotics at different levels of 10^7, 10^8 and 10^9 cfu (kg diet)$^{-1}$ (respectively denoted as MD7, MD8, and MD9) (Table 1). Formulations of the experimental diets were prepared according to Liu et al. [5]. The viability of the probiotic powders was analyzed prior to diet process. Diets were prepared based on the protein (37%) and lipid (7%) requirements of white shrimp as per the method of Liu et al. [5]. Briefly, Feed stuffs were ground up in a hammer mill to pass through a 60-mesh screen. For

the diet preparation, ingredients were thoroughly mixed with 4.5% fish oil and then adding 35% distilled water in a mixer until a stiff dough resulted. Dough was then passed through a food grinder with a die diameter of ~1 mm, and the resulting pellets were cut to ~2 mm in length and dried using an air blower at room temperature to a moisture level less than 10%. The dry pellets were preserved in plastic bins at 4 °C until use. Proximate compositions of the experimental diets were analyzed based on the methods of the Association of Official Analytical Chemists (AOAC) [25]. Crude protein was analyzed by the Kjeldahl procedure using the Kjeltec System from Tecator (Höganäs, Sweden). Crude lipids were determined using the method of Folch et al. [26]. A moisture analyzer (MX-50, A&D, Tokyo, Japan) was used for the analysis of moisture.

The viabilities of probiotics in the experimental diets were evaluated before growth trial and at the end of experiment by differencing bacterial morphologies on culture medium or by using the selective media. *B. subtilis* E20 were opaque, dull, circular with an irregular circumference, and cream-colored with a darker center on nutrient agar. De Man, Rogosa, and Sharpe (MRS) containing 0.5% $CaCO_3$ was used for the *Lactobacillus* counting. *Lactobacillus* colonies were enumerated by acid production which showed a clear zone surrounding [27]. However, the method cannot different *Lac. pentosus* BD6 and *Lac. fermentum* LW2 when they were incorporated in diets. They were counted together. For yeast counting, a yeast selective medium, Dichloran Rose Bengal Chloramphenicol (DRBC) agar was used. Yeast colonies appeared pink were counted due to the uptake of rose bengal [28]. The determining probiotic levels in the experimental diets were listed in Table 2. After 60 days of storage, the results clearly showed that the probiotics were quite stable in the experimental diet, which might be due to the storage of the experimental diets at 4 °C.

2.3. Experimental design and animal rearing

In order to compare the efficiencies of single strains and multiple probiotics to improve the growth performance and health status of shrimp, a control diet (CD), four experimental diets containing a single bacterium (E20D, BD6D, LW2D, and P13D) and three experimental diets containing multiple probiotics (MD7, MD8, and MD9) at different levels were fed to shrimp juveniles (with an initial weight of 0.21 ± 0.01 g) for 56 days. Before the experiment, shrimp at intermolt stage (C stage) were randomly assigned to cement tanks (1.2 × 1.9 × 1.2 m) that contained 50 shrimp each. Each treatment was carried out in triplicate. Shrimp were fed twice daily at a rate of 3%–5% of their body weight. Feed supplements were adjusted based on the mean weights of the shrimp, which were measured once per 2 weeks. Uneaten feed were collected after 1 h of feeding, and faecal matters were segregated from uneaten feed samples by using a dropping pipette. Then, uneaten feed was dried in an oven at 80 °C for the calculation of feed consumption by subtracting the uneaten portion from the amount fed, and recorded daily. Shrimp weights were measured at the beginning of the experiment and at 2-week intervals until the end of the experiment. During the trial, the water temperature, dissolved oxygen (DO), and pH were measured daily using a thermometer, DO meter, and pH meter, respectively. Ammonia-N and nitrite-N were measured every 2 weeks. After the growth trial, shrimp were harvested and individually weighed, and then body composition, and health status were evaluated. The feed efficiency, percent weight gain and survival were calculated as follows:

Feed efficiency = weight gain / total feed intake;

Percentage weight gain (%) = ((final weight - initial weight)/initial weight) × 100; and

Survival (%) = (final number of animals / initial number of animals) × 100.

Table 2
The viability of probiotic in the experimental diets at the beginning and after growth trial. LW2: *Lactobacillus fermentum*; BD6: *Lac. pentosus*, P13: *Saccharomyces cerevisiae*; E20: *Bacillus subtilis*; MD7, MD8 and MD9 mean the diet contained the mixture of probiotics at levels of 10^7, 10^8 and 10^9 cfu (kg diet)$^{-1}$ diet, respectively.

Experimental diets	Probiotic levels (cfu (kg diet^{-1})) in the experimental diets during the growth trial	
	Initial	The end of experiment
LW2D	0.9×10^9	0.7×10^9
BD6D	4.1×10^9	0.9×10^9
P13D	1.6×10^9	1.1×10^9
E20D	1.9×10^9	0.9×10^9
MD7: LW2 + BD6	4.7×10^7	1.3×10^7
P13	0.8×10^7	0.3×10^7
E20	0.6×10^7	0.1×10^7
MD8: LW2 + BD6	2.4×10^8	1.4×10^8
P13	0.9×10^8	0.7×10^8
E20	0.8×10^8	0.9×10^8
MD9: LW2 + BD6	4.9×10^9	1.3×10^9
P13	3×10^9	0.3×10^9
E20	2.9×10^9	1.1×10^9

2.4. Measurement of shrimp body composition

After termination, six shrimp at the intermolt stage (C stage) from each group were euthanized on ice and then used for an analysis of proximate carcass composition. The proximate composition analysis were carried out according to standard methods of the AOAC [25] as with the above description of the composition analysis of the experimental diets. The moult stages of shrimp was characterized based on the method of Chan et al. [29].

2.5. Analysis of immune response

After the growth trial, the immune responses of the shrimp, including total hemocyte count (THC), phenoloxidase (PO) activity, respiratory bursts (RBs), superoxide dismutase (SOD) activity, lysozyme (LYZ) activity, and phagocytosis of hemocytes were evaluated. For immune response analysis, 12 shrimp from each group were used. Six of the selected shrimp were used to measure THC, and PO, RB, SOD, and LYZ activities of hemocytes, and the other six shrimp were used for the phagocytosis analysis. Only shrimp at the intermolt stage (C stage) were used.

One hundred microliter of freshly withdrawn hemolymph from shrimp were mixed with 900 µl of an anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, and 10 mM EDTA, at pH 7.55, with the osmolality adjusted with glucose to 780 mOsm kg^{-1}).

A hemocytometer was used for THC determination. Briefly, a drop of the anticoagulant-hemolymph mixture was placed on a hemocytometer to measure the THC using an inverted phase-contrast microscope (Leica DMIL, Leica Microsystems, Wetzlar, Germany). Each sample was measure for two times.

The detection of PO activity in hemocyte was carried out kinetically using a spectrophotometer by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) [30]. One ml of anticoagulant-hemolymph mixture was centrifuged at 300 × *g* and 4 °C for 10 min, and then the supernatant fluid was discarded. The pellet was rinsed and gently re-suspended in 1 ml cacodylate-citrate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, and 0.10 M trisodium citrate; pH 7.0), and centrifuged again. Thereafter, the pellet was re-suspended in 200 µl cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.01 M calcium chloride, and 0.26 M magnesium chloride; pH 7.0). The cell suspension, 100 µl, was incubated with 50 µl trypsin for 10 min at 25 °C, and 50 µl of L-DOPA was added,

followed by 800 µl of cacodylate buffer 5 min later. Absorption at 490 nm was measured immediately recorded by using a UV–Vis spectrophotometer V-630 (Jasco, Tokyo, Japan). One hundred µl cell suspension in the case of control incubated with 50 µl cacodylate buffer instead of trypsin, and 50 µl of L-DOPA was used for the background PO activity in all test conditions.

The analysis of RBs in hemocytes were performed using the reduction of nitro blue tetrazolium (NBT) to formazan as a measure of superoxide anion (O_2^-) production [31]. One hundred diluted hemolymph solutions were added into a 96-well plate previously coated with 100 µl of a poly-lysine solution (0.2%) to improve cell adhesion. Microplates were then centrifuged at 300 × *g* for 15 min. Plasma was removed, and then hemocyte was inclubation with 100 µl of zymogen solution and allowed to react for 30 min at room temperature. The suspension was discarded, and hemocytes were washed three times with 100 µl of Hank's solution, and then 100 µl of the NBT solution (0.3%) in the appropriate medium was immediately distributed to each well. Following 30 min incubation, NBT solution was removed and hemocytes were fixed by 200 µl absolute methanol, washed three times with 70% methanol, then dried. The formazan deposits were solubilized by the addition of 120 µl of 2 M KOH and 140 µl of DMSO. The OD at 630 nm was measured in triplicate using a microplate spectrophotometer (Spectramax® 190, Sunnyvale, CA, USA).

One hundred microliter diluted hemolymph solutions were centrifuged at 300 × *g* and 4 °C for 30 min to collect hemocytes, and then the pellet was washed twice with phosphate-buffered saline (PBS, pH 7.5). Thereafter, the pellet was homogenized in PBS, and centrifuged at 10^4 × *g* for 30 min at 4 °C. The supernatant (HLS) was transferred to a new tube previously placed on ice and used immediately for the SOD and LYZ analysis.

SOD activity was measured by its ability to inhibit superoxide radical-dependent reactions using a Ransod kit. (Randox, Crumlin, UK). The OD was measured at 505 nm and 37 °C, and the rate of the reaction was estimated from the absorbance readings 0.5 and 3 min after adding xanthine oxidase. The units of SOD activity were calculated from a standard curve of the reference SOD standard supplied with the Ransod kit. The specific activity was expressed as units (mg protein)$^{-1}$. The concentration of protein in the HLS was analyzed by the Bradford method [32] using bovine serum albumin as a standard and the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Mississauga, ON, Canada).

Hemocyte LYZ activity was analyzed by a modification of turbidimetric method [33]. Two hundred microliter of *Micrococcus lysodeikticus* (Sigma, St. Louis, MO, USA) (0.02% w/v) in 0.05 M sodium phosphate buffer (pH 6.2) was mixed with 10 µl of HLS. Next, the mixture was incubated at 25 °C, and the optical density (OD) was detected after 1 and 6 min at 530 nm using a UV–Vis spectrophotometer V-630 (Jasco). The units of LYZ activity were calculated from a standard curve of LYZ from chicken egg white (L6876, Sigma). The concentration of protein in the HLS was analyzed by the Bradford method as described above.

The procedure of phagocytic activity was described previously [31]. Twenty microliters of fluorescent latex beads (5 × 10^6 fluorescent latex beads in 1 ml phosphate-buffered saline (PBS) at pH 7.5) resulting in 10^5 beads shrimp^{-1} was injected into the ventral sinus. After being injected, the shrimp were kept in separate tanks containing 40 L of seawater (25‰) for 2 h at 27 ± 1 °C. Hemolymph (100 µl) from six shrimp, which was withdrawn similarly to that described above, was mixed with 900 µL of an anticoagulant solution. One hundred microliters of a diluted hemolymph sample was mixed with 100 µL of 0.1% paraformaldehyde for 30 min at 4 °C to fix the hemocytes. Then, 50 µL of the suspension was spread on a glass slide. The slide was placed in a cytospin centrifuge and centrifuged at 113 × *g* for 3 min. The slide was then air-dried, stained with 0.1% propidium iodide for 10 min, and observed using a fluorescence microscope (Leica DM2500, Leica Microsystems, Wetzlar, Germany). Two hundred hemocytes were counted. Phagocytic activity (PA) was expressed as:

PA (%) = (Phagocytic hemocytes / Total hemocytes) × 100

2.6. Challenge test

Vibrio alginolyticus was isolated from diseased shrimp on a private farm in Pingtung, Taiwan [34]. The bacteria used for the challenge test were grown in tryptic soy broth (TSB, Difco) supplemented with 2% NaCl (TNB) for 24 h at 28 °C. Bacteria were collected by centrifugation at 7000 × *g* for 15 min at 4 °C, and then the pellet was resuspended in 0.85% NaCl and adjusted to 10^7 cfu ml^{-1} as a stock solution by measuring the optical density (OD) at 595 nm (OD of 1.0 = 10^9 cfu ml^{-1}). Thirty shrimp (mean weights: 1.43 ± 0.51) from each group were injected with 10 µl of a bacterial solution into the ventral sinus resulting in 10^5 cfu (g shrimp)$^{-1}$. Shrimp from the control group were injected with 10 µl of 0.85% NaCl and served as the unchallenged control. Each treatment was carried out in triplicate. Each replicate consisted of 10 shrimp. After the injection, shrimp were held in a separate 60-L glass aquarium with 40 L of seawater (25‰) and aeration. The experiments were carried out for 7 days.

2.7. Statistical analysis

Statistical analysis of the data was performed using SAS software (Cary, NC, USA). Homogeneity of variance and normality were tested using Levene's test and Shapiro-Wilk's test, respectively, prior to analysis of variance (ANOVA). Data, including survival, percentage of weight gain, body compositions, phagocytic activity, and challenge test were square-root arcsine-transformed before ANOVA. Difference between data were statistically analyzed by a one-way ANOVA. Post hoc analysis among groups after finding significant differences were carried out by Duncan's multiple-range test. The level of significance was accepted at $p < 0.05$.

3. Results

3.1. Growth performance

The growth performance of shrimp fed the experimental diets for 56 days is shown in Table 2. Survival ranged from 90% to 98.9% and did not significantly differ among the control and various groups. The shrimp's growth rate was significantly affected by dietary probiotics, especially the mixed probiotics (Fig. 1). A lower level of the mixed probiotics (10^8 cfu (kg diet)$^{-1}$) and shrimp fed the single probiotic at 10^9 cfu (kg diet)$^{-1}$ significantly promoted shrimp growth. Significantly increased body weight (Fig. 1) and percentage weight gain (Table 2) of

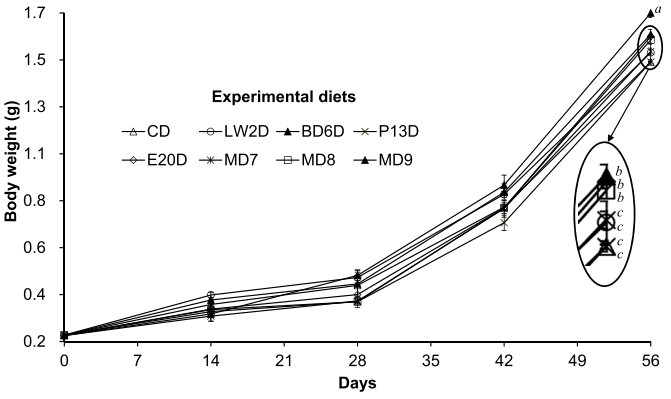

Fig. 1. Changes in body weight of white shrimp fed the experimental diets for 56 days. Data (mean ± SD) with different letters significantly differ among treatments at the end of the growth trial ($p < 0.05$). Data are mean from three replicates of shrimp (n = 3).

Table 3
Growth response of white shrimp fed the experimental diets for 56 days. PWG, percentage weight gain. Values are mean from three replicates of shrimp (n = 3). Within rows, values followed by different letters are significantly different ($p < 0.05$, One-way ANOVA, Duncan's multiple comparisons test).

Parameters	Probiotic diets							
	CD	LW2D	BD6D	P13D	E20D	MD7	MD8	MD9
Survival (%)	97.8 ± 1.36	94.4 ± 3.6	93 ± 4.08	92 ± 5.93	94.4 ± 2.72	90 ± 4.71	98.9 ± 1.36	93.3 ± 2.36
Feed efficiency	0.55 ± 0.01^{c}	0.57 ± 0.01^{bc}	0.6 ± 0.02^{ab}	0.55 ± 0.04^{c}	0.61 ± 0.02^{ab}	0.55 ± 0.03^{c}	0.6 ± 0.01^{ab}	0.65 ± 0.02^{a}
PWG (%)	626.9 ± 1.6^{c}	637.5 ± 19.3^{c}	671.1 ± 10.9^{b}	617.6 ± 4.8^{c}	669.1 ± 1.9^{b}	640.1 ± 7.8^{c}	666.1 ± 6.9^{b}	726.8 ± 10.4^{a}

Table 4
Body composition (% in wet weight) of white shrimp fed different experimental diets in this experiment. The results represent means (± S.D.) of 6 shrimp/treatment. There was no significant different among treatment means ($p > 0.05$).

Body composition (% in wet weight)	Probiotic diets							
	CD	LW2D	BD6D	P13D	E20D	MD7	MD8	MD9
Moisture	76.65 ± 0.39	75.66 ± 1.23	76.68 ± 0.82	75.98 ± 0.29	76.13 ± 0.81	77.01 ± 1.09	76.19 ± 0.68	76.28 ± 0.62
Crude protein	19.22 ± 0.33	19.68 ± 0.85	20.06 ± 0.47	19.68 ± 0.66	19.38 ± 0.91	19.49 ± 0.66	18.89 ± 0.81	20.01 ± 0.73
Crude lipid	0.84 ± 0.03	0.79 ± 0.04	0.79 ± 0.09	0.74 ± 0.04	0.79 ± 0.08	0.81 ± 0.06	0.81 ± 0.09	0.76 ± 0.06
Ash	1.62 ± 0.06	1.65 ± 0.15	1.59 ± 0.21	1.67 ± 0.11	1.62 ± 0.03	1.58 ± 0.07	1.61 ± 0.09	1.65 ± 0.11

shrimp were recorded in the groups of single (BD6D and E20D) and mixed probiotics (MD8 and MD9). The better growth rates of shrimp in the BD6D, E20D, MD8, and MD9 groups resulted from significantly improved feed efficiencies (Table 3).

There were no significant differences in body composition of shrimp among the control and various treatment groups. Moisture, crude protein, crude lipids, and ash in shrimp bodies ranged 75.66%–77.01%, 18.89%–20.06%, 0.74%–0.84%, and 1.58%–1.67%, respectively (Table 4).

3.2. Immune response

The health status of shrimp significantly improved with dietary probiotics, and the mixed probiotics seemed to more effectively promote the immune response of shrimp. No significant differences in the THC of shrimp were recorded among the control and various treatment groups (Fig. 2A), but PO activities of shrimp had significantly increased in groups of single probiotics at a level of 10^{9} cfu (kg diet)$^{-1}$ (BD6D and E20D), and the multiple probiotic strains at the levels of 10^{8} cfu (kg diet)$^{-1}$ (MD8) and 10^{9} cfu (kg diet)$^{-1}$ (MD9) (Fig. 2B). Similarly, results of RBs also revealed the better probiotic efficiency in groups of mixed probiotics in which RBs significantly increased in all groups of the probiotic mixture, whereas no change in RBs was recorded among the CD and all groups of single probiotics (Fig. 3A). Regardless of whether shrimp were fed probiotic diets containing single or multiple strains of probiotic, no significant changes in SOD activity (Fig. 3B) or phagocytic activity (Fig. 4B) were detected, but significant increases in LYZ activities in the groups fed probiotic-containing diets were seen except for the P13D group (Fig. 4A).

3.3. Challenge test

No dead shrimp were recorded in the unchallenged control during the challenge test. Dead shrimp in all groups were first recorded at 12 h after pathogen injection. Shrimp in the control group had the highest cumulative mortality, and the cumulative mortality in the CD group was significantly higher than those of groups fed the probiotics except for group P13D at 24 h after pathogen injection. At the end of the challenge trial, cumulative mortalities of shrimp after pathogen injection did not significantly differ between the control and P13D groups, but the cumulative mortality of shrimp in the CD group was significantly higher than those of the LW2D, BD6D, and E20D groups, and all groups of the probiotic mixture (Fig. 5). The cumulative mortalities

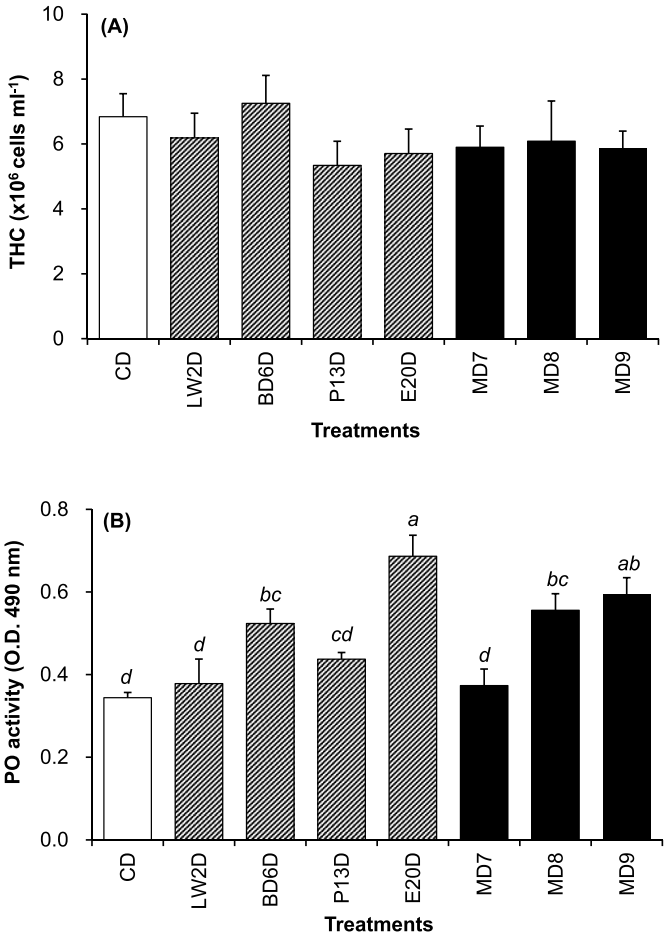

Fig. 2. Total hemocyte count (THC) (A) and phenoloxidase (PO) activity (B) of white shrimp fed the experimental diets for 56 days. Data (mean ± SD) with different letters significantly differ among treatments ($p < 0.05$). The results represent means (± S.D.) of 6 shrimp/treatment.

of shrimp in the CD, LW2D, BD6D, P13D, E20D, MD7, MD8, and MD9 groups were 73.3% ± 4.1%, 40%, 407% ± 12.2%, 53.3% ± 8.2%, 43.3% ± 10.8%, 47% ± 10.8%, 40% ± 7.1%, and 50% ± 7.1%, respectively, at the end of the trial.

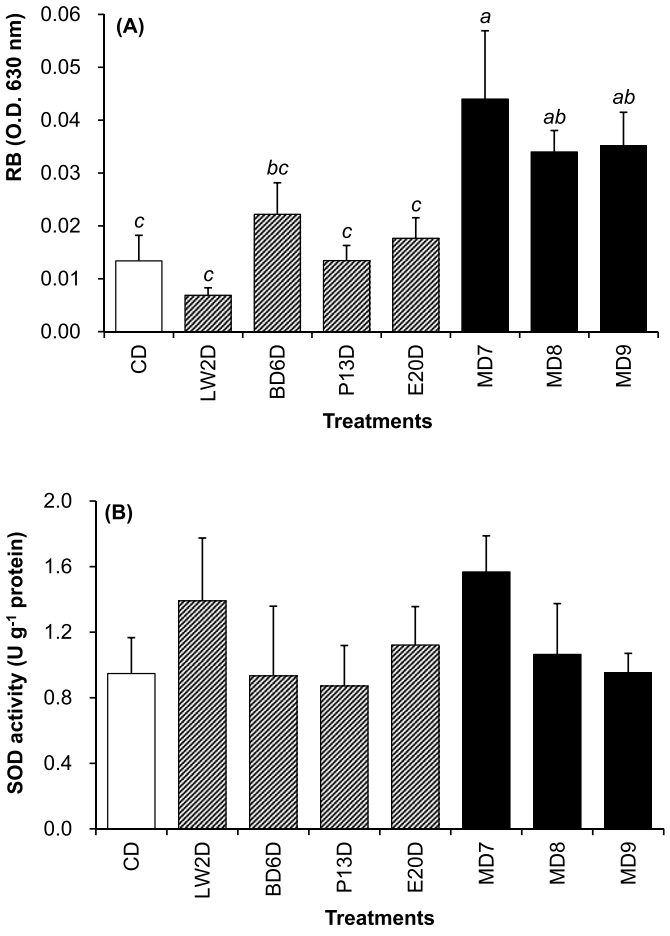

Fig. 3. Respiratory bursts (RBs) (A) and superoxide dismutase (SOD) activity (B) of white shrimp fed the experimental diets for 56 days. Data (mean ± SD) with different letters significantly differ among treatments ($p < 0.05$). The results represent means (± S.D.) of 6 shrimp/treatment.

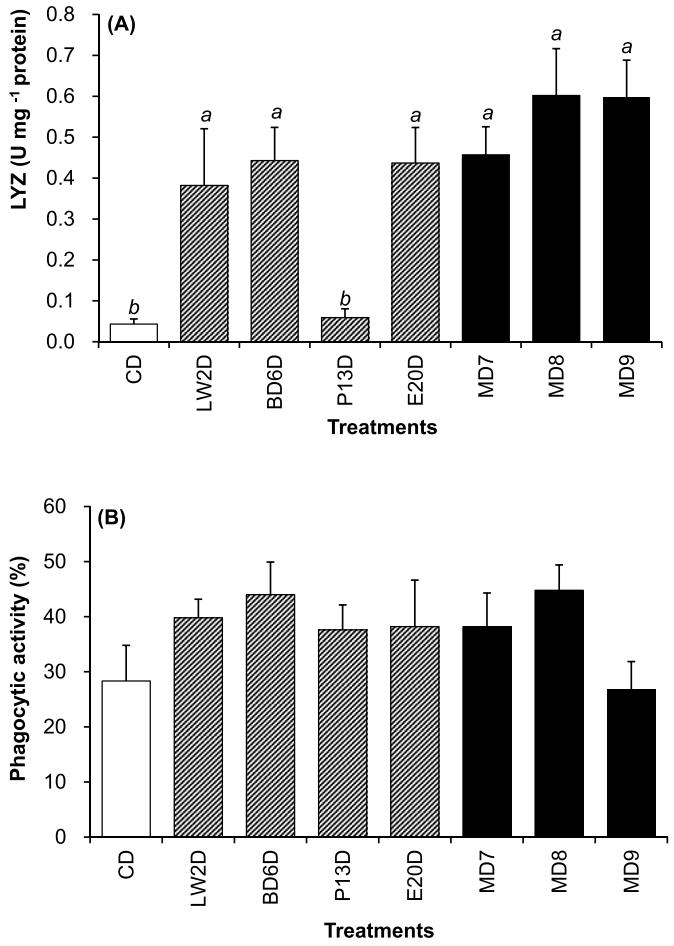

Fig. 4. Lysozyme activity (LYZ) (A) and phagocytic activity (B) of white shrimp fed the experimental diets for 56 days. Data (mean ± SD) with different letters significantly differ among treatments ($p < 0.05$). The results represent means (± S.D.) of 6 shrimp/treatment.

4. Discussions

Probiotics are suggested to be a new tool in disease control, growth promotion, and water quality improvement in aquaculture. Probiotics have become fashionable in the worldwide market as dietary supplements [35]. The positive effects of probiotics include nutrient contribution, survival improvement, growth promotion, and increases in host immune responses and disease resistance against pathogens [2,36]. The effective concentration of a single probiotic in the diet for aquatic animals to improve their health status and growth performance was usually $10^8 \sim 10^{10}$ cfu (kg diet)$^{-1}$ or even higher. Zheng and Wang [37] used *Lac. pentosus* AS13 as a probiotic at a level of 10^7 cfu g^{-1} for white shrimp, and it effectively enhanced the growth performance, feed utilization, digestive enzymes, and disease resistance of white shrimp. Observations of our previous study were that significantly better growth performance [5], immune responses, and disease resistance of white shrimp against *V. alginolyticus* [38] were achieved with the dietary probiotic, *B. subtilis,* at a suggested level of $> 10^8$ cfu (kg diet)$^{-1}$. Zokaeifar et al. [39] suggested that *B. subtilis* L10 and G1 at 10^8 cfu g^{-1} were able to significantly improve the growth performance and disease resistance against *V. harveyi* through an enhanced immune response in white shrimp. In our previous study, *B. subtilis* E20 and *Lac. pentosus* BD6, and *S. cerevisiae* P13 and *Lac. fermentum* LW2 respectively improved either the growth performance or disease resistance of Asian seabass, *Lates calcarifer,* as individual probiotics incorporated into the diet at a level of 10^9 cfu (kg diet)$^{-1}$ [22]. Therefore, the experimental design of the concentration of a single probiotic in the diet was 10^9 cfu (kg diet)$^{-1}$ in this study.

Most studies on probiotics applied in aquaculture used single probiotic strains. Less is known about the effect of mixtures of probiotics, especially if multiple strains of probiotics result in additive or even synergistic effects, or reduced effects due to mutual inhibition. In our previous study, diets incorporating individual or combined species of the probiotics, *B. subtilis* E20 and *Lac. plantarum* 7–40, were fed to the mud crab, *Scylla paramamosian* for 28 days, and results indicated that a reduced efficiency of combined species of probiotics was found due to an antagonistic effect of growth between two probiotics [19]. Therefore, it is considered that the effects of interactions between probiotics and their unique and specific biological effects must be assayed before being used together. In addition, knowledge of the specific effects of each probiotic strain will allow the development of probiotic mixtures adapted to particular cases or pathogens. In many previous studies, multiple strains of probiotic were considered more beneficial for animal growth than a single probiotic, such as a multi-strain probiotic containing *B. subtilis*, *E. faecium*, *Pediococus acidilactici,* and *Lac. reuteris* was developed for rainbow trout, *Oncorhynchus mykiss,* to improve the growth performance and antioxidant activity [40]. A product of multiple probiotic, PrimaLac consisted of *Lac. acidophilus*, *Lac. casei*, *Enterococcus faecium,* and *Bifidobacterium bifidium*) was able to significantly enhanced broiler performance by improving body weight and decreasing the feed conversion ratio [41]. In order to develop a mixture of probiotics with synergistic efficiency, six bacteria and one yeast were screened as probiotic candidates for a mixture of probiotics to enhance the growth performance and health status of Asian seabass. Results

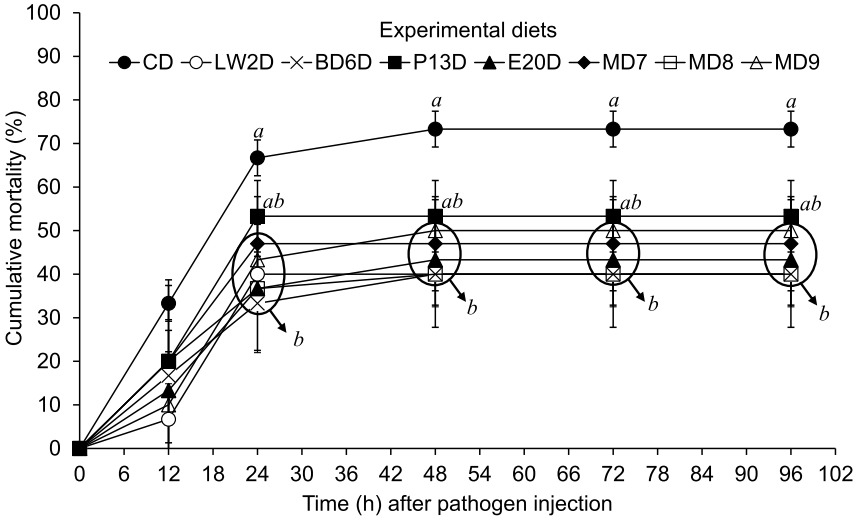

Fig. 5. Cumulative mortality of white shrimp fed the experimental diets for 56 days and then challenged with the pathogen *Vibrio alginolyticus*. Data (mean ± SD) with different letters significantly differ among treatments ($p < 0.05$). Data are mean from three replicates of fish (n = 3).

clearly showed that *B. subtilis* E20 and *Lac. pentosus* BD6, and *S. cerevisiae* P13 and *Lac. fermentum* LW2, respectively improved either the growth performance or disease resistance of Asian seabass, and synergistic effects of the probiotics were found as the four probiotics were mixed to prepare a probiotic mixture for Asian seabass. In this study, a probiotic mixture from our previous study [22] was also used to feed white shrimp for 56 days, and the results indicated that the growth performance of shrimp significantly improved. The beneficial effects of the probiotic mixture were also better than single probiotics, and the effective concentration of the probiotic mixture for growth performance of white shrimp was 10^8 cfu (kg diet)$^{-1}$ in this study, but the single probiotics (*Lac. pentosus* BD6, *S. cerevisiae* P13 and *Lac. fermentum* LW2) added to the diet at this lower level did not improve the growth performance of white shrimp in a preliminary study (data not show).

Data of the carcass composition in this study showed no significant differences in crude protein, crude lipid, ash, or moisture of shrimp after the oral administration of the probiotic mixture in this study. The findings in the present study are in agreement with those of Allameh et al. [42] in which Javanese carp, *Puntius gonionotus,* fed single- and multiprobiotic strains of *E. faecalis*, *Lac. fermentum,* and *Leuconostoc mesenteroides* had no significant change in the carcass composition of fish. In addition, no change in the carcass composition of shrimp after being fed with probiotics indicates that the probiotic did not influence the shrimp's nutritional value.

In addition to improvements in growth performance, white shrimp fed a diet containing a mixture of probiotics at 10^7 cfu (kg diet)$^{-1}$ had better disease resistance against *V. alginolyticus,* compared to the single probiotic in this study. Similar results of improved disease resistance were also recorded in Asian seabass fed the same probiotic mixture [22]. Probiotics are recognized as being alternatives to antibiotics and chemicals and function as alarm molecules to activate the immune system [20,38]. Many studies have used probiotics to study the feasibility of disease prevention in animals. Chang et al. [21] found that multiple strains of probiotics appeared to be the most feasible and effective strategy for preventing NEC and reducing mortality in preterm very-low-birth-weight neonates. Lema et al. [43] investigated the effects of probiotics on *Escherichia coli* O157:H7 infections in lambs and cattle, and the results found that a five-strain probiotic mixture including *Lac. acidophilus*, *Streptococcus faecium*, *Lac. casei*, *Lac. fermentum,* and *Lac. plantarum* led to an increase in shedding of *E. coli* O157:H7 compared to either a two-strain mixture (*Lac. acidophilus* and *S. faecium*) or two of the component strains used individually. In aquaculture, Giri and Oviya [44] reported that rohu, *Labeo rohita*, fed an effective dose (10^8 cfu (g diet)$^{-1}$) of the probiotic, *Lac. plantarum* VSG3, had a significantly better immune response and post-challenge survival after an *A. hydrophila* infection. Similarly, oral administration of the heat-inactivated probiotics, *Lac. delbrüeckii* spp. *lactis* and *B. subtilis,* had stimulatory effects, both local and systemic, on the immune system of the gilthead seabream, *Sparus aurata*, and the multi-strain formulation was more effective than any of the single-bacterium experimental diets [45]. Zheng and Wang [37] indicated that feeding supplemented diets containing *Lac. pentosus* AS13 significantly reduced the mortality rate of white shrimp caused by pathogenic *Vibrio* species (*V. vulnificus*, *V. rotiferianus,* and *V. campbellii*). Vieira et al. [46] also found that the survival of white shrimp significantly increased after a *V. harveyi* infection when white shrimp were fed the probiotic, *Lac. plantarum*.

Lin et al. [22] indicated that probiotics play an important role in stimulating Asian bass immune responses, including RBs, SOD activity, phagocytic activity of leucocytes, and a viral defender, Mx gene expression, after they fed Asian seabass juveniles a diet containing a probiotic mixture for 56 days. Panigrahi et al. [47] demonstrated that dietary *Lac. rhamnosus* JCM 1136 in rainbow trout enhanced superoxide anion production for up to 20 days of feeding. Rengpipat et al. [48] mentioned that the use of *Bacillus* sp. (strain S11) provided disease protection by activating both cellular and humoral immune defenses in the tiger shrimp, *Penaeus monodon*. A mixture of probiotics composed of *Bacillus* and *Vibrio* sp. positively influenced the growth and survival of juvenile white shrimp and presented a protective effect for the immune system, by increasing phagocytosis and antibacterial activity [49]. In this study, treated shrimp also exhibited significant upregulation of immunity after dietary probiotic supplementation, except with P13D. It is thought that the improvement in the disease resistance of shrimp by probiotics is also due to the immune-regulation after dietary administration.

In the market, there is increasing tendency to work with multiple probiotic because many bacteria have symbiotic relationships with each other, such the adhesion of *Bifidobacterium lactis* Bb12 to a mucus model was more than doubled when coupled with either *Lac.* GG or *Lac. bulgaricus* [50]. It is well known that the ability of adhere to mucosal surfaces is related to many probiotic health effects. So, the increased ability of adhesion of *B. lactis* coupled with *Lactobacillus* indicated more health effects to host than *B. lactis* was used alone. In addition, in cases where probiotics have some inhibitory effects on each other [19,51], multiple probiotic are still considered more effective at dealing with pathogens than single-strain probiotic [51,52]. Chapman et al. [51]

reported that probiotic mixture was statistically more effective than single strains on pathogen inhibition, and the results was similar to the observation of Lin et al. [53]. This is considered that multi-species probiotic can have a greater inhibitory effect on pathogen than single strains included in that mixture. In the future, the ability of adhesion of multiple probiotic and single probiotic in intestine of shrimp, and their abilities of pathogen inhibition should be analyzed to clarify the possible reason why multiple probiotic used in this study have better probiotic efficiencies than single probiotic.

In conclusion, oral administration of a probiotic mixture containing E20, BD6, LW2, and P13 was able to improve the shrimp's growth performance and health status, and the probiotic efficiency of the probiotic mixture was significantly greater than those of single probiotics in the diet. Based on the results of shrimp growth and health status measurements, the recommended concentration is 10^8 cfu (kg diet)$^{-1}$.

Acknowledgments

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