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# Effects of *Musa* (ABB group) prebiotic and *Bacillus subtilis* S11 probiotic on growth and disease resistance of cultivated Pacific white shrimp (*Litopenaeus vannamei*)

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**Abstract:** Extract of ripe banana (BN) with a prebiotic activity score of 1.77 were demonstrated to support the growth of probiotic *Bacillus subtilis* S11 (BS11). BN and BS11, separate and combined, were then fed to the Pacific white shrimp (*Litopenaeus vannamei*) over 90 days in the rainy season and in the dry (hot) season. In both seasons, the highest production was found in shrimp fed with BS11 plus 10% BN. After challenging with *Vibrio harveyi* 639, the lowest cumulative mortality was found in shrimp fed with BS11 and 10% BN in the rainy season, indicating a higher shrimp production following the synbiotic use of the BS11 probiotic combined with the BN prebiotic.

**Keywords:** Pacific white shrimp, *Litopenaeus vannamei*, probiotic, *Bacillus subtilis* S11, prebiotic, *Musa* (ABB group), banana

## INTRODUCTION

The farming of Pacific white shrimp (*Litopenaeus vannamei*) has recently become a major economic industry in Thailand. However, from 2013 onwards the industry has faced a severe blow due to outbreaks of acute hepatopancreatic necrosis disease [1]. *Bacillus subtilis* S11 (BS11) has been proposed as a probiotic bacterium for the black tiger shrimp (*Penaeus monodon*) [2, 3] and also for the Pacific white shrimp [4] due to the bacterium's ability to survive the passage through the gastrointestinal tract of these shrimp and enhance the growth and defense against pathogens including *Vibrio harveyi.*

The inclusion of inulin-derived fructo-oligosaccharide (FOS) into regular animal feed has also been shown to have a broad antibacterial effect against such pathogens as *Escherichia coli*, *Salmonella* spp., *Clostridium* spp., enterobacteria, eubacteria and coliforms [5]. Inulin and

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oligofructose have been reported to occur in several fruits including the banana, in which inulin content of 0.3-0.7% of fresh weight were reported [6]. Conversion of inulin to oligofructose normally occurs through controlled partial enzymatic hydrolysis which resembles that of the processing of chicory roots [7]. The most popular banana cultivar in Thailand, *Musa* (ABB group) or Kluai Namwa in Thai, is a hybrid of *Musa acuminata* Colla and *M. balbisiana* Colla [8]. Both of the parental plants typically contain a considerable amount of dietary fibre [8], which has been claimed to have a prebiotic activity that promotes the growth of bacteria within the small intestine leading to the colon and provides potential health benefits [9,10].

In this study the use of a probiotic coupled with a prebiotic, an approach known as synbiotics, is undertaken in order to evaluate the effects of the BS11 probiotic and *Musa* (ABB group) prebiotic supplemented to the regular feed on the growth and disease resistance of the Pacific white shrimp

## MATERIALS AND METHODS

### Bacterial Strains

BS11 was isolated from the gastrointestinal tract of *Penaeus monodon* caught from the Gulf of Thailand as previously described [2, 3] and shown to be a good probiotic for *Litopenaeus vannamei* [4]. *Vibrio harveyi* 639, a pathogenic bacterium, also isolated from *P. monodon* that had died from luminescent disease, was kindly provided by the Shrimp Culture Research Centre, Charoen Pokphand Feedmill, Samutsakorn, Thailand. The bacterium was cultured in tryptic soya agar (TSA), tryptic soya broth (TSB) with 1.5% (w/v) agar supplemented with 2% (w/v) NaCl, or in thiosulphate-citrate-bile-salt-sucrose agar (TCBS) and was used for inducing *V. harveyi* infection in the shrimp. All culture media were purchased from Difco (USA).

*Escherichia coli* ATCC 25922 and *Bifidobacterium animalis* subsp. *lactis* BB12 were provided by the Department of Microbiology and the Department of Food Science, Faculty of Science, Chulalongkorn University respectively. Lactic acid bacteria (LAB), namely *Lactobacillus (Lb.) acidophilus* IAM 10074, *Lb. casei* subsp. *casei* IAM 1045, *Lb. fermentum* IAM 1148, *Lactococcus (Lc.) lactis* subsp. *cremoris* IAM 1150 and *Lc. lactis* subsp. *lactis* IAM 1198 were obtained from Riken Bioresource Centre, Japan.

### Preparation of *Musa* (ABB group) Extract

The ripe (yellow skin) fruits of *Musa* (ABB group) were peeled and sliced. The sliced flesh (10 g) was homogenised in 100 mL of hot water (100ºC) and left to stand for 10 min. Suspended particles were removed by centrifugation at 10160 *g* and 4ºC for 10 min., and the supernatant was harvested [11] and used as the ripe banana extract (BN) in subsequent experiments. Larger scales of BN preparation was performed as desired and kept at 4°C before use.

### Prebiotic Activity Score

Prebiotic activity score (PAS) was determined as previously reported [12]. In brief, an overnight culture of *B. animalis* subsp. *lactis* BB12, *Lb. acidophilus* IAM 10074, *Lb. casei* subsp. *casei* IAM 1045, *Lb. fermentum* IAM 1148, *Lc. lactis* subsp. *cremoris* IAM 1150 and *Lc. lactis* subsp. *lactis* IAM 1198 were transferred at 1% (v/v) in triplicate into individual tubes containing manually prepared lactobacillus de Man, Rogosa and Sharpe broth without dextrose (mMRS-D) (10 mL) supplemented with either 1% (w/v) glucose, 1% (w/v) inulin, or 1% (w/v) BN. The tubes were then incubated at 37ºC under an anaerobic condition in candle jars [13]. An overnight culture of

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either BS11 or *E. coli* ATCC 25922 (0.1 mL) was transferred in triplicate into culture tubes containing manually prepared TSB without dextrose (mTSB-D) (10 mL) and also into the same broth but with either 1% (w/v) glucose, 1% (w/v) inulin or 1% (w/v) BN and incubated aerobically at 37°C [13]. After 24 hr of incubation, samples were harvested and enumerated on MRS agar (Difco, USA) for LAB and on TSA for BS11 and *E. coli*. All chemicals for manually prepared culture media were obtained from Merck (Germany). Inulin was from Sigma-Aldrich (USA).

The PAS was determined using the following formula [12]:

$$PAS = \frac{P_{P_{24}} - P_{P_0}}{P_{g_{24}} - P_{g_0}} - \frac{E_{P_{24}} - E_{P_0}}{E_{g_{24}} - E_{g_0}}$$

where P*p* and P*g* are the probiotic log colony forming units (CFU)/mL when grown on the prebiotic and glucose respectively; E*p* and E*g* are the enteric log CFU/mL when grown on the prebiotic and glucose respectively; and the subscript numbers represent the culture time (0 and 24 hr). By definition, substrates with a high PAS support a good growth of the probiotic bacteria, with cell densities (CFU/mL) comparable with BS11 grown on mTSB-D with glucose.

**Preparation of Shrimp Feed**

Commercial feed (Charoen Pokphand Foods Public Co., Thailand) was used as the regular feed and then supplemented with the probiotic and/or prebiotic before use. The feed mixed with BN at 10%, 20% and 30% (v/w) (designated as 10%BN-, 20%BN- and 30%BN-feeds respectively) were prepared. BS11 was cultured in TSB at 30°C with shaking at 200 rpm for 24 hr and then washed three times with sterile normal saline solution by centrifuging at 10160 *g* and 4ºC for 10 min. The cell pellet was harvested and mixed into the feed. The wet cells were thoroughly mixed with the regular feed at a rato of 1:3 respectively (~2.5% of bacteria by weight) and designated as BS11-feed. In addition, the BS11 was co-supplemented into the 10%, 20% and 30% BN-feeds (designated as BS11&10%BN-, BS11&20%BN- and BS11&30%BN-feeds respectively). Each mixture was spread out, dried in an oven for 1-2 hr at 37°C and then stored in a clean plastic bag at 4ºC until use. Each batch was examined for viable BS11 cells (CFU/g).

**Cultivation of Shrimp**

The post-larvae of the Pacific white shrimp (*Litopenaeus vannamei*), aged 20 days after hatching, were obtained from a commercial shrimp farm in Pathumthani province and acclimatised in an aerated tank with a closed recirculating water system (containing 25 mg/L NaCl) at 30°C until they reached 1-2 g in weight. They were then transferred into a 200-L high-density polyethylene culture tank (0.64 x 1.05 x 0.56 m). Suspended solids were removed with a filtering unit consisting of three layers: sand at the bottom, oyster-shells in the middle and fibre filler on the top. Water was transferred into the filter unit by an air-lift pump and passed through the unit by gravity into the culture tank. All the culture tanks were placed outdoors under a roof and sun-shade net cover. A total of eight different feeding regimes, viz. the regular feed (control) and the BS11-, 10%BN-, 20%BN-, 30%BN-, BS11&10%BN-, BS11&20%BN- and BS11&30%BN-feeds were evaluated. Each treatment was performed in triplicate using 30 shrimp in each tank. The shrimp were fed three times daily at 09.00 am, 1.00 pm and 6.00 pm at 10% of their net body weight. This set of experiment was performed twice, one in the rainy season and one in the hot season. The shrimp body weight and survival, total bacteria, BS11 and *Vibrio* spp. counts from the cultivation tank and

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intestines of moribund shrimp, together with total haemocyte count (THC) and phenoloxidase (PO) activity of live shrimp were monitored before and after challenging with *V. harveyi* 639.

Water samples (100 mL) were collected from the centre of each tank every 15 days and their pH, alkalinity, dissolved oxygen, salinity, temperature, and ammonium and nitrite contents were monitored as described previously [14].

### *Vibrio harveyi* Challenge

*Vibrio harveyi* 639 was cultured in TSB with 2% (w/v) NaCl or on TCBS and used for inducing the infection. After feeding for 90 days, the shrimp in each treatment were exposed to pathogenic *V. harveyi* 639 at ~$10^7$ CFU/mL by immersion in tank water. Ten shrimp were randomly transferred to each 40-L water tank (three tanks per treatment). The remaining shrimp were kept and fed with the same regime as before. No water was exchanged between the tanks thereafter for 3 days. During the trial, the number of live shrimp was measured and the moribund shrimp were removed every 24 hr, and from this the cumulative mortality was recorded. The hepatopancreas and intestine were dissected from each moribund shrimp and examined for the presence of *V. harveyi* 639 initially by isolation on TCBS agar, and subsequent identification as *V. harveyi* was performed as previously described [15].

### Defense Parameters from Shrimp Hemolymph

Shrimp haemolymph (100 µL) was collected from the ventral-sinus using a 26-gauge needle and a 1-mL syringe containing 200 µL of anticoagulant solution (10% w/v sodium citrate). A 20-µL drop of the mixture was placed in a haemocytometer and the THC determined under a light microscope at 400 x magnification.

After collecting the haemolymph and pelleting the cells by centrifugation at 800 *g* and 4ºC for 10 min., the pellet was washed and collected in ice-cold cacodylate buffer (Sigma-Aldrich, USA) at pH 7.0 and then homogenised with a sonicator for 10 sec. The homogenate was centrifuged at 16000 *g* and 4ºC for 20 min. to obtain the hemocyte lysate supernatant. The PO activity of the supernatant was measured spectrophotometrically at 490 nm using L-3,4-dihydroxyphenylalanine (Sigma-Aldrich, USA) as the substrate as described previously [4, 16, 17]. One unit of enzyme activity was defined as an increase in absorbance per min. per µg protein [18]. The protein content in the hemocyte lysate supernatant was measured by Bradford's method [19] using bovine serum albumin (Sigma-Aldrich, USA) as standard.

### Statistical Analysis

The data on shrimp growth, survival and disease resistance are presented as mean ± standard deviation (SD). The significance of differences between means was tested by analysis of variance and Duncan's multiple range tests with accepted significance at $p < 0.05$ level [20].

## RESULTS AND DISCUSSION

### Prebiotic Activity Score

BS11 grown in mTSB-D with 1% inulin or 1% BN has a reasonably high PAS of 1.42 ± 0.20 and 1.77 ± 0.35 respectively, with 1.25-fold higher growth in BN- than inulin-supplemented mTSB-D, but this is not statistically significant (Figure 1). In contrast, *B. animalis* subsp. *lactis* BB12 growth in mMRS-D with 1% inulin is markedly lower than that in mMRS-D with 1% BN giving a low PAS of -0.18 ± 0.08 and -0.36 ± 0.02 respectively. The inability of *B. animalis* subsp.

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*lactis* BB12 to grow well in the presence of inulin or BN is in agreement with that previously reported for a different strain of *B. bifidum* NCI on inulin-S with a PAS of -1.11 ± 0.08 [12]. Among the other tested LAB, the highest PAS of 1.94 ± 0.40 and 1.97 ± 0.43 in mMRS-D with 1% inulin and 1% BN respectively was detected in *Lc. lactis* subsp. *cremoris* IAM 1150. These are 1.4- and 1.1-fold higher than PAS for BS11 on the media supplemented with inulin and BN respectively. None of the other tested LAB grew as well in mMRS-D with 1% BN compared to that with 1% inulin (PAS < 1), except for *Lc. lactis* subsp. *cremoris* IAM 1150. However, the LAB evaluated in this study seem to grow well in the presence of inulin but not in the presence of BN (Figure 1). Similar to that reported previously [12], the PAS is observed to be dependent on both the bacterial strain and the prebiotic type/dose. Apparently, BN can replace inulin and support BS11 growth.

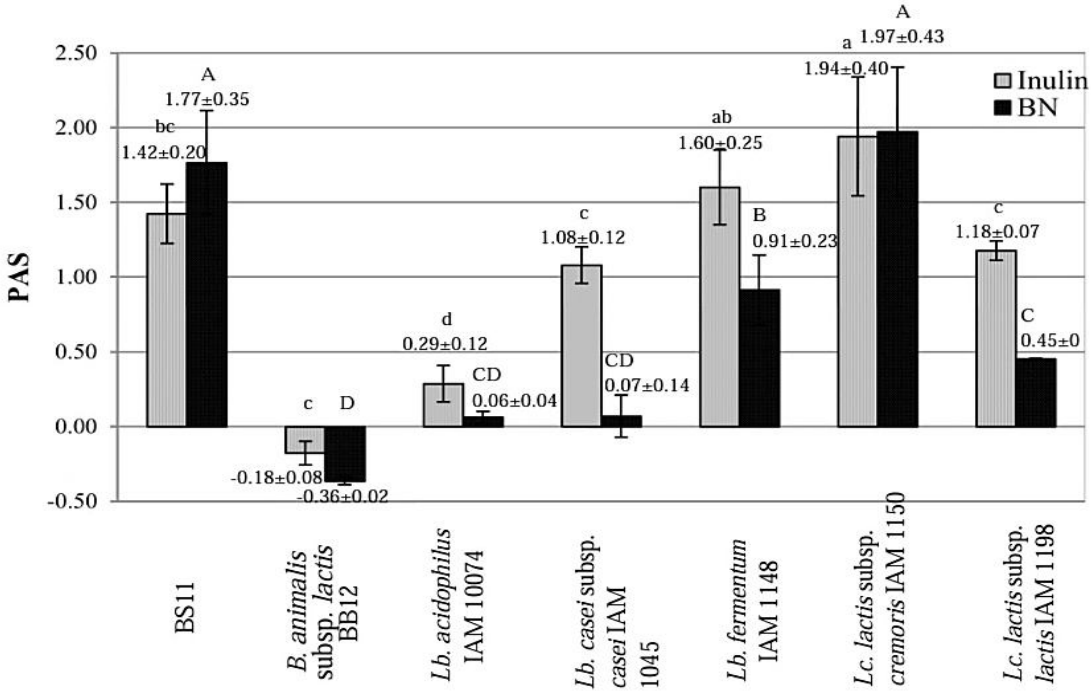


**Figure 1.** PAS of BS11 grown in mTSB-D and various LAB grown in mMRS-D supplemented with either 1% inulin or 1% BN as a prebiotic. Data are shown as mean ± SD from triplicate analysis, and means with different lowercase letters (for inulin) or uppercase letters (for BN) are significantly different ($P < 0.05$).

**Effects of BS11- and BN-Feeds on Shrimp Weight, Survival and Production**

The weights of *L. vannamei* among the eight treatments were not significantly different after 30 days of culture in the rainy season (Figure 2a). The average water quality in all treatments (4-6 mg/L dissolved oxygen, 25-29 mg/L salinity, pH 7.5-8.4, 0-0.50 mg/L ammonia, 80-150 ppm alkalinity and 0-0.50 mg/L nitrite) was within safe limits for shrimp culture [21]. The temperature varied only slightly, ranging between 29.0-31.0ºC in the rainy season. The water quality in all treatments in the hot season was similar, except for a narrower pH range (7.36-8.25) and broader temperature range (28.5-35.5ºC), which included a markedly high climate temperature of up to 41.5ºC between 11am-1pm for a one-week period after 30 days of culture. This elevated temperature was likely to have induced significant heat stress in the surviving shrimp.

After 90 days of culture in the rainy season, the highest average shrimp weight (15.7 ± 0.75 g) was found in those fed with 20%BN-feed, which was significantly higher than that in the control

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(Figure 2a). In contrast, the shrimp survival after 90 days of culturing with feed supplemented with BS11&10%BN (95.6 ± 3.9%) or BS11&20%BN (94.4 ± 5.1%) were significantly greater than that of the control (Figure 2c). Thus, there appears to be a marked synbiotic function of the BS11 probiotic and BN prebiotic on the shrimps' survival but not on their weight. The highest average shrimp production occurred in shrimp fed with BS11&10%BN-feed (380.9 ± 36.2 g), which was significantly higher than that in the control (Figure 2e).

In the trials performed in the hot season the highest increase in the average shrimp weight after 90 days of culture was observed in shrimp fed with BS11&20%BN-feed (15.7 ± 0.74 g) (Figure 2b). On the other hand, the highest shrimp survival was detected in shrimp fed with BS11-feed (81.1 ± 11.7%) (Figure 2d). A lower survival was found in the hot season than in the rainy season, apparently due to the high water temperature fluctuations in the hot season, which is above the optimum temperature for shrimp growth (28-30$^{o}$C). This may also account for a larger variance seen in the average values in the hot season than in the rainy season. Likewise, the net average production in the hot season was lower than in the rainy season, with the highest production level in the hot season being found in shrimp fed with the BS11&10%BN-feed (291.1 ± 0.44 g) (Figure 2f). However, this is still 1.3-fold lower than the best production level found in the rainy season. The results are in agreement with the known effects of temperature stress, which can increase the shrimp's susceptibility to pathogens, limit growth and reduce survival rate and production [22, 23].

The higher net production of shrimp fed with BS11&10%BN-feed than with BS11-feed implies the possibility of a synbiotic effect of the combination of the BS11 probiotic and the BN prebiotic on production. In both seasons a reduced shrimp production level was found mostly when shrimp were fed only with BN-feed and this is significantly different from those fed with BS11- and BS11&BN-feeds (Figures 2e and 2f). This is in agreement with previous reports that the prebiotic function of FOS did not affect the weight gain and survival of the Pacific white shrimp [24], whilst Bio-Mos® and β-1,3-D-glucan did not affect the growth of the western king prawn *P. latisulcatus* [25]. In contrast, mannan oligosaccharides at 3.0 g/kg were found to significantly enhance the weight and survival of *P. semisulcatus* PL [26], whilst short chain FOS significantly enhanced the survival and weight of juvenile white shrimp in a dose-dependent manner [27]. In addition, the survival of Indian white shrimp larvae ($M_1$-$M_3$) after administration of inulin (Raftilline ST) + Easy DHA Selco®-enriched *Artemia* was significantly higher than that fed with either Easy DHA Selco®-enriched *Artemia* or inulin-enriched *Artemia* [28].

Without the prebiotic function of BN, the probiotic BS11 alone seems to significantly enhance the shrimp production level (Figures 2e and 2f), consistent with its previously reported probiotic properties [8] including growth enhancement, induction of healthy appearance [29, 30] and increased survival [31, 32]. It is probable that BS11 can produce some anti-microbial substances that negatively affect other pathogens [2, 3] and/or it can produce useful enzymes or metabolites to increase the uptake of useful nutrients, provide a competitive exclusion effect on pathogens and provide some benefits to indigenous induction [2, 3, 25, 33]. Many other *Bacillus* spp. have previously been reported to act as good probiotic candidates for Pacific white shrimp by supplementation into the diet [34-36] or culture water [31, 37]. Here, prebiotic (BN) feeds are demonstrated to confer benefits on the BS11 growth and may improve gastrointestinal microflora of the host as described previously [9, 24, 38].

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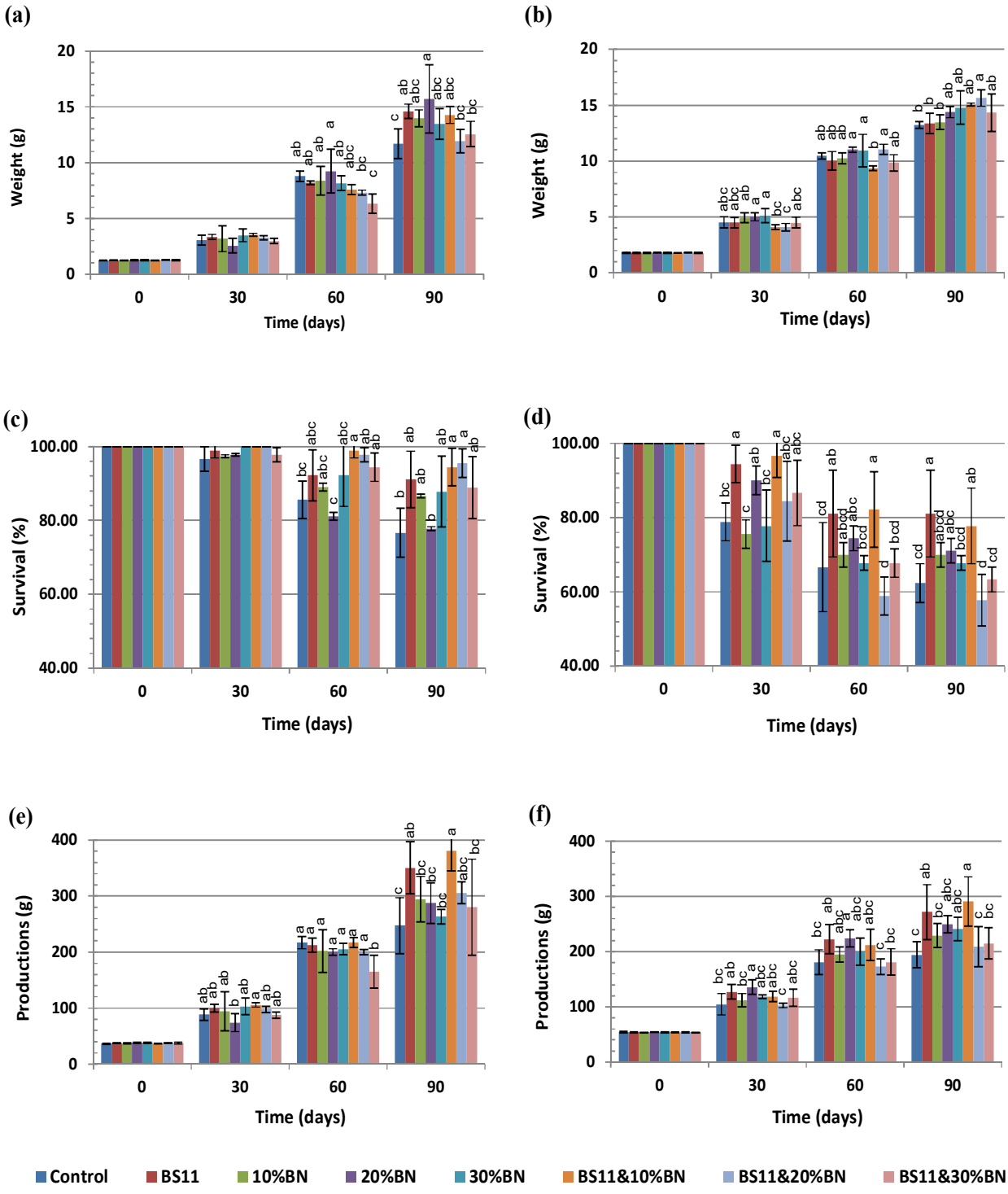


**Figure 2.** Average live (wet) weights (a, b), survival (c, d) and production (e, f) of *L. vannamei* during 90-day culture period when fed with regular feed (control) or with BS11-, 10%BN-, 20% BN-, 30%BN-, BS11&10%BN-, BS11&20%BN- or BS11&30%BN-supplemented feeds in rainy season (a, c, e) and hot season (b, d, f). Data are shown as mean ± SD from triplicate analysis, and means with different letters are significantly different between treatments within the respective time points.

The total bacterial and *Vibrio* spp. counts in the culture water of all treatments ranged between ~4 x $10^5$ - ~3 x $10^7$ and ~2 x $10^2$ - ~2 x $10^4$ CFU/mL respectively in rainy season, and between ~3 x $10^4$ - ~$10^6$ and ~1 x $10^3$ - ~2 x $10^4$ CFU/mL respectively in hot season. As expected,

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BS11 was only found in the culture water from tanks of shrimp fed with BS11-supplemented feeds and was at ~7 x $10^2$ - ~2 x $10^3$ CFU/mL in rainy season and ~9 x $10^2$ - ~3 x $10^3$ CFU/mL in hot season.

**Challenge Test and Defense Parameters from Shrimp Hemolymph**

From Figures 3a and 3b, the THCs in shrimp fed with different supplemented feeds before and after being challenged by *V. harveyi* 639 are numerically higher than those in shrimp fed with regular feed in both seasons, but are only significantly higher in shrimp fed with BS11- and BS11&10%BN-feeds. The reduction in THC after being challenged with *V. harveyi* 639 for all eight treatments ranges between 36-56% and 52-64% in the rainy and hot seasons respectively. The lowest THC reduction is observed in shrimp fed with BS11&10%BN-feed in both seasons, whereas the highest THC reduction is found in shrimp fed with regular feed. The control shrimp give the lowest average THC after *V. harveyi* 639 challenge, at 1.6- and 2.0-fold lower than those from shrimp fed with BS11&10%BN- and BS11-feeds respectively in the rainy season (Figure 3a), and at 1.4- and 2.3-fold lower in the hot season (Figure 3b).

From Figures 3c and 3d, the highest reduction in hemocyte PO activity (84% and 70% in rainy and hot seasons respectively) after challenge with *V. harveyi* are observed in shrimp fed with regular feed, whilst BS11- and BS11&%BN-feeds give 55-68% and 51-63% reduction in rainy and hot seasons respectively. The lowest average hemocyte PO activity after *V. harveyi* 639 challenge occurs in the control shrimp, being more than 2.0-fold lower than that in the BS11&10%BN- and BS11-feeds in both seasons.

From Figures 3e and 3f, mortality is evident from the first day onwards in all the treatments after challenge with *V. harveyi* 639. However, the shrimp fed with control feed suffer a significantly higher mortality from day 2 and reach 90% mortality 3 days after *V. harveyi* exposure in rainy season and 42% in hot season. The improved shrimp survival, taken to imply disease resistance as compared to control, is clearly evident in the shrimp fed with all BN- and BS11-feeds, with the highest survival of 66.7% in those fed with BS11&10%BN-feed in rainy season, 90.0% survival in those fed with 20%BN- or 30%BN-feeds in hot season, and 86.7% survival in those fed with BS11&10%BN-feed. BS11-, BN- or BS11&BN-feed thus gives an enhanced survival after an external *V. harveyi* exposure. In agreement with these results, a decreased shrimp mortality after infection with *V. parahaemolyticus* was previously reported in shrimp that were fed with feed containing *V. alginolyticus* UTM 102, *B. subtilis* UTM126, *Roseobacter gallaeciensis* SLV03 and *Pseudomonas aestumarina* SLV22 [36]. Overall, a lower mortality was observed in the hot season during the 3-day exposure period to *V. harveyi* than in the rainy season. This may well reflect the ~$10^3$ CFU/mL lower viable *Vibrio* spp. levels in shrimp water in the hot season compared with those in the rainy season.

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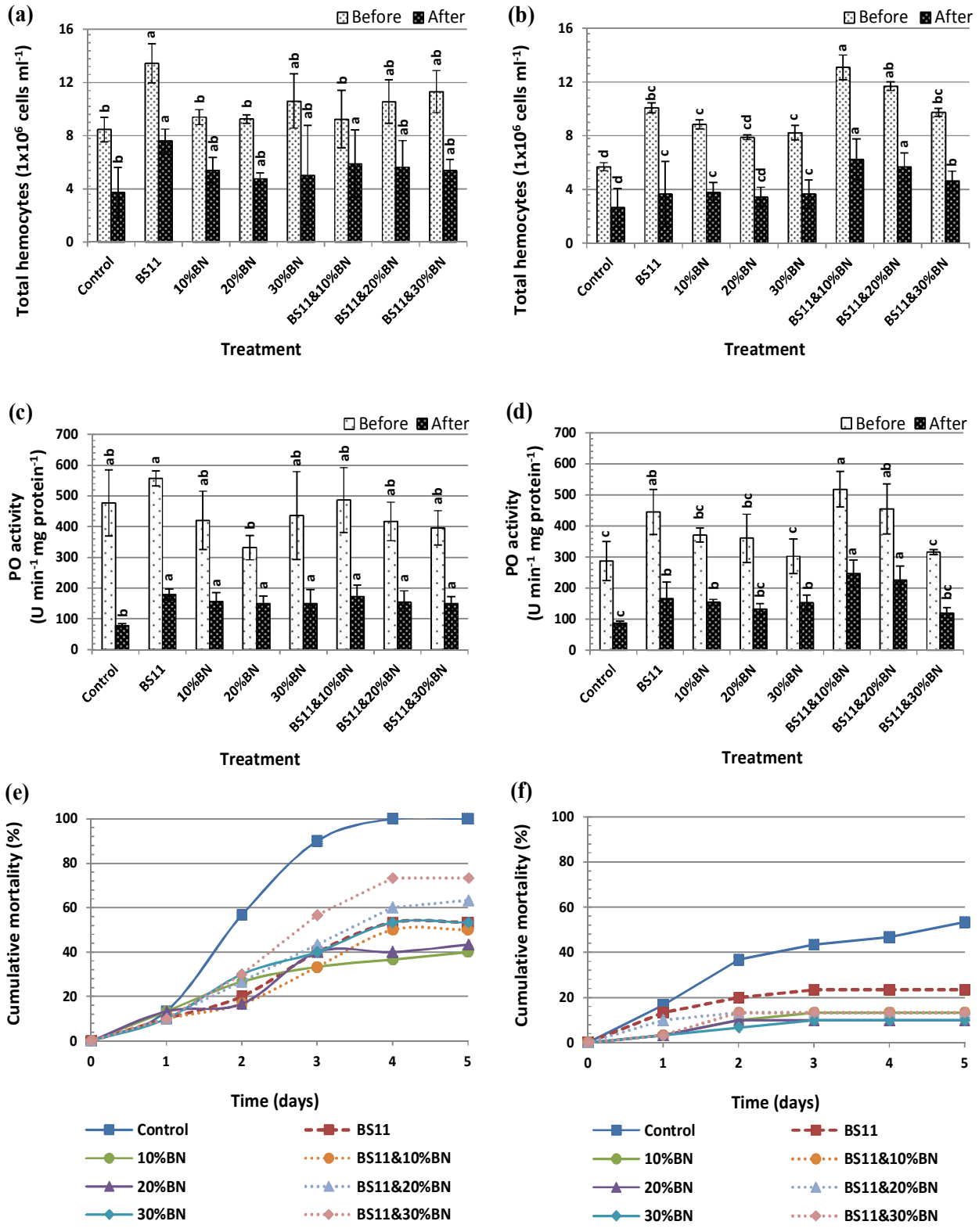


**Figure 3.** Mean shrimp THCs (a, b) and PO activities (c, d) before and after challenge for 3 days by *Vibrio harveyi* 639, and shrimp cumulative mortality (e, f) during 3-day challenge for shrimp fed with regular feed (control) or BS11-, 10%BN-, 20%BN-, 30%BN-, BS11&10%BN-, BS11&20%BN- or BS11&30%BN-supplemented feeds in rainy season (a, c, e) and hot season (b, d, f). Data are shown as mean ± SD, derived from nine shrimp. Different letters represent significant difference among treatments before or after challenge by *Vibrio harveyi* 639.

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The *Vibrio* counts in the intestines of the moribund shrimp from all the treatments after challenge with *V. harveyi* 639 for 3 days ranged between 3.9-8.2 x $10^7$ CFU/g in the rainy season and 3 x $10^5$ - 1.15 x $10^7$ CFU/g in the hot season, which were significantly higher than those before *V. harveyi* challenge. Lower *Vibrio* counts from the intestines of shrimp fed with BS11-feed than with normal or BN-feeds were noted. The decrease in total *Vibrio* counts in the intestines of BS11-fed shrimp in this study agrees with previous results on shrimp fed with probiotic *Halomonas* sp. B12 [39], although it does not exactly match the shrimp mortality rates observed here.

## CONCLUSIONS

A synbiotic effect using *Bacillus subtilis* S11 probiotic bacteria and banana prebiotic on enhancing the production and luminous vibriosis resistance of the Pacific white shrimp*, Litopenaeus vannamei*, has been demonstrated. The results from this study support the possibility of using *Bacillus subtilis* S11 and/or banana prebiotic as alternatives to antibiotics in the shrimp farming process, which could lead to a more sustainable and environmentally friendly industry as well as a healthier food product of higher economic value.

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