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FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Nutritional and immunological studies of Pacific white shrimp (*Litopenaeus vannamei)* fed *Bacillus* spp. as a dietary supplement

Chorong Lee

Department of Marine Life Science
GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

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Chorong Lee
(Supervised by Professor Kyeong-Jun Lee)

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This dissertation has been examined and approved.

.....
Jee Eun Han, Prof. of Veterinary Medicine,
Kyungpook National University

.....
Se-Jin Lim, Neo Environmental Business Company

.....
Kwang-Sik Choi, Prof. of Marine Life Science

.....
Sukgeun Jung, Prof. of Marine Life Science

.....
Kyeong-Jun Lee, Prof. of Marine Life Science

2019. 12
Date

Department of Marine Life Science
GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

CONTENTS

요약문

양식새우에서 발생하는 대표적 질병인 급성간췌장증후군(AHPND, acute hapatopancreas necrosis disease), 전염성근괴사증(IMN, infectious myonecrosis), 흰점바이러스(WSSV, white spot syndrome virus), 백변병(WFD, white feces syndrome) 등은 새우 양식산업에 막대한 경제적 손실을 끼치고 있다. 질병 치료를 위한 항생제의 과다사용은 생태계 오염, 내성균 발현, 생물 체내의 잔류로 인한 식품 안전성 문제 등과 같은 심각한 문제를 야기시킨다. 본 연구는 항생제의 사용을 줄일 수 있는 방안의 하나로, 프로바이오틱스 중 가장 많이 사용되고 있는 *Bacillus* 균주(*B. subtilis*, *BS*; *B. pumilus*, *BP*; *B. licheniformis*, *BL*)를 흰다리새우 사료에 단독으로 혹은 적절히 혼합 첨가하여 총 3회에 걸쳐 사육실험을 실시하였다.

사육실험에서는 공통으로 사료 내 *Bacillus* spp.의 단독 또는 혼합 첨가 시 흰다리새우의 성장, 사료효율 및 비특이적 면역력에 미치는 영향에 대하여 연구하였으며, Chapter 2에서는 사료 소화율 및 수질개선 평가, Chapter 3에서는 AHPND에 대한 질병저항성 평가에 초점을 두어 연구를 진행하였다. Chapter 4에서는 Chapter 2와 3의 연구를 보완하여 AHPND, WSSV 각각에 대한 질병저항성을 평가하기 위해 연구를 진행하였다.

Chapter 2에서는 단독 또는 혼합된 *Bacillus* ($1x10^{10}$ CFU/g)를 사료 내 각각 0.1, 0.2%씩 첨가하여 5가지 실험사료를 제작하였다(Control, *BS*0.1, *BS*0.2, *BS*/*BP*0.1 & *BS*/*BP*0.2). 새우(초기평균무게: 0.14g)는 총 20개의 96L 수조에 각 25마리씩 4반복으로 배치하였고, 사양실험은 8주간 진행되었다. 단독 또는 혼합된 *Bacillus*를 사료 내 각각 0.2% 첨가한 실험구에서 대조구에 비해 유의적으로 높은 성장률과 사료효율이 관찰되었다. 비특이적 면역력, 사료 소화율은 대조구에 비해 *Bacillus* 첨가 모든 실험구에서 유의적으로 높았다. 사육수 무교환 실험에서는

Bacillus 첨가 모든 실험구에서 대조구에 비해 유의적으로 낮은 암모니아 농도가 측정된 것으로 보아, *Bacillus*는 사육수질 개선에 긍정적인 영향을 끼치는 것으로 판단된다.

Chapter 3에서는 *Bacillus* ($1x10^{10}$ CFU/g)를 단독 또는 혼합 첨가하여 4가지 실험사료를 제작하였다(Control, *BS*, *BS*/*BP* & *BS*/*BP*/*BL*). 새우(초기평균무게: 0.51g)는 총 16개의 120L 수조에 각 30마리씩 4반복으로 배치하였고, 사양실험은 33일간 진행되었다. *Bacillus* 단독 실험구에서 대조구에 비해 유의적으로 높은 성장률이 관찰되었고, *Bacillus* 첨가 모든 실험구에서 대조구에 비해 AHPND에 대한 질병저항성이 유의적으로 높았다. AHPND 독소 분석 결과 사료 내 *Bacillus* 첨가 시 대조구에 비해 AHPND 질병에 대한 빠른 회복력을 보였으며, 병리조직학적 분석 결과에서도 이와 유사한 경향이 관찰되었다.

Chapter 4에서는 *Bacillus*를 단독 또는 혼합 첨가하여 5가지 실험사료를 제작하였다(Control, *BS*-A, *BS*-B, *BS*/*BP*/*BL*10^{9} & *BS*/*BP*/*BL*10^{10}). 새우(초기평균무게: 0.15g)는 총 50개의 120L 수조에 각 20마리씩 10반복으로 배치하였고, 사양실험은 51일간 진행되었다. 성장률은 *Bacillus* 혼합 실험구, 비특이적 면역력은 *Bacillus* 첨가 모든 실험구에서 대조구에 비해 유의적으로 높았다. AHPND에 대한 질병저항성은 *Bacillus* 단독 실험구에서 대조구에 비해 경향적으로 높았고, 공격시험 종료 시 대구조에서만 AHPND 독소가 검출된 것으로 보아 사료 내 *Bacillus* 첨가 시 AHPND의 폐사를 줄일 수 있으며, 감염되더라도 빠른 회복을 보일 수 있을 것으로 판단된다. WSSV에 대한 질병저항성은 모든 실험구에서 유의적인 차이는 없었지만, 병리조직학적 분석 결과 대조구에서 *Bacillus* 첨가 실험구에 비해 WSSV의 감염증상(핵 내 봉입체 형성)이 더 많이 관찰되었다.

위 결과들을 종합하여 볼 때, *Bacillus* 균주(*B. subtilis*, *B. pumilus*, *B. licheniformis*)를 사료에 단독 또는 혼합 첨가 시 흰다리새우의 성장, 사료효율 및

사료 소화율을 효과적으로 증진시킬 수 있을 것으로 판단된다. 특히, 비특이적 면역력을 크게 향상시킴으로써 AHPND, WSSV에 대한 질병저항성을 높여 흰다리새우의 폐사율을 크게 줄일 수 있을 것으로 판단된다. 사양실험에 사용된 *B. subtilis*는 새우에서 분리된 균주이기 때문에 실험새우의 장 내에 더욱 쉽게 정착되어 긍정적인 영향을 끼쳤을 것으로 사료된다. 사료 내 *B. subtilis*($1x10^{10}$ CFU/g) 단독, 또는 3종류의 *Bacillus* spp. ($1x10^{9}$ CFU/g)을 혼합하여 첨가 시 적정 첨가 함량은 0.2%인 것으로 판단된다.

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CHAPTER 1: Introduction

1.1. Pacific white shrimp (*Litopenaeus vannamei*)

Aquaculture including shrimp culture is one of the fastest-growing industries in the world. Among cultured shrimp species, Pacific white shrimp is the most cultured species. According to FAO (2017), 75.7% of world shrimp production was gained from *L. vannamei* in 2015. However, disease outbreaks have been a major problem in the shrimp industry in the past decades. Pathogenic (bacteria, virus, parasites and fungi) and non-pathogenic (environmental parameters, nutritional deficiency and algal toxins) disease are responsible for such outbreaks resulting in the most economic losses in the industry (Toledo et al., 2019). Expanding the cultivation scale of shrimp aquaculture have developed high density culture techniques and systems. However, the high density culture for the aquatic animals has caused deterioration of aquatic environment. Frequent occurrence of the diseases has been the issue for the reduction of *L. vannamei* production (Kuebutornye et al., 2019). Hence, dietary supplementation of immunostimulants has been recommended to control the diseases through improving innate immunity of the shrimp (Mohan et al., 2019).

1.2. Probiotic use in aquaculture

Probiotics in aquaculture are defined as the supplements of microbes in a live form that leads to modulation of microbial population in the hosts for advantageous effects on growth, immunity and disease resistance (Fuller, 1989). Reduction of antibiotic use by using probiotics is considered as one of the best therapeutic ways in shrimp farming (Balcazar et al., 2006).

In the case of shrimp aquaculture, intensive culture systems with higher stocking density were trending with the expansion of world aquaculture in the past decades. Intensive culture system, shrimp is vulnerable to bacterial or viral disease which leads to vast losses in yields. Also, acute

hepatopancreatic necrosis disease, AHPND (also named EMS; early mortality syndrome) in Asia and central America (Lightner et al., 2012) has led to an increase in antimicrobial use (Chumpol et al., 2017).

Chemical disinfectants and antibiotics were used for the purpose of disease prevention. The use of antibiotics has continuously developed antibiotic-resistant bacterial strains including *V. parahaemolyticus* (Han et al., 2015a). It has caused accumulation of antibiotic residuals which affect adversely for food safety in aquaculture products (Pandiyan et al., 2013). Probiotics have been found as an alternative remedy to reduce the antibiotic use in shrimp culture (Balcazar et al. 2006). They can be identified as "viable cell preparations" causing positive impacts on host species (Merrifield et al., 2010). They are known to play important roles in removing pathogenic organisms, increasing beneficial microbial balance in the host intestine, assisting in enzymatic digestion and stimulating the innate immune functions (Soltani et al., 2019). If a probiotic strain could be colonized and established well inside the host intestine, it is to be more beneficial (Nayak, 2010).

Probiotics have various mechanisms. They can produce anti-pathogenic substances to enhance the immune function, strength of epithelial shield, mucosal adhesion of intestine and competitive removal or reduction of pathogenic adhesions (Bermudez-Brito et al., 2012). Many beneficial effects of several probiotics such as the species of *Bacillus* (Dong et al., 2014; Sekar et al., 2016; Cai et al., 2019; Cheng et al., 2019; Tepaamorndech et al., 2019; Vogeley et al., 2019), *Dunaliella* (Felix et al., 2017, 2019), *Enterobacter* (Zuo et al., 2019), *Lactobacillus* (Zuo et al., 2019), *Pseudomonas* (Hai et al., 2009) and mixed cultures (Sanchez-Ortiz et al., 2016; Chumpol et al., 2017; Nimrat et al., 2019; Wang et al., 2019; Xie et al., 2019) have been demonstrated in earlier research works and used in shrimp diets successfully.

1.3. *Bacillus* species

One of the reason for using *Bacillus* strains as a probiotic is the ability of them to form spores in the marine environment. They can be isolated from bivalves and crustaceans (Tseng et al., 2009;

Liu et al., 2014). Especially, *Bacillus* spores can stand for a long time unharmed and tolerant to temperature alternations that make them more ideal for industrial uses (Elshaghabee et al., 2017). Antibiotics from *Bacillus* species could easily penetrate through the protective slime layer of gram-negative bacteria because the *Bacillus* itself secretes a number of slime and biofilm degrading enzymes (Moriarty, 1998). *Bacillus* spp. has been proven to have an immunostimulatory effect by producing antimicrobial peptides (Kumar et al., 2008). In the case of shrimp culture, several *Bacillus* species are reported to have great potential as a probiotic when they are supplemented in shrimp diets or rearing water (Liu et al., 2010; Zokaeifar et al., 2014; Cai et al., 2019). Enzymatic activities of *Bacillus* probiotics and their beneficial effects on growth performance of cultured shrimp have been well documented. Moreover, it is stated that *Bacillus* based probiotics can release various enzymes that could improve the digestibility of indigestible nutrients in the host gut when they are germinated. However, there is lack of information for the probiotics on the digestibility of shrimp (Lin et al., 2004; Tsai et al., 2019). *B. subtilis*, *B. pimilus* and *B. licheniformis* species isolated from prawn intestine, seawater and fermented soybean lump are reported to have high active digestive enzymes such as, lipase, trypsin, amylase, protease and high antibacterial activity against pathogens such as *Vibrio harvei*, *Streptococcus iniae*, *Aeromonas salmonicida*, *Edwardsiella tarda* (Ghosh et al., 2002; Balcazar et al., 2007; Zokaeifar et al., 2012; Tsai et al., 2019; Zhao et al., 2019). Also, the effects of *Bacillus* spp. as an immunostimulatory agent for a variety of diseases have been documented (Tseng et al., 2009; Liu et al., 2014; Kumar et al., 2013; Sadat Hoseini Madani et al., 2018; Amoach et al., 2019; Wang et al., 2019).

1.4. Shrimp disease

AHPND and WSSV (white spot syndrome virus) are considered as very serious and rapidly spreading pathogens that could cause huge losses in the world shrimp production.

1.4.1. AHPND

AHPND (also known as early mortality syndrome, EMS) has caused losses in the shrimp

productions in Asia (Lightner et al., 2012) and Central America (Mexico) (Nunan et al., 2014). Generally, AHPND affects shrimp production at post-larvae stage resulting in mass mortality (up to 100%) within a month. The disease was also reported to affect *Penaeus monodon* at the last stage (Leobert et al., 2015). It is well known that *V. parahaemolyticus* (VP_{AHPND}) is the main pathogen for the disease targeting the hepatopancreas (Tran et al. 2013). VP_{AHPND} has only been examined for the case of AHPND and other species of *Vibrio* also have been considered as clinical causes of AHPND (Han et al., 2015a). Histopathological symptoms of AHPND are evident in the hepatopancreas with R- and B- cell dysfunctions, hematopoietic infiltration and hypertrophy (FAO, 2013).

1.4.2. WSSV

WSSV is considered as a serious infectious disease in shrimp culture (Stentiford and Lightner, 2011; Tang et al., 2013; Oakey and Smith, 2018). Although there are many geographical isolates along with genotypic variability, all of them are considered as an individual species within the genus Whispovirus called 'White spot syndrome virus' (Lo et al., 2012). WSSV mainly targets the tissues having ectodermal and mesodermal embryonic origin particularly subcuticular and cuticular connective tissues (Wu et al., 2013). Symptoms of WSSV infection include loss of appetite, visible white spots on the body, lethargic movements and reddish discoloration of the exoskeleton leading a high mortality rate. The susceptibility of all Penaeid shrimp species to WSSV is very high.

1.5. Chapter justification

The study of this dissertation was aimed to evaluate the effects of dietary *Bacillus* probiotics such as *B. subtilis*, *B. pumilus* and *B. licheniformis* on nutritional and physiological performance of *L. vannamei*. In the studies, the effects *Bacillus* spp. supplementation to diets were specifically examined on growth performance, innate immunity, diet digestibility, culture water quality and

disease resistance against AHPND and/or WSSV. All chapters are focused on the effects of dietary *Bacillus* spp. supplementation on growth performance, feed utilization and innate immunity. The justification of each chapter is as follows;

Chapter 2 was conducted to investigate the supplemental effects of single use or combination of the probiotics (10^{10} CFU/g of *B. subtilis* and *B. pumilus*) on digestibility, growth performance, feed efficiency, innate immunity and culture water quality of *L. vannamei*.

Chapter 3 examined the supplemental effects of three different probiotics (10^{10} CFU/g of *B. subtilis, B. pumilus and B. licheniformis*) on growth performance and feed efficiency. In addition, a challenge test against AHPND was conducted to provide data for the development of probiotic agents that could help to reduce the high mortality of *L. vannamei* by VP_{AHPND}.

Chapter 4 focused on the effects of dietary supplementation of individual or mixed Bacillus spp. (10^9 or 10^{10} CFU/g) such as *B. subtillis, B. pumilus* and *B. licheniformis* on growth performance, innate immunity and disease resistance against AHPND and WSSV of *L. vannamei*.

CHAPTER 2

Dietary supplementations of *Bacillus* probiotic improves digestibility, innate immunity, water quality and growth performance of Pacific white shrimp (*Litopenaeus vannamei*)

2.1. MATERIALS AND METHODS

2.1.1. Probiotic bacteria

The tested probiotic was prepared and provided from the Applied Technology Center, CJ CheilJedang Corp. (Suwon, South Korea). The bacterium was cultured on BHI agar (Difco, USA) at 37 ℃ for 24 h and its purity was checked. The strain was found to have the morphological characteristic of gram-positive rod-shaped bacterium. The bacterial strain was identified by analysis of 16s rDNA sequencing and showed 99% homology with *Bacillus* sp.. To distinguish spores and bacterial cells, cultured bacteria were stained and observed under the microscope according to the standard procedure described by Bartholomew and Mittwer (1950). The powder form of the probiotic, a mixture of spore and cell, was prepared by freeze-drying.

2.1.2. Experimental diets and design

Experimental diets were regarded as a control (without probiotic supplementation) and four other diets were prepared by inclusions of *B. subtilis* (*BS*) alone, and a mixture of *BS* and *B. pumilus* (*BP*) at different levels (0.1 x 10^{10}*BS*, 0.2 x 10^{10}*BS*, 0.1 x 10^{10}*BS*/*BP*, 0.2 x 10^{10}*BS*/*BP* diets for Control, *BS*0.1, *BS*0.2, *BS*/*BP*0.1 and *BS*/*BP*0.2, respectively). All the dietary ingredients were thoroughly mixed in a feed mixer and pelleted (SP-50, Gumgang Engineering, Daegu, South Korea) in a proper diameter. Then, the pelleted diets were dried in a dryer at 25℃ for 12 h. After creating the experimental diets, the number of viable bacteria in the pellets was counted. The results of viable cell count were 2.1 × 10^7, 4.4 × 10^7, 1.6 × 10^7 and 3.5 × 10^7 CFU/g feed (*BS*0.1, *BS*0.2,

BS/*BP*0.1 and *BS*/*BP*0.2, respectively). The diets were analyzed for moisture, crude protein and crude ash by the standard methods (AOAC, 2005). Crude lipid concentration was accordingly analyzed (Folch et al., 1957) (Table 2-1).

Table 2-1. Dietary formulation and proximate composition of the five experimental diets for *L. vannamei* (% dry matter).

Ingredients (%)	Experimental diets[1]				
	Control	*BS*0.1	*BS*0.2	*BS*/*BP*0.1	*BS*/*BP*0.2
Fish meal[2]	40.00	40.00	40.00	40.00	40.00
Soybean meal[3]	12.81	12.81	12.81	12.81	12.81
Squid liver powder	10.00	10.00	10.00	10.00	10.00
Wheat flour	25.61	25.61	25.61	25.61	25.61
Amygluten 110	3.00	3.00	3.00	3.00	3.00
Fish oil A/C	2.00	2.00	2.00	2.00	2.00
Amino acid[4]	0.42	0.42	0.42	0.42	0.42
Vitamin/Mineral premix[5]	5.96	5.96	5.96	5.96	5.96
Rice bran	0.20	0.10	0.00	0.10	0.00
BS ($1x10^{10}$)	0.00	0.10	0.20	0.00	0.00
BS/*BP* ($1x10^{10}$)	0.00	0.00	0.00	0.10	0.20
Chemical composition (%, dry mater)					
Moisture	5.59	5.71	5.78	5.74	5.74
Crude protein	47.3	48.9	49.2	48.8	49.0
Crude lipid	7.74	7.70	7.73	7.75	7.72
Crude ash	6.12	6.10	6.20	6.07	6.09

[1]Experimental diets were regarded as a control (without probiotic supplementation) and four other diets were prepared by inclusions of *B. subtilis* alone (*BS*), and a mixture of *BS* and *B. pumilus* (*BP*) at different levels (0.1×10^{10} *BS*, 0.2×10^{10} *BS*, 0.1×10^{10} *BS*/*BP*, 0.2×10^{10} *BS*/*BP* for *BS*0.1, *BS*0.2, *BS*/*BP*0.1and *BS*/*BP*0.2 diets, respectively) of basal diet.

[2]CJ Cheilhedang Co. Ltd., South Korea (crude protein: 67%)

[3]South America (crude protein: 44%)

[4]Amino acid mixture composition (g/100.4 g dry weight mixture; all L-form amino acids unless otherwise indicated): arginine, 8.88; histidine, 3.00; isoleucine, 4.32; leucine, 7.80; lysine hydrochloride, 9.64; methionine, 2.76; phenylalanine, 4.32; threonine, 4.56; tryptophan, 1.50; valine, 6.12; aspartic acid, 10.6; glutamic acid, 19.4; glycine, 10.8; alanine, 6.72.

[5]Vitamin/Mineral premix (g $kg^{-1}$of mixture): retinol, 3.0; cholecalciferol, 1.0; ascorbic acid, 20.0; tocopherol, 20.0; menadione, 2.0; thiamine, 4.0; riboflavin, 6.0; pyridoxine, 5.0; cobalamin, 6.0; inositol, 54.0; panththenic acid, 12.0; biotin, 0.2; niacin amide, 40.0; folic acid, 2.0; ferrous sulfate, 10.0; copper sulfate, 1.0; zinc sulfate, 30; manganous sulfate, 2.0; cobalt chloride, 10.; potassium iodide, 1.0; potassium, 6.0; sodium selenite, 0.01.

2.1.3. Shrimp and feeding trial

L. vannamei at the post larvae stage was purchased in a local shrimp farm (Tamla shrimp, Jeju, South Korea) and transported to the Institute of Marine Sciences of Jeju National University (Jeju, South Korea). The shrimps were fed a commercial shrimp diet (SAJO DongA One, Seoul, South Korea) for a month. Then, the shrimp (initial mean body weight, 0.14±0.00 g) were randomly selected and distributed into 96 L capacity 20 acrylic tanks at a density of 25 shrimp per tank. Quadruplicate groups of shrimp were fed one of the four diets at a ratio of 6 - 12% body weight (four times a day, 08:30, 12:00, 15:30 and 19:00h) for 8 weeks. The salinity was maintained at 30 ppt during the feeding trial. Water quality was maintained within a standard range for *L. vannamei* as follows; temperature (28 – 31 ℃), pH (6.48 – 7.10), dissolved oxygen (6.39 – 7.21 mg L^{-1}) and ammonia (0.035 – 0.154 mg L^{-1}).

2.1.4. Sample collection and analyses

At the end of the feeding trial, all the shrimp in each tank were individually weighed for calculation of final body weight (FBW, g), weight gain (WG, %), specific growth rate (SGR, %), feed conversion ratio (FCR) and survival (%). Seven shrimp per tank (28 shrimp per dietary treatment) were randomly captured and placed in ice water for 3 min to anesthetize before hemolymph sampling. Hemolymph (200 µL) was withdrawn from the ventral sinus of each shrimp into a 1 mL syringe containing 400 µL of precooled (4°C) anticoagulant solution (Alsever's solution, sigma). About 50 µl anticoagulant-hemolymph was used to determine macrophage activity (NBT; nitroblue-tetrazolium activity). The remaining anticoagulant-hemolymph mixture was centrifuged at 800 × g for 20 min at 4°C and the supernatant was stored at −80℃ for non-specific immune responses analyses.

Immunological parameters were assessed accordingly. Oxidative radical production by phagocytes during respiratory burst was measured through NBT assay described by Zhang et al. (2013). PO activity was measured according to Hernandez-Lopez et al. (1996). Antiprotease activity was determined according to the method described by Ellis (1990). A turbid metric assay

was used for the determination of lysozyme level by the method described by Paglia and Valentine (1967). SOD activity was measured by the percentage reaction inhibition rate of an enzyme with WST-1 (water-soluble tetrazolium dye) substrate and xanthine oxidase using a SOD Assay Kit (Sigma, 19160) according to the manufacturer's instructions. Also, GPx and catalase activities were measured using a GPx assay kit (Biovision, Inc., Milpitas, CA, USA) according to the manufacturer's instructions.

2.1.5. Digestibility test

For estimation of apparent digestibility coefficient (ADC) of the experimental diets, chromic oxide (Cr_2O_3) (Sigma-Aldrich, St. Louis, USA) was included in the diets as an inert indicator at a concentration of 1.0% diet. Shrimp after the feeding trial were stocked into the five acrylic tanks of 210 L capacity at a density of 40 shrimp per tank. The shrimp were fed the diets three times a day at 08:30, 12:00 and 17:00 hours. 30 min after feeding, the tanks were siphoned out to remove uneaten feed. Fecal samples were collected three times a day from each tank (11:30, 15:00 and 18:30 h) and collected on filter paper. All feces collected from each tank in each period were pooled and frozen at –20℃ until analysis. After feeding, the tanks and the settling columns were thoroughly cleaned to eliminate all feed waste and fecal residues. Chromium oxide content of diet and feces samples were analyzed by the method described by Divakaran et al. (2002). The ADC of dry matter and protein of the experimental diets were calculated through the following formula:

ADC of nutrients (%) = 100 x [100 - (% Cr_2O_3 in diet/% Cr_2O_3 in feces) x (nutrient in feces/nutrient in diet))

2.1.6. Zero water exchange test

Shrimp (average body weight, 2.87g) were randomly distributed into 96 L capacity 15 acrylic tanks at a density of 12 shrimps per tanks. Shrimp of triplicate groups were fed one of the five experimental diets at a ratio of 10% body weight (four times a day, 08:30, 12:00, 15:30 and 19:00 h)

on zero exchange water system. The water sample was collected from an experimental tank once a day (10:00 h) and NH_4^+ of the sample was analyzed using by Verdouw et al. (1978) for 10 days.

2.1.7. Statistical analysis

The feeding trial was designed to be completely randomized. Data were analyzed one-way analysis of variance (ANOVA) in SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the mean difference was compared with Tukey's HSD multiple range tests. Statistical significance was determined at $P < 0.05$. Data are presented as mean ± SD. The percentage of data was analyzed after transformation into arcsine.

2.2. RESULTS

All the experimental diets were readily accepted by the *L. vannamei* at the start of the feeding trial and they ate aggressively during 8 weeks of the feeding trial. The results of apparent digestibility coefficients for dry matter (ADCd) and protein (ADCp) are shown in Table 2-2. Shrimps fed the test diets exhibited significantly higher ADCd and ADCp than shrimp fed control diet.

The growth performance, feed efficiency and survival of shrimps fed the experimental diets are shown in Table 2-3. The FBW was significantly improved in shrimp fed *BS*0.2 and *BS*/*BP*0.2 diets compared to shrimp fed control diet. Shrimp fed *BS*0.2 diet showed significantly higher WG than shrimp fed control diet. Significantly higher SGR and lower FCR were obtained in shrimp fed *BS*0.1, *BS*0.2 and *BS*/*BP*0.2 diets compared with shrimp fed control diet. The survival rate ranged from 84.0 to 90.7% with no significant difference between dietary treatments ($P>0.05$).

Non-specific immune responses were positively affected by dietary *Bacillus* spp. supplementation (Table 2-4). NBT activity, shrimps fed *BS*0.2, *BS*/*BP*0.1 and *BS*/*BP*0.2 diets were significantly higher than shrimp fed the control diet. The *Bacillus* supplemented diets showed significantly increased PO activity compared to the control diet. Shrimp fed *BS*0.2 diet showed significantly higher antiprotease activity than shrimp fed control diet. Also, GPx activity of shrimp fed *BS*0.1, *BS*0.2 and *BS*/*BP*0.1 diets were significantly higher than shrimp fed control diet. Lysozyme and SOD activities did not show any significant difference among all the shrimp groups.

The zero water exchange test is shown in Figure 2-1. From the 10th day of the experiment, in tanks of test diet groups had significantly lower total ammonia concentration than control diet group.

Proximate compositions of whole-body of shrimp are shown in Table 2-5. Significantly lower dry matter composition found in shrimp fed *BS*0.1 and *BS*0.2 diets than that of shrimp fed control and *BS*/*BP*0.1 diets.

2.3. DISCUSSION

Use of probiotics in aquaculture sector is well documented and it is stated that probiotics have beneficial effects on growth, feed digestion, innate immunity and culture water quality (Liu et al., 2014; Sanchez-Ortiz et al., 2016; Amoach et al., 2019; Tepaamorndech et al., 2019; Wang et al., 2019). Most of the studies have focused on the effect of probiotic supplemented diets on growth and immunity, whereas information on digestibility of nutrients lacks (Lin et al., 2004). Tsai et al. (2019) mentioned that probiotics enhance microbial enzyme activities of digestive tract of microorganisms and thereby improve the digestive process and feed utilization. The present results indicated that dietary supplementation of the *Bacillus* spp. (10^7 CFU/g) can improve dry matter and protein digestibility of *L. vannamei*. Lin et al. (2004) demonstrated a significant increment in dry matter, crude protein, lipid, phosphorus, amino acids and fatty acids digestibility in shrimp fed a diet containing *Bacillus* spp. (10^8 CFU/g) probiotics compared to shrimp fed a non-supplemented control diet. The apparent digestibility coefficients of dry matter and crude protein of *L. vannamei* were improved when they were fed with a diet containing *B. subtilis* (10^9 CFU/g) E20 probiotics as reported by Tsai et al. (2019). The improved digestibility can be explained with the action of probiotics in the gut. Probiotics could enhance the secretion of endogenous digestive enzymes such as lipase, amylase and protease of shrimp when they were supplemented into diets leading to the improved growth through enhanced digestive capacity (Ochoa-Solano and Olmos-Soto, 2006; Zhang et al., 2010; Zokaeifar et al., 2014; Amoach et al., 2019; Cai et al., 2019; Xie et al., 2019). According to previous studies, genus *Bacillus* can secrete enzymes that could efficiently break down a variety of proteins, lipids and carbohydrates (Moriarty 1996, 1998; Ochoa-Solano and Olmos-Soto, 2006). Therefore, the improvement in growth performance with increased digestive enzyme activities could be possible with dietary supplementation of probiotics (Tsai et al., 2019).

In the present study, significant enhancement in growth performance and feed utilization were found at levels of 10^7 CFU/g *Bacillus* spp. of sole or mixed diets. Several studies have reported the favorable effects of various probiotics on shrimp growth and feed utilization. Vogeley et al. (2019) observed improvement in the growth performance of *L. vannamei* (average weight 1.05g) when

shrimp was fed a diet supplemented with *B. subtilis* at a level of 10^6 CFU/g. Zokaeifar et al. (2014) used *B. subtilis* as a probiotic at a level of 10^5 CFU/g or even higher dose for *L. vannamei* (average weight 0.67g), and found that it effectively enhanced the growth performance and survival of shrimp. In addition, many studies have reported that the addition of mixed *Bacillus* species (e.g. *B. licheniformis*, *B. coagulans*, *B. thuringiensis*, *B. megaterium* and *B. polymyxa*) or various probiotics (e.g. *Roseobacter gallaeciensis*, *Rhodobacter sphaeroides*, and *Lactobacillus*) in feeds increased the growth, feed efficiency and survival of *L. vannamei* (Wang et al., 2007; Nimrat et al., 2012; Zokaeifar et al., 2012; Sanchez-Ortiz et al., 2016; Interaminense et al., 2018; Sadat Hoseini Madani et al., 2018; Amoach et al., 2019). Probiotics can synthesize vitamins and cofactors that could improve enzymatic activity. They can lead to better growth performance by increasing the absorption of nutrients (Kumar et al., 2013). Most abundantly used probiotics for the increment of growth of shrimp belong to the genera of *Bacillus* (Lin et al., 2004). Tsai et al. (2019) reported that a probiotic, *B. subtilis* E20, was able to secrete digestive enzymes such as, protease and phytase, which could increase shrimp growth and feed efficiency when added to the shrimp diet and improve the nutritional utilization and values of feed ingredients. By the addition of the probiotics in diets, feed intake could be increased eventually resulting in improved protein conversion and feed efficiency in shrimp (Lara-Flores et al., 2003; Son et al., 2009). The significantly increased growth performance and feed utilization efficiency of shrimp in the probiotic groups were due to the improvements in the ADCs of dry matter and crude protein of the diets by *Bacillus* species. However, Cheng et al. (2019) reported that fermentation of *B. subtilis* did not affect the growth performance, feed utilization and survival of *L. vannamei* (average weight 0.76g). The efficacy of the probiotic may vary depending on various conditions such as type, culture method and concentration. Therefore, it is important to select suitable strains for aquatic animals and properly control the amounts added to the diet.

Crustaceans such as shrimp rely on innate immune responses in contrast to higher vertebrates those having acquired immune responses. Therefore, externally supplied probiotics are expected to stimulate relatively uncomplicated innate immune response in shrimp. Generally, probiotics are

known to enhance the immune response by increasing macrophage activity and improving the formation of immunoglobulin and interferons in blood and local antibodies at surfaces of inner gut wall having mucous layers (Fuller, 1992). Moreover, the probiotic *Bacillus* spp. have been identified to secrete a large number of extracellular substances and antimicrobial peptides that can act against a number of pathogenic microorganisms (Soltani et al., 2019). Phagocyte, phenoloxidase, lysozyme, SOD and GPx activities are playing important roles in the shrimp immune system and considered as useful indicators in measuring the immune activity. The results of this study indicated that dietary supplementation of *Bacillus* spp. diets caused an improvement in the innate immunity and antioxidant enzyme activities of *L. vannamei*. In agreement with the results of the current study, some other authors have reported the positive effects of dietary probiotic on immune responses of shrimps (Tseng et al., 2009; Liu et al., 2014; Kumar et al., 2013; Sadat Hoseini Madani et al., 2018; Amoach et al., 2019; Wang et al., 2019). Tseng et al. (2009) showed elevated phagocyte and PO activities in *L. vannamei* fed *B. subtilis* diets (10^7 or 10^8 CFU/g). Liu et al. (2014) also observed that the supplemented *B. subtilis* (10^6 – 10^8 CFU/g) has significantly enhanced levels of phagocytic and PO activities compared to the control group. Significantly higher GPx activity was observed with the addition of commercial probiotic (Bactocell PA 10) in *L. stylirostris* feed (Castex et al., 2009). In the present study, there were no significant differences in lysozyme and SOD activities of *L. vannamei* among all experimental groups. However, when the mixture of *B. subtilis* and *B. licheniformis* (10^4 or 10^8 CFU/g) was added to the diet, significantly higher lysozyme activity was observed in *L. vannamei* (Sadat Hoseini Madani et al., 2018) and significantly higher lysozyme and SOD activities were observed when *B. coagulans* (10^6-10^8 CFU/g) was added to the *L. vannamei* diets (Amoach et al., 2019). The ability of probiotic bacteria to modulate immune response of the host gut has been revealed by nutrigenomic studies. Furthermore, probiotic bacteria could modulate pattern recognition receptors (PRRs) signaling and immune response pathways (Bron et al., 2012). The interaction between microbe-associated molecular patterns (MAMPs) and PRRs is a key stage in the immune response (Wongpanya et al., 2017). PRRs signaling causes the stimulation of innate immune responses

including expression of antimicrobial factors, antigen presentation and immune activation (Baarlen et al., 2013). Also, in the case of modulation of the immunity and antagonism over pathogens, attachment of probiotics on to the mucosal surface of the intestine plays an important role. Moreover, improving adhesion of gut mucosa strengthening epithelial barrier, restricting adhesion or competitive exclusion of pathogens and production of anti-pathogenic substances are important in immune-modulatory actions of probiotics. That evidence can explain why the innate immunity was improved by dietary supplementation of *Bacillus* spp. in this study. Therefore, *Bacillus* species can be used as an immunostimulant to prevent shrimp from diseases by enhancing the innate immunity and antioxidant capacity of shrimp.

In the present study, the direct addition of *Bacillus* spp. into the tank water was not done. However, the diet pellets are unavoidably stand in the water for a while before they are captured and ingested by shrimp right after a feeding. On that occasion, diet pellets could release *Bacillus* spp. into the water from the supplemented diet groups (Cai et al., 2019). The water quality analysis after the meal revealed that ammonium concentrations were significantly lower in *Bacillus* spp. supplemented groups compared to the control group. Therefore, we can assume that the *Bacillus* spp. could improve the quality of shrimp culture water.

The present study demonstrated that the addition of *Bacillus* spp. such as *B. subtilis* and *B. pumilus* in shrimp diets could improve digestibility, growth performance, feed utilization, innate immunity and culture water quality for *L. vannamei*. The individual or mixed dietary supplementation of the *Bacillus spp.* (10^7 CFU/g) in diets at a level of approximately 0.2% could have such positive effects in *L. vannamei* culture.

Table 2-2. Apparent digestibility coefficients (ADC, %) for dry matter and protein of the five experimental diets for *L. vannamei*. Experimental diets were regarded as a control (without probiotic supplementation) and four other diets were prepared by inclusions of *B. subtilis* (*BS*) alone, and a mixture of *BS* and *B. pumilus* (*BP*) at different levels (0.1 x 10^{10} *BS*, 0.2 x 10^{10} *BS*, 0.1 x 10^{10} *BS*/*BP*, 0.2 x 10^{10} *BS*/*BP* for *BS*0.1, *BS*0.2, *BS*/*BP*0.1and *BS*/*BP*0.2 diets, respectively).

	ADCd (%)[1]	ADCp (%)[2]
Control	85.6±0.68[b]	93.5±0.31[b]
*BS*0.1	87.9±0.38[a]	95.2±0.15[a]
*BS*0.2	86.9±0.49[a]	94.8±0.20[a]
BS/*BP*0.1	87.7±0.26[a]	95.0±0.11[a]
BS/*BP*0.2	87.9±0.29[a]	95.1±0.12[a]

Values are mean of quadruplicates and presented as mean ± SD. Values with different superscripts in the same column are significantly different ($P<0.05$).

[1]Apparent digestibility coefficient of dry matter

[2]Apparent digestibility coefficient of protein

Table 2-3. Growth performance and feed utilization of *L. vannamei* (average body weight: 0.14±0.00 g) fed the five experimental diets for 8 weeks. Experimental diets were regarded as a control (without probiotic supplementation) and four other diets were prepared by inclusions of *B. subtilis* (*BS*) alone, and a mixture of *BS* and *B. pumilus* (*BP*) at different levels (0.1 x 10^{10} *BS*, 0.2 x 10^{10} *BS*, 0.1 x 10^{10} *BS*/*BP*, 0.2 x 10^{10} *BS*/*BP* for *BS*0.1, *BS*0.2, *BS*/*BP*0.1and *BS*/*BP*0.2 diets, respectively).

	FBW[1]	WG[2]	SGR[3]	FCR[4]	Survival (%)
Control	10.2±0.69^{b}	7029±506^{b}	7.62±0.13^{b}	1.53±0.04^{a}	90.7±6.11
*BS*0.1	11.5±0.60^{ab}	8085±464^{ab}	7.86±0.10^{a}	1.13±0.12^{b}	88.0±8.00
*BS*0.2	11.9±0.43^{a}	8381±356^{a}	7.93±0.07^{a}	1.11±0.22^{b}	84.0±17.4
BS/*BP*0.1	11.0±0.91^{ab}	7818±772^{ab}	7.80±0.17^{ab}	1.36±0.06^{ab}	88.0±4.00
BS/*BP*0.2	11.8±0.47^{a}	8151±202^{ab}	7.88±0.04^{a}	1.21±0.05^{b}	85.3±4.62

Values are mean of quadruplicates and presented as mean ± SD. Values with different superscripts in the same column are significantly different (P<0.05). The lack of superscript letter indicates no significant differences among treatments.

[1]Final body weight (g)

[2]Weight gain (%) = [(final mean body weight - initial mean body weight)/initial mean body weight] × 100

[3]Specific growth rate (%/day) = 100× [(ln (final body weight)-ln (initial body weight)]/days × 100

[4]Feed conversion ratio = dry feed fed/wet weight gain

Table 2-4. Non-specific immune parameters of *L. vannamei* fed the five experimental diets for 8 weeks. Experimental diets were regarded as a control (without probiotic supplementation) and four other diets were prepared by inclusions of *B. subtilis* (*BS*) alone, and a mixture of *BS* and *B. pumilus* (*BP*) at different levels (0.1 x 10^{10} *BS*, 0.2 x 10^{10} *BS*, 0.1 x 10^{10} *BS*/*BP*, 0.2 x 10^{10} *BS*/*BP* for *BS*0.1, *BS*0.2, *BS*/*BP*0.1and *BS*/*BP*0.2 diets, respectively).

	NBT[1]	PO[2]	Antiprotease[3]	Lysozyme[4]	SOD[5]	GPx[6]
Control	1.65±0.15^{b}	0.21±0.01^{b}	31.6±2.35^{b}	6.55±1.28	68.2±5.28	28.5±1.86^{b}
*BS*0.1	1.79±0.13^{ab}	0.25±0.01^{a}	36.1±3.41^{ab}	7.92±1.48	72.8±7.72	36.7±2.30^{a}
*BS*0.2	1.85±0.03^{a}	0.25±0.01^{a}	36.6±1.83^{a}	7.74±1.23	72.0±7.65	36.4±1.73^{a}
BS/*BP*0.1	1.89±0.03^{a}	0.27±0.02^{a}	34.8±1.18^{ab}	7.70±1.16	72.6±10.3	34.3±2.59^{a}
BS/*BP*0.2	1.86±0.03^{a}	0.25±0.01^{a}	36.1±2.14^{ab}	7.89±1.02	69.6±4.40	32.8±3.26^{ab}

Values are mean of quadruplicates and presented as mean ± SD. Values with different superscripts in the same column are significantly different (P<0.05).

[1]Nitro blue tetrazolium activity (absorbance)

[2]Phenoloxidase activity (absorbance)

[3]Antiprotease (% inhibition)

[4]Lysozyme activity (μg ml^{-1})

[5]Superoxide dismutase (% inhibition)

[6]Glutathione peroxidase (mU ml^{-1})

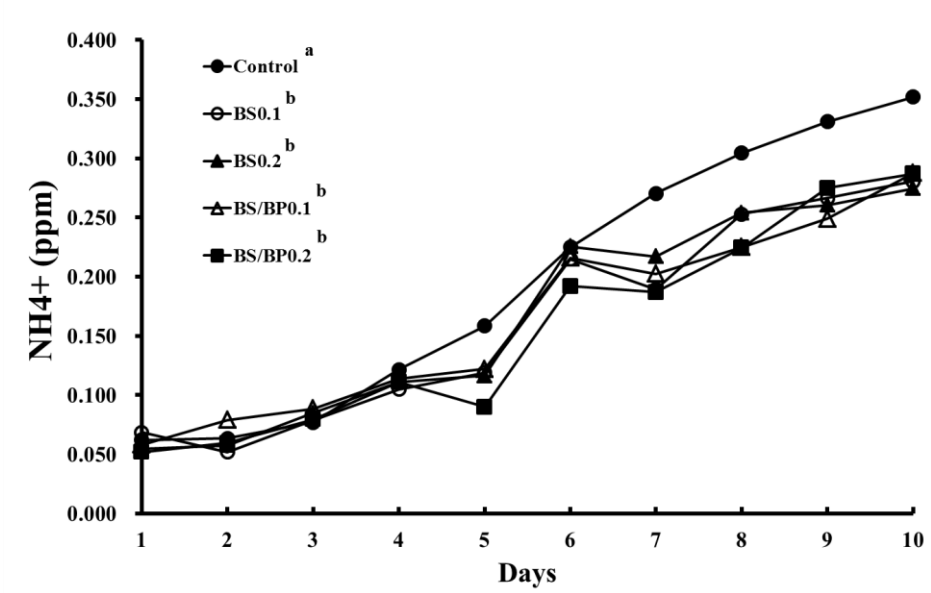

Figure 2-1. Ammonium concentration in the zero water exchange test for 10 days. Triplicate groups of shrimp were hand-fed with one of the test diets four times a day during the zero water exchange period. Experimental diets were regarded as a control (without probiotic supplementation) and four other diets were prepared by inclusions of *B. subtilis* (*BS*) alone, and a mixture of *BS* and *B. pumilus* (*BP*) at different levels (0.1×10^{10} *BS*, 0.2×10^{10} *BS*, 0.1×10^{10} *BS*/*BP*, 0.2×10^{10} *BS*/*BP* for control, *BS*0.1, *BS*0.2, *BS*/*BP*0.1and *BS*/*BP*0.2 diets, respectively).

Table 2-5. Whole-body proximate composition (%) of *L. vannamei* fed the five experimental diets for 8 weeks. Experimental diets were regarded as a control (without probiotic supplementation) and four other diets were prepared by inclusions of *B. subtilis* (*BS*) alone, and a mixture of *BS* and *B. pumilus* (*BP*) at different levels (0.1 x 10^{10} *BS*, 0.2 x 10^{10} *BS*, 0.1 x 10^{10} *BS*/*BP*, 0.2 x 10^{10} *BS*/*BP* for control, *BS*0.1, *BS*0.2, *BS*/*BP*0.1and *BS*/*BP*0.2 diets, respectively).

	Dry matter	Crude protein	Crude lipid	Crude ash
Control	24.9±0.35[a]	76.5±3.46	5.37±0.96	12.9±2.34
*BS*0.1	23.7±0.35[b]	83.9±2.31	5.26±1.05	14.5±0.22
*BS*0.2	23.7±0.39[b]	84.3±2.85	5.09±0.28	12.3±0.33
BS/*BP*0.1	25.1±0.36[a]	79.4±2.23	5.29±0.12	14.8±3.47
BS/*BP*0.2	24.3±0.23[ab]	82.0±3.58	5.40±0.53	13.9±1.46

Values are mean of quadruplicates and presented as mean ± SD. Values with different superscripts in the same column are significantly different (P<0.05). The lack of a superscript letter indicates no significant differences among treatments.

CHAPTER 3

Effect of dietary supplementation of *Bacillus* spp. on growth performance, and resistance of Pacific white shrimp (*Litopenaeus vannamei*) to acute hepatopancreatic necrosis disease (A manuscript submitted to a journal, Israeli journal of the aquaculture – Bamidgeh, IJA_71.2019.1582)

3.1. MATERIALS AND METHODS

3.1.1. Experimental diets and design

Experimental diets were prepared by supplementing powder forms of *Bacillus* spp. (1×10^{10} CFU g^{-1}) with a combination at 0.2% (*BS, B. subtilis* only), 0.4% (*BS/BP,* a mixture of *B. subtilis* and *B. pumilus*) and 0.6% (*BS/BP/BL,* a mixture of *B. subtilis, B. pumilus* and *B. licheniformis*) into a fish meal-based control diet with no supplement. The tested *Bacillus* spp. (1×10^{10} CFU g^{-1}) was provided from the Applied Technology Center, CJ CheilJedang Corp. (Suwon, South Korea). The bacteria were originally isolated from intestinal microflora of shrimp. The ingredients were mixed in a feed mixer (NVM-14, Gyeonggido, South Korea) and pelleted (SP-50, Gumgang Engineering, Daegu, South Korea) after the addition of fish oil and 15% distilled water. The pelleted diets were dried at 23-26 ℃ for 18h, and stored at –20 ℃ until use. The proximate composition of the diets was analyzed by AOAC (2005) (Table 3-1).

Table 3-1. Dietary formulation and proximate composition of the four experimental diets used in the feeding trial of *L. vannamei* (% dry matter).

Ingredients	Experimental diets[1]			
	Control	*BS*	*BS*/*BP*	*BS*/*BP*/*BL*
Fish meal[2]	40.00	40.00	40.00	40.00
Soybean meal[3]	12.81	12.81	12.81	12.81
Squid liver meal	10.00	10.00	10.00	10.00
Wheat flour	25.61	25.61	25.61	25.61
Amygluten 110	3.00	3.00	3.00	3.00
Fish oil A/C	2.00	2.00	2.00	2.00
Amino acid[4]	0.42	0.42	0.42	0.42
Vit/Min premix[5]	5.96	5.96	5.96	5.96
Rice bran	0.20	0.00	0.00	0.00
BS ($1x10^{10}$)	0.00	0.20	0.00	0.00
BS/*BP* ($1x10^{10}$)	0.00	0.00	0.40	0.00
BS/*BP*/*BL* ($1x10^{10}$)	0.00	0.00	0.00	0.60
Proximate composition (% dry matter)				
Moisture	5.68	5.60	5.53	5.42
Crude protein	47.3	47.4	47.1	47.6
Crude lipid	7.31	7.49	7.39	7.38
Crude ash	6.01	6.12	6.14	6.11

[1]Experimental diets were prepared by supplementing powder forms of *Bacillus* spp. (1×10^{10} CFU g^{-1}) with a combination at 0.2% (*BS*, *B. subtilis* only), 0.4% (*BS/BP*, a mixture of *B. subtilis* and *B. pumilus*) and 0.6% (*BS/BP/BL*, a mixture of *B. subtilis*, *B. pumilus* and *B. licheniformis*), and a fish meal-based diet was prepared as a control diet (no supplement).

[2]CJ Cheilhedang Co. Ltd., South Korea (crude protein: 67%)

[3]South America (crude protein: 44%)

[4]Amino acid mixture composition (g/100.4 g dry weight mixture; all L-form amino acids unless otherwise indicated): arginine, 8.88; histidine, 3.00; isoleucine, 4.32; leucine, 7.80; lysine hydrochloride, 9.64; methionine, 2.76; phenylalanine, 4.32; threonine, 4.56; tryptophan, 1.50; valine, 6.12; aspartic acid, 10.6; glutamic acid, 19.4; glycine, 10.8; alanine, 6.72.

[5]Vitamin/Mineral premix (g $kg^{-1}$of mixture): retinol, 3.0; cholecalciferol, 1.0; ascorbic acid, 20.0; tocopherol, 20.0; menadione, 2.0; thiamine, 4.0; riboflavin, 6.0; pyridoxine, 5.0; cobalamin, 6.0; inositol, 54.0; panththenic acid, 12.0; biotin, 0.2; niacin amide, 40.0; folic acid, 2.0; ferrous sulfate, 10.0; copper sulfate, 1.0; zinc sulfate, 30; manganous sulfate, 2.0; cobalt chloride, 10.; potassium iodide, 1.0; potassium, 6.0; sodium selenite, 0.01.

3.1.2. Shrimp and feeding trial

L. vannamei at the post larvae stage were purchased at a local shrimp farm (Tamla shrimp, Jeju, South Korea) and transported to the Institute of Marine Sciences of Jeju National University (Jeju, South Korea). The shrimps were fed a commercial shrimp diet (SAJO DongA One, Seoul, South Korea) for a month. Then, the shrimp (initial mean body weight, 0.51±0.01 g) were randomly selected and distributed into 110 L capacity 16 acrylic tanks at a density of 30 shrimp per tank. Four replicate groups of shrimp were fed one of the four diets at a ratio of 4-12% body weight (four times a day, 08:30, 12:00, 15:30 and 19:00h) for 33 days. At the end of the feeding trial, all the shrimp in each tank were individually weighed for calculation of final body weight (FBW), weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR) and survival.

The salinity was maintained at 30 ppt during the feeding trial. Water quality was maintained within a standard range for *L. vannamei* as follows; temperature (30 - 32 ℃), pH (7 - 8), dissolved oxygen (6.5 - 7.0 mg L^{-1}) and ammonia (0.05 - 0.10 mg L^{-1}).

3.1.3. Bacterial immersion challenge test

The causative strain associated with AHPND was selected by conventional PCR targeting pirA- and pirB-like genes (Han et al. 2015b). A preliminary test was conducted to verify the right time of exposure and proper concentration of the strain (VP_{AHPND}) in tanks prior to the challenge test. After the feeding trial, each eleven shrimp (average weight, 3.6 g) was randomly selected from the respective dietary tanks and distributed into another sets of 110 L capacity 16 acrylic tanks keeping with the four replicates per dietary treatment in a quarantine room. For the infection, *V. parahaemolyticus* was cultured in TSB^{+} overnight (30h) with shaking (150 rpm) to reach 2 x 10^5 CFU ml^{-1} water. The bacterial culture solution of 50 ml (OD_{600}, 2.1) was then added into each tank for the immersion challenge. Shrimp were monitored for mortality every 1 hour. After immersion for 10h, rearing water of each tank was exchanged by 50%. Four replicate groups of shrimp were fed their own respective diet at a ratio of 10% body weight (three times a day, 08:30, 13:30 and 18:30h) and monitored for 193h.

3.1.4. DNA extraction and AHPND qPCR assays

We used AHPND-affected *L. vannamei* in this study to determine the quantities of virulence plasmid. The hepatopancreas was sampled before the challenge (0h), during the challenge (10h and 24h), and at the end of challenge (193h). The qPCR analysis was used to detect the pirA gene from the laboratory bioassay (Table 3-2). DNA of tissue was extracted using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). We used the TaqMan Faster Universal PCR Master Mix (Life Technologies) on 1 cycle at 95 ℃ for 20 sec, 95 ℃ for 3 sec and 40 cycles at 60 ℃ for 30 sec. All samples were analyzed by Qiagen Rotor-Gene Q real-time PCR Detection System (Qiagen, Hilden, Germany).

Table 3-2. Sequences of primers used for quantitative real time PCR.

Primers/probe	Target	Sequence (5′–3′)	Size (bp)	Reference
VpPirA-F	*Pir*A	TTG GAC TGT CGA ACC AAA CG	135	Han et al. (2015c)
VpPirA-R		GCA CCC CAT TGG TAT TGA ATG		
Probe		(FAM)- AGA CAG CAA ACA TAC ACC TAT CAT CCC GGA -(TAMRA)	-	

3.1.5. Histological analysis

The sampled (0, 10, 24 and 193h) hepatopancreas were used for histological analysis by haematoxylin and eosin (H & E) staining method. To minimize tissue damage, Davidson's alcohol-formalin-acetic acid (AFA) was injected into the hepatopancreas of the sampled shrimp using a 1 ml syringe right before the tissue sampling. The dissected hepatopancreas was fixed in 1.5 ml eppendorf tube containing Davidson's AFA for 24h and stored in ethyl alcohol (70%). The fixed tissue was cut into a shape of suitable size for tissue specimen preparation (about 2-3 mm), placed in a cassette for 13h and then followed the standard method. After completion of the staining, the slides were photographed 200X using a microscope program (TCapture, Tucen Photonics) and a phase contrast microscope (BX50, Olympus, Japan).

3.1.6. Statistical analysis

The feeding trial was designed to be completely randomized. Data were analyzed one-way analysis of variance (ANOVA) in SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the mean difference was compared with Duncan's multiple range tests. Statistical significance was determined at $P < 0.05$. Data are presented as mean ± SD. Percentage data were analyzed after transformation into arcsine.

3.2. Results

The growth, feed efficiency and survival of shrimps fed the diets are shown in Table 3-3. The growth (FBW, WG and SGR) was significantly improved in shrimp fed *BS* diet compared to those of shrimp fed other diets. FCR was numerically lower in shrimp fed *BS* diet than that of shrimp fed other diets although it was not significant. No significant difference was observed in survival among all the groups.

During the AHPND challenge studies, the survival was significantly affected by the dietary supplementation of *Bacillus* spp. (Fig. 3-1). The shrimp became lethargy and showed erratic swimming and less diet consumption immediately after the infection of *V. parahaemolyticus*. The *Bacillus* supplemented diets showed significantly increased cumulative survival compared to the control diet. All the shrimp fed the control diet were dead 37h after the infection.

The results of the qPCR analysis of AHPND toxin in the hepatopancreas are shown in Table 3-4. The cycle threshold (Ct) was compared in all treatments. The lower the Ct value means the greater the amount of toxin. AHPND toxin was not detected in all the shrimp before the challenge test (0h). At 10h after the infection, DNA extracted from pooled samples was proved to be AHPND-positive in all diet groups and their Ct values were 25.7, 31.3, 24.4 and 28.3 for the control, *BS*, *BS*/*BP* and *BS*/*BP*/*BL* diets, respectively. At the last sampling (193h), no hepatopancreas samples were collected due to 100% mortality in the control group, while AHPND was not detected in shrimp fed *BS* diet. The Ct value was over 30.0 in shrimp fed the *BS*/*BP* and *BS*/*BP*/*BL* diets (30.3±1.49 and 30.3±0.93, respectively) at 193h after the infection.

The bacterial counts in the shrimp hepatopancreas (Table 3-5) indicated that higher number of beneficial microbes (*Bacillus* spp.) was found in shrimp fed *Bacillus* supplemented diets than in shrimp fed the non-supplemented control diet. The shrimp fed BS/BP diet showed almost 7 fold greater numbers of the microbes than shrimp fed the control diet.

A G-grading system was adapted to classify the severity of the sampled hepatopancreas (Lightner 1996). The G-grading system uses from G0 as a negative to G4 as the highest severity of AHPND. Normal tissue morphology (G0) was observed in all the treatment groups sampled before

the challenge test (Fig. 3-2a and 3-2b). The sampled hepatopancreas at 10h showed the most severe damage (G4) in shrimp fed the control diet (Fig. 3-2c), whereas sloughing and lack of B and R cells in hepatopancreas tubule epithelial cells were found in shrimp fed all the *Bacillus* supplemented diets (G2-3, G1-2 and G1 for *BS*, *BS*/*BP* and *BS*/*BP*/*BL*, respectively) (Fig. 3-2d). At 24h, sloughing and lack of B and R cells in hepatopancreas tubule epithelial cells were observed in shrimp fed *BS*/*BP* and *BS*/*BP*/*BL* diets (G2-3) (Fig. 3-2e and 3-2f). At the end of the challenged point (193h), only a few inflammatory cells were observed instead of the tissue necrosis by AHPND toxin in shrimp fed the *Bacillus* supplemented diets indicating almost recovered hepatopancreas (Fig. 3-2g and 3-2h).

3.3. Discussion

The shrimps in this study grew on very well the experimental diets, and even showed a better growth rate than those reported for similar sized *L. vannamei* (initial mean body weight, 0.5 - 0.6g) (Zhu et al., 2018). The growth performance of *L. vannamei* (from 0.67 to 4.0g size) was significantly improved by the supplementation of two different *B. subtilis* strains strains (Zokaeifar et al., 2012) and a mix of *Bacillus* spp. (*B. licheiformis* and two different strains of *B. subtilis* at a ratio of 1:1:1) in diets (Sanchez-Ortiz et al., 2016) compared to those fed a non-supplemented control diet. However, in the present study, the sole supplementation of *B. subtilis* itself positively affected the growth of shrimp rather than when mixed with other *Bacillus* spp. A number of studies have demonstrated that *Baciilus* spp. as a probiotic in the form of dietary supplements improves growth performances of several shrimp species (NavinChandran et al., 2014). Probiotics are known to assist the digestion processes of shrimps by either producing extracellular enzymes or providing some growth factors (Arellano-Carbajal and Olmos-Soto, 2002). Probiotics can also help to establish a balanced microbial flora in shrimp intestines and thereby improve the digestive process and function. These beneficial effects of probiotics enhance the growth, feed utilization efficiency and eventually the innate immunity of the host animals including shrimps (Shen et al., 2010). And, Balcazar et al. (2006) reported that the improved growth and feed efficiency of *L. vannamei* might be due to prevention of intestinal disorders or lowering levels of some anti-nutritional factors in formulated feeds. In this regard, the improved growth performance of the shrimp fed *B. subtilis* in the present study could be explained. However, differences in the performance of aquatic animals could happen by different preparation processes or strain of *B. subtilis* and by the different application methods in the forms of either dietary additives or direct treatments to the culture water.

The *Bacillus* spp. showed great potential as a dietary therapeutic probiotic for *L. vannamei* in the present study. It is well known that shrimps are completely relying on their innate immunity to protect them from pathogens because they are lack of adaptive immunity (Tassanakajon et al., 2013). In recent years, by the reasons, immune-stimulants and probiotics have been used as dietary supplements for shrimp aquaculture to improve their innate immunity. The decrease in the

immunity is the decisive cause of diseases and the improvement in immunity is directly related to the enhancement of disease resistance. In the present study, significantly higher disease resistance against *V. parahaemolyticus* was observed in shrimp fed the *Bacillus* spp. in either sole or mixed forms. Improved disease resistance of shrimp to *Vibrio* species has been reported by the addition of *Bacillus* spp. into diets. Balcazar et al. (2007) reported the resistance against *V. parahaemolyticus* was increased when *L. vannamei* were fed four different probiotics (*V. alginolyticus*, *B. subtilis*, *P. aestumarina* and *Roseobacter gallaeciensis*). In other studies, the disease resistance of *L. vannamei* to *V. parahaemolyticus* was increased when a mixed probiotic of *R. sphaeroides* and *Afifella marina* (Chumpol et al., 2017) or *Dunaliella* sp. (Felix et al., 2017) was added to their diets. The *B. subtilis* has also been reported to improve the disease resistance of *L. vannamei* aginst *V. alginolyticus* and *V. harveyi* (Tseng et al., 2009; Liu et al., 2014).

The increased resistance to AHPND found in the present study can be explained with the AHPND toxin level and histopathologic changes in hepatopancreases. Quantitative PCR has become a method that can quantitatively compare disease infections (Han et al., 2015c). In the present study, qPCR analysis was used to confirm the anti-AHPND ability of probiotic in the challenge test. The highest mortality was observed at 10h after the infection at which the toxin was observed in all shrimp groups. The shrimp fed *BS* diet seemed to be recovered from the AHPND at 24h and 193h after the infection because the AHPND toxin was not detected in the hepatopancreas of the shrimp.

Histological examination can easily confirm the AHPND bacteria in hepatopancreas by showing damages in the infected tissues. In the present study, normal hepatopancreas tissues were identified in shrimp before the challenge test against *V. parahaemolyticus*. At 10h after the infection, damages in the hepatopancreas were clear and the most severe damages were found in shrimp fed the control diet while tissue necrosis was being progressed in shrimp fed all the *Bacillus* supplemented diets. The hepatopancreas is responsible for immune response and is a target organ for bacteria and virus in shrimp. Aguirre-Guzmán et al. (2010) reported that *V. parahaemolyticus* was detected in the gills and hepatopancreas when the shrimp was challenged. Also, *V.*

parahaemolyticus was concentrated in hepatopancreas and intestine rather than other organs (Khimmakthong and Sukkarum, 2017). The innate immunity which eventually improves resistance to AHPND can be activated by pattern recognition receptors (PRRs) when pathogens were detected in hepatopancreases (Takeuchi and Akira, 2010).

Probiotics can modulate the microflora in the gastrointestinal tracts of host animals leading to beneficial microbe being dominant (Burr et al., 2005). Gram-positive bacteria including *Bacillus* spp. are known to have some specific molecules present in their cell walls. The molecules possess microbe-associated molecular patterns (MAMPs) which can interact with PRR for immune responses (Bron et al., 2012). The PRR signaling can trigger the innate immune responses of the host animals (Baarlen et al., 2013). Recent genomic approaches and analyses already proved the fact that probiotic microorganisms are closely involved in a modulation of the immune system. Lectin, a type of PRRs, facilitates recognition and phagocytosis of a pathogen by an opsonization in crustaceans. Many studies have reported that lectins in AHPND-survived prawns and in the stomach of shrimp after challenged with *V. parahaemolyticus* are upregulated (Thepnarong et al., 2015) and have indicated that lectins are involved in immune response activity in shrimp stomach and hepatopancreas (Ge et al., 2017). Therefore, another reason for the increased resistance to AHPND in the present study is likely to be due to the presence of some specific molecules such as peptidoglycans and capsular polysaccharides in the cell walls of the *Bacillus* spp. and increased beneficial microbes (Table 3-5) in the host microflora. Besides, the *Bacillus* spp. used in this study was derived from marine waters and these probiotics could have settled in the shrimp intestine more easily. The beneficial microbes such as *Bacillus* spp. compete with pathogenic microbes and eventually inhibit their proliferation in the host intestines (Nayak, 2010).

In conclusions, the addition of *Bacillus* spp. such as *B. subtilis, B. pumilus* and *B. licheniformis* as a mixture in shrimp diets can increase the disease resistance of the shrimp to AHPND. Moreover, the sole dietary supplementation of *B. subtilis* at level of approximately 0.2% could be a promising practice to improve growth and feed efficiency of *L. vannamei*.

Table 3-3. Growth performance and feed efficiency of *L.vannamei* (average body weight: 0.51g) fed four experimental diets for 33 days. Experimental diets were prepared by supplementing powder forms of *Bacillus* spp. (1×10^{10} CFU g^{-1}) with a combination at 0.2% (*BS*, *B. subtilis* only), 0.4% (*BS/BP*, a mixture of *B. subtilis* and *B. pumilus*) and 0.6% (*BS/BP/BL*, a mixture of *B. subtilis*, *B. pumilus* and *B. licheniformis*), and a fish meal-based diet was prepared as a control diet (no supplement).

	FBW[1]	WG[2]	SGR[3]	FCR[4]	Survival (%)
Control	3.49±0.12[b]	592±18.9[b]	6.04±0.09[b]	1.30±0.26	80.0±3.33
BS	3.86±0.18[a]	677±31.9[a]	6.37±0.13[a]	1.19±0.12	71.1±6.94
BS/*BP*	3.48±0.17[b]	589±38.3[b]	6.03±0.17[b]	1.34±0.20	75.6±5.09
BS/*BP/BL*	3.57±0.12[b]	607±24.2[b]	6.11±0.11[b]	1.39±0.06	80.0±3.33

Values are mean of quadruplicates and presented as mean ± SD. Values with different superscripts in the same column are significantly different ($P<0.05$). The lack of superscript letter indicates no significant differences among treatments.

[1]Final body weight (g)

[2]Weight gain (%) = [(final mean body weight-initial mean body weight)/initial mean body weight] × 100

[3]Specific growth rate (%) = 100× [(ln(final body weight)-ln(initial body weight)]/days

[4]Feed conversion ratio = dry feed fed/wet weight gain

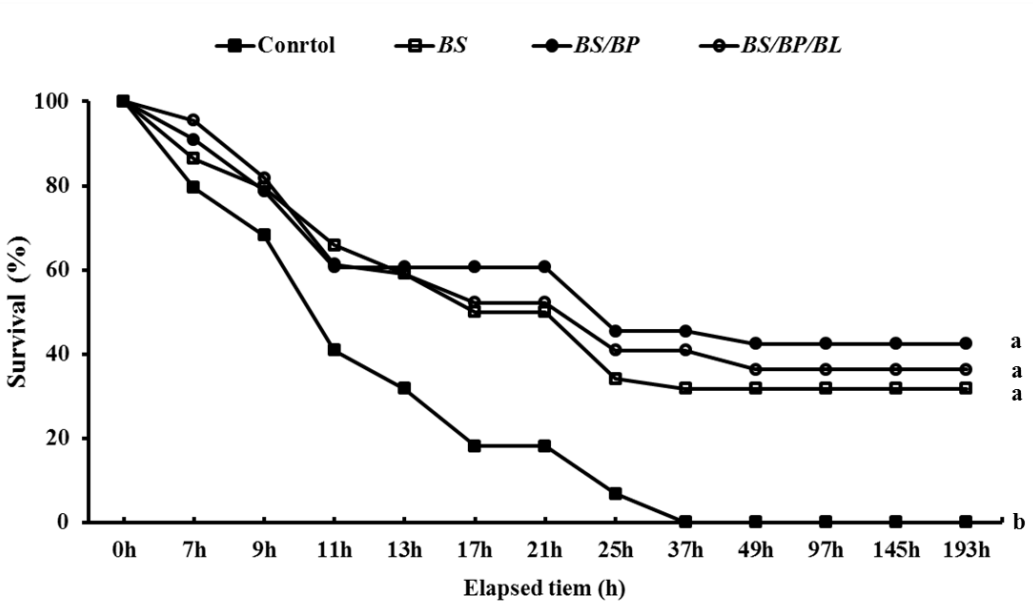

Figure 3-1. Survival of *L. vannamei* after challenged against *V. parahaemolyticus*. The shrimp were immerged with *V. parahaemolyticus* suspension containing 2×10^5 CFU mL^{-1}. Quadruplicate groups of shrimp were hand-fed with one of the test diets three times a day during the challenge period. Experimental diets were prepared by supplementing powder forms of *Bacillus* spp. (1×10^{10} CFU g^{-1}) with a combination at 0.2% (*BS, B. subtilis* only), 0.4% (*BS/BP*, a mixture of *B. subtilis* and *B. pumilus*) and 0.6% (*BS/BP/BL*, a mixture of *B. subtilis*, *B. pumilus* and *B. licheniformis*), and a fish meal-based diet was prepared as a control diet (no supplement). Different letters indicate significant differences in survival at the end of the trial ($P<0.05$).

Table 3-4. The cycle threshold (Ct) values of the hepatopancreas of shrimp sampled at 0, 10, 24, and 193 (final) h after the *V. parahaemolyticus* infection. Experimental diets were prepared by supplementing powder forms of *Bacillus* spp. (1×10^{10} CFU g^{-1}) with a combination at 0.2% (*BS*, *B. subtilis* only), 0.4% (*BS/BP*, a mixture of *B. subtilis* and *B. pumilus*) and 0.6% (*BS/BP/BL*, a mixture of *B. subtilis*, *B. pumilus* and *B. licheniformis*), and a fish meal-based diet was prepared as a control diet (no supplement).

Treatments	Ct values			
	0h	10h	24h	193h
Control	nd[1]	25.7±0.50[c]	24.6±0.87[c]	-[2]
BS	nd[1]	31.3±0.12[a]	nd[1]	nd[1]
BS/*BP*	nd[1]	24.4±0.20[d]	33.6±0.27[a]	30.3±1.49
BS/*BP*/*BL*	nd[1]	28.3±0.10[b]	27.9±1.01[b]	30.3±0.93

Values are mean of quadruplicates and presented as mean ± SD. Values with different superscripts in the same column are significantly different ($P<0.05$).

[1]nd, not detected of toxin

[2]At the last sampling (193h), no hepatopancreas sample was obtained due to 100% mortality in the control group.

Table 3-5. The bacterial counts in the hepatopancreas of *L. vannamei* fed with four experimental diets. The hepatopancreas of *L. vannamei* was sampled at the end of the feeding trial. Experimental diets were prepared by supplementing powder forms of *Bacillus* spp. (1×10^{10} CFU g^{-1}) with a combination at 0.2% (*BS, B. subtilis* only), 0.4% (*BS/BP*, a mixture of *B. subtilis* and *B. pumilus*) and 0.6% (*BS/BP/BL*, a mixture of *B. subtilis*, *B. pumilus* and *B. licheniformis*), and a fish meal-based diet was prepared as a control diet (no supplement).

	(CFU/g)
	Bacillus (TSA^{+})
Control	3.0×10^{3}
BS	1.6×10^{4}
BS/*BP*	2.0×10^{4}
BS/*BP*/*BL*	7.0×10^{3}

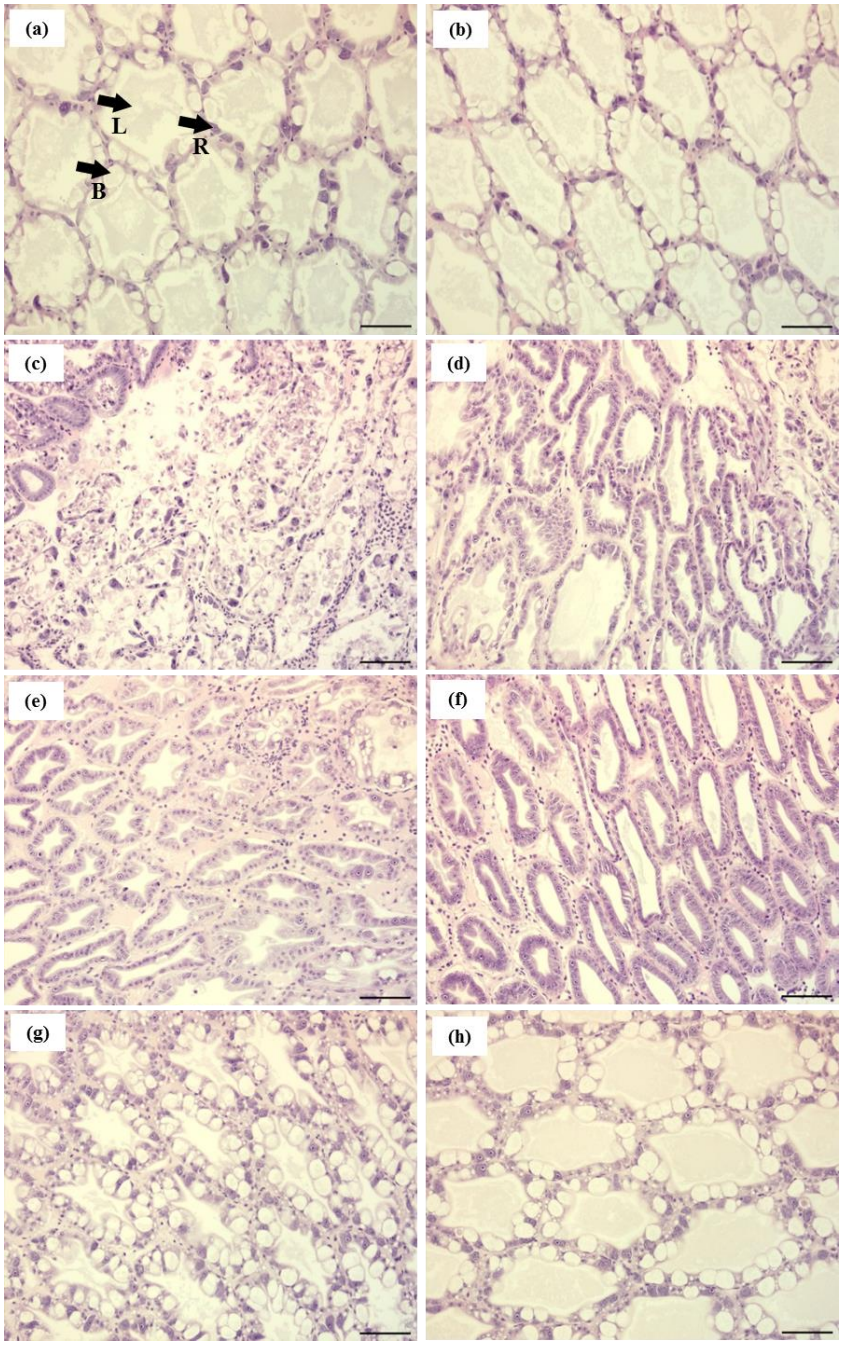

Figure 3-2. Detection of *V. parahaemolyticus* in hepatopancreas tissues in the shrimp at (a) 0h control, (b) 0h *BS*/*BP*/*BL*, (c) 10h control, (d) 10h *BS*, (e) 24h *BS*/*BP*, (f) 24h *BS*/*BP*/*BL* and (g) 193h *BS*, (h) 193h *BS*/*BP*. Two representative figures are listed by sampling time. Hepatopancreas was stained with H & E method. Scale bars, 200 μm. At the last sampling (193h), no hepatopancreas sample was obtained due to 100% mortality in shrimp fed the control diet.

CHAPTER 4

Dietary supplementation of *Bacillus* spp. improves growth performance, innate immunity and disease resistance of Pacific white shrimp *Litopenaeus vannamei* against acute hepatopancreatic necrosis disease or white spot syndrome virus

4.1. MATERIALS AND METHODS

4.1.1. Probiotic bacterium

Bacillus spp. was cultured in the same protocols as chapter 2.

4.1.2. Experimental diets and design

Experimental diets were regarded as a control (without probiotic supplementation) and four other diets were prepared by inclusions of *B. subtilis* (*BS*) A and B species alone, and a mixture of *BS*, *B. pumilus* (*BP*) and *B. licheniformis* (*BL*) at different levels (0.2 x 10^{10} *BS*-A, 0.2 x 10^{10} *BS*-B, 0.2 x 10^{9} *BS*/*BP*/*BL*, 0.2 x 10^{10} *BS*/*BP*/*BL* diets for the Control, *BS*-A, *BS*-B, *BS*/*BP*/*BL*10^{9} and *BS*/*BP*/*BL*10^{10}, respectively). All the dietary ingredients were thoroughly mixed in a feed mixer and pelleted (SP-50, Gumgang Engineering, Daegu, South Korea) in a proper diameter. Then, the pelleted diets were dried in a dryer at 25℃ for 12 h. The diets were analyzed for moisture, crude protein and crude ash by the standard methods (AOAC, 2005). Crude lipid concentration was accordingly analyzed (Folch et al., 1957) (Table 4-1).

Table 4-1. Dietary formulation and proximate composition of the five experimental diets for *L. vannamei* (% dry matter).

Ingredients	Experimental diets[1]				
	Control	*BS*-A	*BS*-B	*BS*/*BP*/*BL*10^9	*BS*/*BP*/*BL*10^{10}
Fish meal (Sardine)	15.0	15.0	15.0	15.0	15.0
Fish meal (Tuna)	15.0	15.0	15.0	15.0	15.0
Squid liver powder	10.7	10.7	10.7	10.7	10.7
Blood meal	1.00	1.00	1.00	1.00	1.00
Soy bean meal	19.0	19.0	19.0	19.0	19.0
Wheat flour	32.2	32.0	32.0	32.0	32.0
Amygluten 110	1.00	1.00	1.00	1.00	1.00
Fish oil	2.50	2.50	2.50	2.50	2.50
Vit/Min premix[2]	3.60	3.60	3.60	3.60	3.60
BS-A ($1x10^{10}$)	0.00	0.20	0.00	0.00	0.00
BS-B ($1x10^{10}$)	0.00	0.00	0.20	0.00	0.00
BS/*BP*/*BL* ($1x10^9$)	0.00	0.00	0.00	0.20	0.00
BS/*BP*/*BL* ($1x10^{10}$)	0.00	0.00	0.00	0.00	0.20
Proximate composition (% dry matter)					
Moisture	6.90	6.58	6.43	6.81	6.66
Crude protein	43.0	42.8	43.1	43.3	43.0
Crude lipid	8.44	8.25	8.45	8.66	8.34
Crude ash	10.1	10.2	10.2	10.2	10.2

[1]A control was prepared without probiotic supplementation and four other diets were prepared by inclusions of *B. subtilis* (*BS*) A and B species alone, and a mixture of *BS*, *B. pumilus* (*BP*) and *B. licheniformis* (*BL*) at different levels (0.2 x 10^{10} *BS*-A, 0.2 x 10^{10} *BS*-B, 0.2 x 10^9 *BS*/*BP*/*BL*, 0.2 x 10^{10} *BS*/*BP*/*BL* diets for the control, *BS*-A, *BS*-B, *BS*/*BP*/*BL*10^9 and *BS*/*BP*/*BL*10^{10}, respectively).

[2]Vitamin/Mineral premix (g $kg^{-1}$of mixture): retinol, 3.0; cholecalciferol, 1.0; ascorbic acid, 20.0; tocopherol, 20.0; menadione, 2.0; thiamine, 4.0; riboflavin, 6.0; pyridoxine, 5.0; cobalamin, 6.0; inositol, 54.0; panththenic acid, 12.0; biotin, 0.2; niacin amide, 40.0; folic acid, 2.0; ferrous sulfate, 10.0; copper sulfate, 1.0; zinc sulfate, 30; manganous sulfate, 2.0; cobalt chloride, 10.; potassium iodide, 1.0; potassium, 6.0; sodium selenite, 0.01.

4.1.3. Shrimp and feeding trial

L. vannamei at the post larvae stage was purchased in a local shrimp farm (Tamla shrimp, Jeju, South Korea) and transported to the Institute of Marine Sciences of Jeju National University (Jeju, South Korea). The shrimps were fed a commercial shrimp diet (CJ Cheiljedang, Incheon, South Korea) for 3 weeks. Then, the shrimp (initial mean body weight, 0.15 g) were randomly selected and distributed into 120 L capacity 50 acrylic tanks at a density of 20 shrimp per tank. Ten replicate groups of shrimp were fed one of the four diets at a ratio of 5 - 18% body weight (five times a day, 08:30, 11:30, 14:30, 17:30 and 20:00h) for 51 days. Water quality was maintained within a standard range for *L. vannamei*.

4.1.4. Sample collection and analyses

At the end of the feeding trial, all the shrimp in each tank were individually weighed for calculation of final body weight (FBW, g), weight gain (WG, %), specific growth rate (SGR, %), feed conversion ratio (FCR) and survival (%). Three shrimp per tank (30 shrimp per dietary treatment) were randomly captured and placed in ice water for 3 min to anesthetize before hemolymph sampling. Hemolymph (200 μL) was withdrawn from the ventral sinus of each shrimp into a 1 mL syringe containing 400 μL of anticoagulant solution (Alsever's solution, sigma). Then, anticoagulant-hemolymph (50 μl) was used to determine macrophage activity (NBT; nitroblue-tetrazolium activity). The remaining anticoagulant-hemolymph mixture was centrifuged at 800 × g for 20 min at 4℃ and the supernatant was stored at −80℃ for non-specific immune responses analyses. Non-specific immune response was performed in the same protocol as chapter 2.

4.1.5. Challenge test

4.1.5.1. AHPND

The causative strain associated with AHPND was selected by conventional PCR targeting pirA- and pirB-like genes (Han et al. 2015c). After the feeding trial, each twelve shrimp (average weight, 5.9 g) was randomly selected from the respective dietary tanks and distributed

into another set of 120 L capacity 16 acrylic tanks keeping with the four replicates per dietary treatment in a quarantine room. For the infection, *V. parahaemolyticus* was cultured in TSB^{+} overnight (30h) with shaking (150 rpm) to reach 2 x 10^{9} CFU ml^{-1} water. The bacterial culture solution of 50 ml (OD_{600}, 2.1) was then added into each tank for the immersion challenge. Shrimp were monitored for mortality every 1 hour. After immersion for 12h, the rearing water of each tank was exchanged by 95%. During the challenge test, the shrimp were fed their own respective diet at a ratio of 10% body weight (three times a day, 08:30, 13:30 and 18:30h) and monitored for 150h.

4.1.5.2. WSSV

For WSSV stock, moribund shrimp (*L. vannamei*) showing white spots on cuticles were collected from an anonymous shrimp farm. After the feeding trial, each sixteen shrimp (average weight, 5.9 g) was randomly selected from the respective dietary tanks and distributed into another set of 120 L capacity 20 acrylic tanks keeping with the four replicates per dietary treatment in a quarantine room. The shrimp were injected intramuscularly with WSSV suspension containing 4.1 x 10^{7} CFU mL^{-1}. After injection for 52h, the rearing water of each tank was exchanged by 95%. During the challenge test, the shrimp were fed their own respective diet at a ratio of 10% body weight (three times a day, 08:30, 13:30 and 18:30h) and monitored for 162h.

4.1.6. DNA extraction and qPCR assays

For qPCR, DNA was extracted from gill and hepatopancreas of the frozen samples using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Then, extracted DNA from the gill was used for the WSSV qPCR assay (Han et al., 2019), hepatopancreas was used for the VP_{AHPND} qPCR assay (Han et al., 2015c). We used the TaqMan Faster Universal PCR Master Mix (Life Technologies) on 1 cycle at 95 ℃ for 20 sec and 40 cycles at 95 ℃ for 1 sec, 60 ℃ for 20 sec. All samples were analyzed by Real Time System TP950 Thermal Cycler Dice^{tm} (TaKaRa).

4.1.7. Histopathology examination of the hepatopancreas tissues

The sampled (0, 20 and 150h) hepatopancreas were used for histological analysis by hematoxylin and eosin (H & E) staining method. After completion of the staining, the slides were photographed 200X using a microscope program (TCapture, Tucen Photonics) and a phase contrast microscope (BX50, Olympus, Japan).

4.1.8. Immunohistochemistry (IHC) examination of the gill tissues

For the IHC examination of WSSV samples (0, 48 and 162h), standard methods (Bell and Lightner, 1988) were followed as the gill tissues of Davidson's AFA-fixed shrimp were processed, embedded in paraffin, and sectioned (4-μm thick). After that, sections were deparaffinized, rehydrated, and blocked using peroxidase blocking reagent (Dako A/S, Glostrup, Denmark). Then, they were incubated at 37 °C for 1 h with 1 μg mL−1 of monoclonal antibody (Envirogen Technologies, Inc.,) and, raised against WSSV envelope protein VP_{28} (Poulos et al., 2001). Biotinylated secondary antibodies and ABC reagents were applied following instructions from the manufacturer (Vectastain ABC kit, Vector Labs, Burlingame, CA, USA). Three min incubation was done with the 3,3′-diaminobenzidine substrate for the color development. Sections were counterstained with hematoxylin, and finally WSSV-positive cells were observed with light microscopy.

4.1.9. Statistical analysis

The feeding trial was designed to be completely randomized. Data were analyzed one-way analysis of variance (ANOVA) in SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the mean difference was compared with Duncan's multiple range tests. Statistical significance was determined at $P<0.05$. Data are presented as mean ± SD. The percentage of data was analyzed after transformation into arcsine.

4.2. Results

Growth performance, feed utilization and survival of shrimp fed the diets are shown in Table 4-2. The growth (FBW, WG and SGR) was significantly improved in *BS*/*BP*/*BL* groups compared to shrimp fed control and *BS*-A diets. Significantly lower FCR and higher PER were obtained in shrimp fed *BS*-B and *BS*/*BP*/*BL*10^9 diets compared to shrimp fed control and *BS*-A diets. No significant difference was observed in survival among all the groups.

Shrimp fed the test diets had significantly higher NBT (except for *BS*-A diet), lysozyme and SOD (except for *BS*/*BP*/*BL*10^{10} diet) activities than shrimp fed control diet (Table 4-3). Regarding GPx activity, *BS*/*BP*/*BL* groups were significantly higher than shrimp fed control and *BS*-A diets.

During the AHPND challenge studies, All the *Bacillus* spp. supplemented diet groups showed numerically increased survival rate compared to the control group even though it was not significant (Fig. 4-1). During the WSSV challenge studies, no significant difference was observed in the survival rate among all the groups (Fig. 4-2). All shrimp fed the control diet were dead within 144h after the infection.

The results of the qPCR analysis of AHPND toxin in the hepatopancreas are shown in Table 4-4. AHPND toxin was not detected in all the shrimp before the challenge test (0h). At 20h after the infection, DNA extracted from pooled samples was proved to be AHPND-positive in all diet groups and their Ct values were 19.2, 28.7, 21.9 and 21.0 for the Control, *BS*-A, *BS*-B and *BS*/*BP*/*BL*10^{10} diets, respectively. At the last sampling (150h), AHPND was not detected in *Bacillus* spp. supplemented groups. However, AHPND has detected in shrimp fed the control diet (Ct value, 25.1).

Any abnormality in tissue morphology was not observed in all the treatment groups sampled before the AHPND challenge test (Fig. 4-3 (a)). The sampled hepatopancreas at 20h showed the most severe damage in all the treatment groups (Fig. 4-3 (b)), whereas sloughing and lack of B and R cells in hepatopancreas tubule epithelial cells were found in shrimp fed all the *Bacillus* supplemented diets. At the end of the challenge point (150h), sloughing and lack of B and R cells in hepatopancreas tubule epithelial cells were observed in the shrimp fed control diet (Fig. 4-3 (c)).

Only a few inflammatory cells were observed instead of the tissue necrosis by AHPND toxin in shrimp fed the *Bacillus* supplemented diets indicating almost recovered hepatopancreas (Fig.4-3 (c)).

Any abnormality in tissue morphology was not observed in all the treatment groups sampled before the WSSV challenge test (Fig. 4-4 (a)). At 48h after the infection, indicative WSSV reactions, brown coloration against pink cytoplasm and purple nuclei, were visualized in the shrimp fed all diets. It was more intensively observed in shrimp fed the control diet than the Bacillus supplemented diet groups (Fig. 4-4 (b)). At the end of the challenge point (162h), only a few inflammatory cells were observed instead of the tissue necrosis by WSSV toxin in shrimp fed the *Bacillus* supplemented diets indicating almost recovered gill (Fig.4-4 (c)). Histopathology analysis was not carried out due to a lack of samples in the control group.

4.3. Discussion

Supplementation of probiotics directly into the feeds leads to enhanced growth and innate immunity of host animals through balancing gut microflora by live cells of probiotics in the intestine. Beneficial effects of dietary supplementations of probiotics especially the *Bacillus* spp. have been demonstrated in several ways (Soltani et al., 2019). Various advantages effects of dietary *Bacillus* supplementation include triggering the activity of digestive enzymes to break down ingested feeds and increasing the gut surface area through improving the structure of microvilli (Ochoa-Solano and Olmos-Soto et al., 2006; Zokaeifar et al., 2012; Chai et al., 2016; Kewcharoen and Srisapoome, 2019). According to Kewcharoen and Srisapoome (2019), doses of *B. subtilis* (10^7 or 10^9 CFU/kg) can positively alter villus height and epithelial cell width of the intestine for the better absorption of the ingested nutrients. The mucosal structure of the intestine plays a major role in nutrient absorption and digestion and especially in immune function by acting as a barrier to the pathogenic colonization. It is reported that the supplementation of *B. subtilis* could enhance such structural development in the intestine (Amoch et al., 2019; Kewcharoen and Srisapoome, 2019; Xie et al., 2019). In the present study, the addition of mixed *Bacillus* spp. in the diets might have increased the digestive absorption of the diet which led to the increased shrimp growth. Also, higher activity of digestive enzymes, such as amylase, protease and lipase which might be stimulated by the probiotics in the gut may be another reason for the improved growth performance of shrimp. *Bacillus* spp. are capable to produce a wide range of extracellular substances, such as trypsin, lipase, amylase and antimicrobial peptides. They may be compensating the growth performance along with reducing infections (Cheng et al., 2013; Xie et al., 2019). In the present study, the mixed supplementation of *Bacillus* spp. such as *B. subtilis*, *B. pumilus* and *B. licheniformis* positively affected the growth of shrimp rather than *B. subtilis* alone. Wang et al. (2019) mentioned that a mixture of probiotics could improve growth, health status and probiotic efficiency in shrimp rather than a single use of probiotic in the diet. Furthermore, many studies have reported that the growth performance, feed efficiency and survival rate of shrimp were increased by adding mixed *Bacillus* species (10^4 – 10^{10} CFU/g) such as *B. subtilis, B. licheniformis,*

B. thuringiensis, *B. megaterium*, *B. polymyxa* (Dong et al., 2014; Sanchez-Ortiz et al., 2016; Sekar et al., 2016; Interaminense et al., 2018; Sadat Hoseini Madani et al., 2018; Cai et al., 2019; Nimrat et al., 2019).

Probiotics play many important roles in aquaculture, however, protections mechanism from pathogenic organisms by competitive exclusion, production of inhibitory compounds and triggering antiviral actions is prominent under the immunity improving function (Pandiyan et al. 2013). Since the disease outbreaks are crucial in the shrimp aquaculture, strengthening the immune functions and increasing the survival rate of shrimp is essential issue because better health status could indirectly lead to better growth performance. Probiotics also could control and inhibit pathogenic organisms in shrimp by production of antipathogenic substances (Duenas-Decamp et al., 2008), enhancement of immunostimulatory functions (Tseng et al., 2009) and manipulation of gut microflora (Luis-Villasenor et al., 2013). Raaijmakers et al. (2010) reported that the *Bacillus* bacteria can produce anti-microbial substances and an immunostimulatory action. Our results have confirmed that dietary supplementation of the *Bacillus* spp. can improve innate immunity and antioxidant enzyme activities of *L. vannamei*. Lysozyme is an enzyme that capable of acting against the cell wall pathogens and it is considered as a key immune parameter in shrimp (Burge et al., 2007). In the present study, lysozyme in the plasma was significantly enhanced in sole or mixed *Bacillus* spp. treated groups compared to the non-supplemented control group. Xie et al. (2019) reported that addition of dietary complex probiotics at levels of 0.2 - 0.8% for *L. vannamei* significantly increased lysozyme activity. Dong et al. (2014) also observed significantly enhanced lysozyme activity compared to the control group when a mixture of *B. subtilis* and *B. licheniformis* (10^{10} CFU/g) was supplemented in the diets. In addition, many studies have reported that lysozyme activity is significantly increased when sole or mixed probiotics, such as *B. flexus*, *L.actobacillus pentosus*, *L. Fermentum* and *Saccharomyces cerevisiae* are added in shrimp diets (Sadat Hoseini Madani et al., 2018; Cai et al., 2019; Wang et al., 2019). In this study, the significant increase in SOD and GPx activities might be dud to the supplemented probiotics. In the process of phagocytosis, reactive oxygen species (ROS) are produced and SOD is acting as the main O_2

scavenger. This reaction produces H_2O_2 and O_2 as the products. Therefore, SOD is considered as an important antioxidant enzyme in the innate immune reactions of crustaceans. The level of antioxidant enzyme activities, such as SOD and GPx can be used as a parameter to estimate the level of pathogenic infection (Sekar et al., 2016). *L. vannamei* fed with mixed *Bacillus* spp. (*B. licheniformis* and *B. subtilis*, $6x10^6$ CFU/g) showed a higher level of SOD gene expression (Sanchez-Oriz et al., 2016). Likewise, dietary administration of 10^6 - 10^9 CFU/g *B. licheniformis* increased SOD activity of *M. japonicas* (Kumar et al., 2013). Similarly, enhanced activities of antioxidant enzymes by probiotics have been demonstrated (Amoach et al., 2019; Xie et al., 2019). Along with those results, it can be stated that the probiotic fed shrimp are more immunocompetent against several pathogens.

Antibiotics and chemical drugs are cost-effective but tend to be toxic to the animals. Probiotics are considered as an environmentally friendly solution in controlling various pathogens in aquaculture through dietary supplementation (Kewcharoen and Srisapoome, 2019). However, there is little information on the effects of probiotics and/or prebiotics to prevent diseases of *L. vannamei* or other Penaeid shrimp species. The *Bacillus* spp. showed great potentials as a dietary therapeutic probiotic for *L. vannamei* in the present study. Although there was no significant difference in all the experimental groups, a numerically increased survival rate was observed in shrimp fed *Bacillus* spp. against VP_{AHPND} disease. In our previous study, significantly higher disease resistance against VP_{AHPND} was observed in shrimp fed the *Bacillus* spp. (10^{10} CFU/g) as either sole or mixed forms (Lee et al., 2019). Kewcharoen and Srisapoome (2019) reported that the supplementation of 10^7 or 10^9 CFU/g of *B. subtilis* in feed increased disease resistance to AHPND in *L. vannamei*. Other studies have reported that the supplementation of sole or mixed probiotics such as *B. licheniformis, B. flexus, B. circulans, B. aryabhattai, L. pentosus* to shrimp feed can control diseases of *Vibrio* species such as *V. parahaemolyticus, V. harveyi, V. alginolyticus* (Liu et al., 2014; Zokaeifar et al., 2014; Amoch et al., 2019; Cai et al., 2019; Tepaamorndech et al., 2019; Vogeley et al., 2019). The enhanced resistance to AHPND in the present study can be elaborated with the AHPND toxin level and histopathologic changes in hepatopancreas. The hepatopancreas is

considered as the main organ for the reserve and detoxification of xenobiotics in crustaceans. The sensitivity of hepatopancreas to physiological and environmental changes is relatively higher. In the present study, confirmation of the anti-AHPND ability of probiotic was demonstrated using qPCR analysis in the challenge test (Han et al. 2015c). The first mortality was observed at 12h after the infection, and AHPND toxin was detected in all experimental groups at 20h. AHPND toxin was not detected in the hepatopancreas of the shrimp fed *Bacillus* spp. supplemented diets at 150h after the infection which indicates that they were recovered from the AHPND. AHPND toxin was detected only in the hepatopancreas of shrimp fed the control diet at the time. Similar to the AHPND toxin results, tissue necrosis was observed in all hepatopancreas tissues at 20h after the infection. However, hepatopancreas tissues had not been recovered in the control group even at 150h after the infection whereas those were already recovered in *Bacillus* spp. fed shrimp groups.

In this study, there was no positive effect on the cumulative survivals of shrimp against WSSV infection even in the *Bacillus* spp. fed shrimp groups. According to the result of confirming WSSV infection by IHC method, there was a markedly different level between the control group and the *Bacillus* spp. fed groups at 48h after the infection. Brown coloration against pink cytoplasm and purple nuclei, indicative WSSV-positive reactions, were visualized in the shrimp of all groups at 48h after the infection. In the control group, the analysis was not able to be performed because of 100% mortality at 144h after the infection. However, the gill tissue of the *Bacillus* spp. fed groups was gradually recovered. When *L. vannamei* was challenged with WSSV, 73.7% and 70.1% of shrimp were reported to survive in 10^7 or 10^9 CFU/g of *Bacillus* spp. groups, respectively (Chai et al., 2016). As a result of the challenge test for 96h after WSSV infection, the cumulative survival was significantly enhanced in shrimp fed probiotics of 10^7 CFU/g *Enterobacter hominis* and *Lactobacillus* (Zuo et al., 2019). Decreased immunity can be used as a criterion for disease determination and an enhancement of immune function is directly connected to the improved disease resistance. Therefore, the results of this study suggest that the increased innate immunity of *L. vannamei* by the supplementation of *Bacillus* spp. in diets could have a positive effect on the increase of disease resistance against AHPND or WSSV infections.

In conclusion, the present study showed that the addition of *Bacillus* spp. such as *B. subtilis*, *B. pumilus* and *B. licheniformis* into diets could improve growth performance, feed utilization, innate immunity and disease resistance against AHPND of *L. vannamei*. A proper dose of the mixed dietary supplementation of the three *Bacillus spp.* (10^9 CFU/g) in diets would be approximately 0.2%.

Table 4-2. Growth performance and feed efficiency of *L.vannamei* (average body weight: 0.15g) fed five experimental diets for 51 days. A control was prepared without probiotic supplementation and four other diets were prepared by inclusions of *B. subtilis* (*BS*) A and B species alone, and a mixture of *BS*, *B. pumilus* (*BP*) and *B. licheniformis* (*BL*) at different levels (0.2 x 10^{10} *BS*-A, 0.2 x 10^{10} *BS*-B, 0.2 x 10^{9} *BS*/*BP*/*BL*, 0.2 x 10^{10} *BS*/*BP*/*BL* diets for the control, *BS*-A, *BS*-B, *BS*/*BP*/*BL*10^{9} and *BS*/*BP*/*BL*10^{10}, respectively).

	FBW[1]	WG[2]	SGR[3]	FCR[4]	PER[5]	Survival (%)
Control	5.92±0.23^{b}	3826±148^{bc}	7.49±0.08^{bc}	1.30±0.05^{a}	1.79±0.07^{b}	82.5±8.58
BS-A	5.85±0.14^{b}	3768±103^{c}	7.46±0.05^{c}	1.30±0.06^{a}	1.80±0.08^{b}	87.1±6.36
BS-B	6.30±0.50^{ab}	4095±338^{ab}	7.62±0.17^{ab}	1.18±0.10^{b}	1.98±0.16^{a}	89.4±6.35
BS/BP/BL10^{9}	6.49±0.44^{a}	4228±310^{a}	7.68±0.15^{a}	1.17±0.13^{b}	1.99±0.21^{a}	89.0±6.52
BS/*BP*/*BL*10^{10}	6.43±0.55^{a}	4166±376^{a}	7.65±0.18^{a}	1.21±0.11^{ab}	1.93±0.17^{ab}	82.8±9.39

Values are mean of ten replicate and presented as mean ± SD. Values with different superscripts in the same column are significantly different (P<0.05). The lack of superscript letter indicates no significant differences among treatments.

[1]Final body weight (g)

[2]Weight gain (%) = [(final mean body weight-initial mean body weight)/initial mean body weight] × 100

[3]Specific growth rate (%) = 100× [(ln(final body weight)-ln(initial body weight)]/days

[4]Feed conversion ratio = dry feed fed/wet weight gain

[5]Protein efficiency ratio = wet weight gain/ total protein given

Table 4-3. Non-specific immune response of *L. vannamei* fed the five experimental diets for 51 days. A control was prepared without probiotic supplementation and four other diets were prepared by inclusions of *B. subtilis* (*BS*) A and B species alone, and a mixture of *BS*, *B. pumilus* (*BP*) and *B. licheniformis* (*BL*) at different levels (0.2 x 10^{10} *BS*-A, 0.2 x 10^{10} *BS*-B, 0.2 x 10^{9} *BS*/*BP*/*BL*, 0.2 x 10^{10} *BS*/*BP*/*BL* diets for the control, *BS*-A, *BS*-B, *BS*/*BP*/*BL*10^{9} and *BS*/*BP*/*BL*10^{10}, respectively).

	NBT[1]	Lysozyme[2]	PO[3]	SOD[4]	GPx[5]
Control	1.85±0.15^{b}	1.02±0.22^{b}	0.21±0.04	73.8±9.90^{b}	64.7±7.35^{c}
BS-A	2.18±0.33^{ab}	1.68±0.53^{a}	0.26±0.13	82.3±5.55^{a}	64.0±6.44^{c}
BS-B	2.29±0.45^{a}	2.24±0.80^{a}	0.30±0.17	82.2±4.45^{a}	69.0±7.80^{bc}
BS/BP/BL10^{9}	2.33±0.24^{a}	2.27±0.70^{a}	0.25±0.09	82.9±3.62^{a}	80.4±8.42^{a}
BS/*BP*/*BL*10^{10}	2.43±0.50^{a}	1.86±0.69^{a}	0.25±0.12	81.0±4.53^{ab}	73.3±6.25^{ab}

Values are mean of ten replicate groups and presented as mean ± S.D. Values with different superscripts in the same column are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.

[1]Nitro blue tetrazolium activity (absorbance)

[2]Lysozyme activity (μg ml^{-1})

[3]Phenoloxidase activity (absorbance)

[4]Superoxide dismutase (% inhibition)

[5]Glutathione peroxidase (mU ml^{-1})

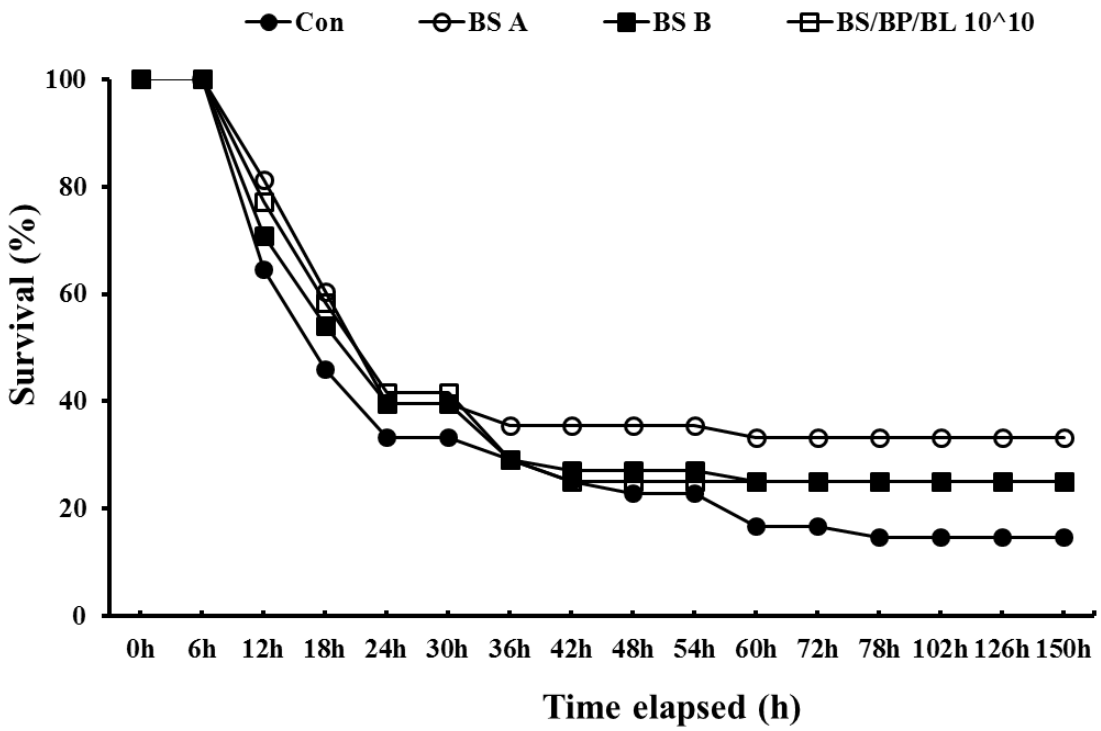

Figure 4-1. Survival rate of *L. vannamei* after challenged against *V. parahaemolyticus*. The shrimp were immerged with *V. parahaemolyticus* suspension containing 2×10^9 CFU mL^{-1}. Quadruplicate groups of shrimp were hand-fed with one of the test diets three times a day during the challenge period. A control was prepared without probiotic supplementation and four other diets were prepared by inclusions of *B. subtilis* (*BS*) A and B species alone, and a mixture of *BS, B. pumilus* (*BP*) and *B. licheniformis* (*BL*) at different levels (0.2×10^{10} *BS*-A, 0.2×10^{10} *BS*-B, 0.2×10^9 *BS*/*BP*/*BL*, 0.2×10^{10} *BS*/*BP*/*BL* diets for the control, *BS*-A, *BS*-B, *BS*/*BP*/*BL*10^9 and *BS*/*BP*/*BL*10^{10}, respectively).

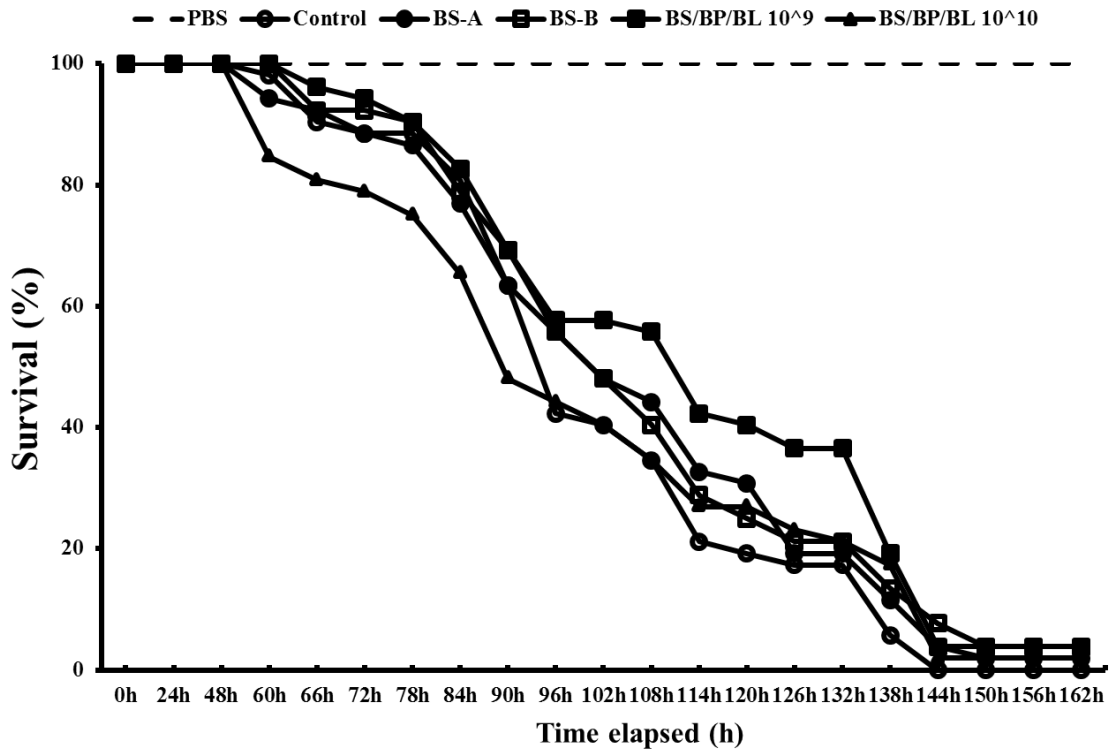

Figure 4-2. Survival rate of *L. vannamei* after challenged against WSSV. The shrimp were injected with WSSV suspension containing 4.1 x 10^7 CFU mL^{-1}. Quadruplicate groups of shrimp were hand-fed with one of the test diets three times a day during the challenge period. A control was prepared without probiotic supplementation and four other diets were prepared by inclusions of *B. subtilis* (*BS*) A and B species alone, and a mixture of *BS*, *B. pumilus* (*BP*) and *B. licheniformis* (*BL*) at different levels (0.2 x 10^{10} *BS*-A, 0.2 x 10^{10} *BS*-B, 0.2 x 10^9 *BS*/*BP*/*BL*, 0.2 x 10^{10} *BS*/*BP*/*BL* diets for the control, *BS*-A, *BS*-B, *BS*/*BP*/*BL*10^9 and *BS*/*BP*/*BL*10^{10}, respectively).

Table 4-4. The cycle threshold (Ct) values of the hepatopancreas of shrimp sampled at 0, 20, and 150 (final) h after the VP_{AHPND} infection. A control was prepared without probiotic supplementation and four other diets were prepared by inclusions of *B. subtilis* (*BS*) A and B species alone, and a mixture of *BS*, *B. pumilus* (*BP*) and *B. licheniformis* (*BL*) at different levels (0.2×10^{10} *BS*-A, 0.2×10^{10} *BS*-B, 0.2×10^{9} *BS*/*BP*/*BL*, 0.2×10^{10} *BS*/*BP*/*BL* diets for the control, *BS*-A, *BS*-B, *BS*/*BP*/*BL*10^{9} and *BS*/*BP*/*BL*10^{10}, respectively).

Treatments	0h	20h	150h
Control	nd[1]	19.2	25.1
BS-A	nd[1]	28.7	nd[1]
BS-B	nd[1]	21.9	nd[1]
BS/*BP*/*BL*10^{10}	nd[1]	21.0	nd[1]

Values are mean of quadruplicates and presented as mean ± SD. Values with different superscripts in the same column are significantly different ($P<0.05$).

[1]nd, not detected of toxin

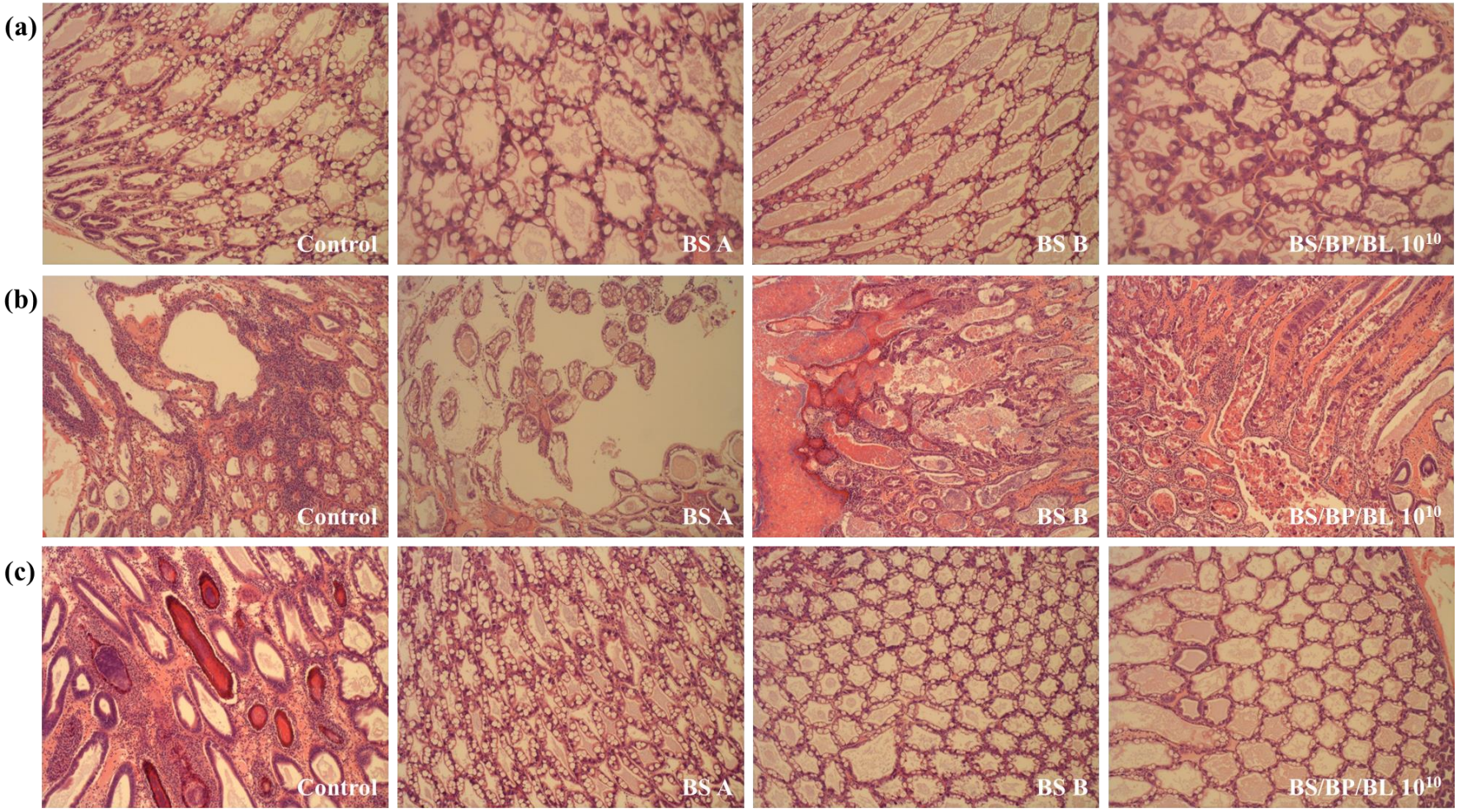

Figure 4-3. Histopathological features of the hepatopancreas of *L. vannamei*. Hepatopancreas was stained with H&E method. The shrimp were challenged by *V. parahaemolyticus*. (a) 0h, (b) 20h, (c) 150h.

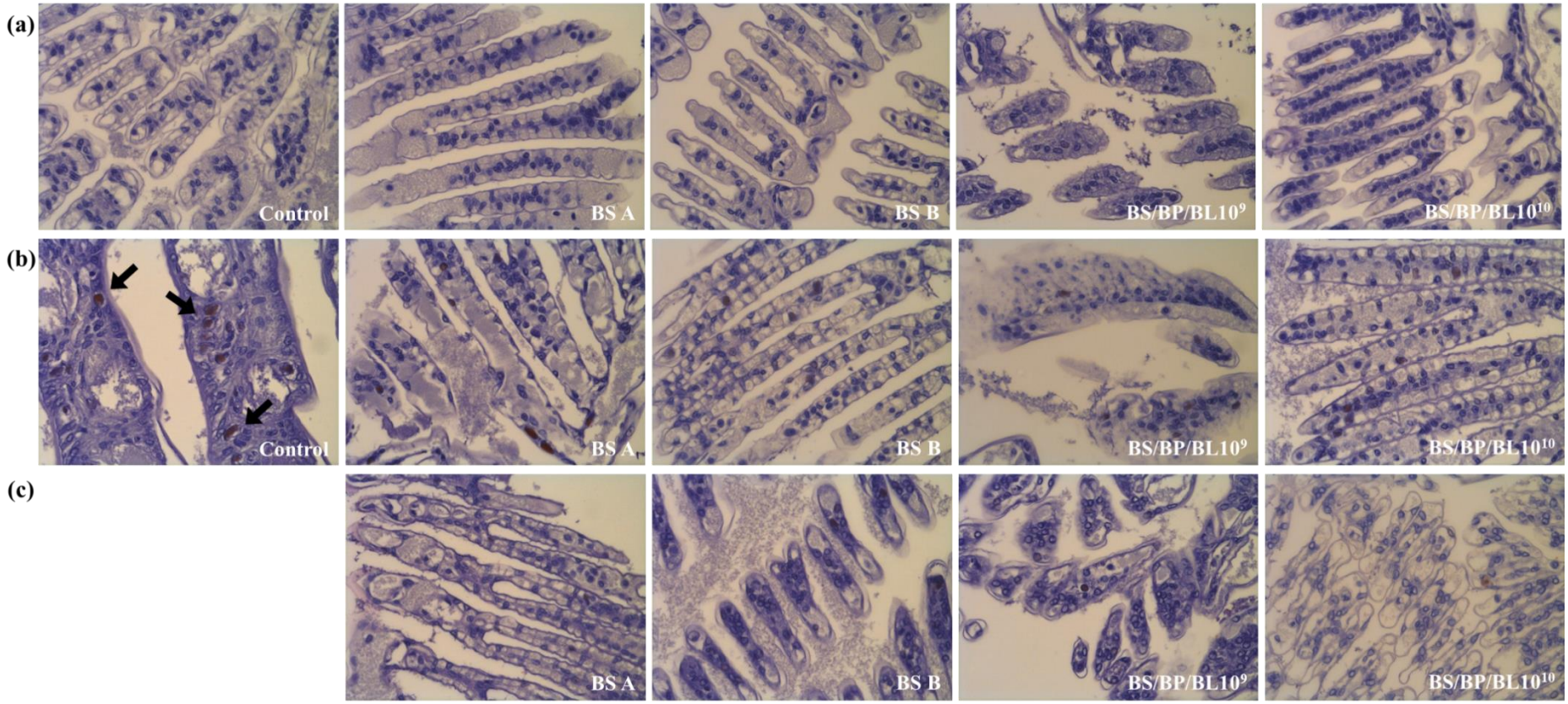

Figure 4-4. Histopathological features of the gill of *L. vannamei*. Gill was stained with IHC method. The shrimp were challenged by WSSV. (a) 0h, (b) 48h, (c) 162h. At the last sampling, no gill sample was obtained due to 100% mortality in the control group.

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LIST OF PUBLICATIONS

International Journals

1. **Chorong Lee**, Ji-Hoon Cha, Min-Gi Kim, Jaehyeong Shin, Seo Hyung Woo, Sung Hun Kim, Jae Won Kim, Seung-Cheol Ji, Kyeong-Jun Lee. (2019) The effects of dietary *Bacillus subtilis* on innate immune response, hematological parameters, growth performance and resistance of juvenile olive flounder (*Paralichthys olivaceus*) against *Streptococcus iniae*. Journal of the world aquaculture society (early view).
2. Jee Eun Han, Ji-Eun Kim, Hayun Jo, **Chorong Lee**, Kyeong-Jun Lee, Jong-Su Eun, Jae-Won Kim. (2019) Increased susceptibility of white spot syndrome virus-exposed *Litopenaeus vannamei* to *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease. Aquaculture, 512, 734333.
3. G. L. B. E. Gunathilaka, Min-Gi Kim, **Chorong Lee**, Jaehyeong Shin, Bong-Joo Lee and Kyeong-Jun Lee. (2019) Effects of taurine supplementation in low fish meal diets for red seabream (*Pagrus major*) in low water temperature season. Fisheries and Aquatic Sciences, 22:23.
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