A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Supplemental effects of biofloc powder on growth performance, innate immunity and disease resistance of white shrimp *Litopenaeus vannamei*

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Department of Marine Life Science

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

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Supplemental effects of biofloc powder on growth performance, innate immunity and disease resistance of white shrimp *Litopenaeus vannamei*

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This dissertation has been examined and approved.

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요약문

이 연구는 바이오플락 기술을 통해 생산된 양식사육수 내 유용유기물(Biofloc, BF)의 사료 내 첨가가 흰다리새우의 성장, 비특이적면역력, 질병저항성 및 항생제 대체 가능성을 조사하기 위해 수행되었다. 총 3개의 실험이 실시되었으며(Exp-Ⅰ, Ⅱ, Ⅲ), Exp-Ⅰ의 실험사료는 총 7개가 되도록 설계하였다. 7개의 실험사료 중 BF이 첨가되지 않은 대조구를 제외한 6개의 실험사료에는 BF를 단계별(0.5, 1.0, 2.0, 4.0, 6.0, 8.0%)로 첨가하였다. 사양실험은 3반복씩 21개, 92L 아크릴수조에서 진행되었으며, 실험새우 초기 평균무게는 1.01 ± 0.01 g으로 수조 당 18마리씩 무작위로 선택하여 배치되었다. 사료공급은 어체중의 8~10%로 제한공급 하였고, 1일 4회(08:00, 12:00, 16:00, 18:00)에 나누어 8주간 공급 하였다. 성장실험 결과, 최종 무게와 일간성장률 모두 대조구와 비교하여 BF 4% 그룹에서 유의적으로 높은 경향을 보였다. 사료전환효율과 단백질전환효율에서도 BF 4% 그룹이 대조구에 비해 유의적으로 높았다. 사료섭취량은 대조구와 비교하여 BF 8% 그룹이 유의적으로 낮았으며, 생존율은 BF 0.5% 첨가그룹이 모든 실험구와 비교하여 유의적으로 높았다. 비특이적 면역분석결과, 대식세포 활성에서는 BF 0.5%와 4.0% 그룹이, Glutathione peroxidase (GPx) 활성에서는 BF 6.0% 그룹이 대조구에 비해 유의적으로 높았으며, Phenoloxidase (PO) 활성에서는 BF 6.0% 그룹이 타 실험구에 비하여 유의적으로 높았다. 병원성 균주(*Vibrio harveyi*)를 이용한

공격실험 결과, BF 4.0% 그룹이 대조구에 비해 유의적으로 높은 생존율을 보였다.

Exp-Ⅱ의 실험사료는 총 7개가 되도록 설계하였다. 7개의 실험사료 중 BF가 첨가되지 않은 대조구를 제외한 5개의 실험사료에는 BF를 단계별(0.25, 0.5, 1.0, 2.0, 4.0%)로 첨가하였고, 나머지 1개의 실험사료에는 항생제 대체 가능성을 조사하기 위하여 oxytetracyline (Antibiotic, OTC)을 0.5% 함량으로 첨가하였다. 사양실험은 3반복씩 21개, 120L 아크릴수조에서 진행하였으며, 새우 초기 평균무게는 0.70 ± 0.00 g으로 수조 당 20마리씩 무작위로 선택하여 배치하였다. 사료공급은 어체중의 8~13%로 제한공급 하였고, 1일 4회(08:00, 12:00, 16:00, 18:00)에 나누어 6주간 공급 하였다. 성장실험 결과, 모든 실험구에서 유의적인 차이가 없었다. 비특이적 면역분석 결과 대식세포 활성에서는 BF 1.0% 그룹이 2.0% 그룹보다 유의적으로 높았으며, immunoglobulin 농도는 BF 0.25% 첨가그룹이 대조구에 비해 유의적으로 높게 나타났다. *V. harveyi*를 이용한 공격실험 결과 항생제를 첨가한 OTC 실험구가 BF 0.5% 실험구에 비해 높은 생존율을 보였다.

Exp-Ⅲ의 실험사료는 총 8개가 되도록 설계하였다. 8개의 실험사료 중 BF가 첨가되지 않은 대조구를 제외한 5개의 실험사료에는 BF을 단계별(0.25, 0.5, 1.0, 2.0, 4.0%)로 첨가하였고, 나머지 2개의 실험사료에는 항생제 대체가능성을 조사하기 위하여 OTC와 penicillin (Antibiotic, Amp)을 0.5% 함량으로 첨가하였다. 사양실험은 3반복씩 24개, 120L 아크릴수조에서

진행하였으며, 새우 초기 평균무게는 1.00 ± 0.00 g으로 수조 당 15마리씩 무작위로 선택하여 배치하였다. 사료공급은 어체중의 8~13%로 제한공급 하였고, 1일 4회(09:00, 13:00, 17:00, 19:30)에 나누어 7주간 공급 하였다. 성장실험 결과, 사료전환효율에서는 대조구와 BF 0.25%, Amp 실험구가 BF 4.0% 실험구보다 유의적으로 높은 값을 보였으며, 단백질전환효율에서는 대조구와 Amp 그룹이 BF 4.0% 그룹보다 유의적으로 높았다. 사료섭취량은 BF 4.0% 실험구가 대조구에 비해 유의적으로 높았다. *V. harveyi*를 이용한 공격실험 결과 대조구와 BF 0.25% 그룹이 Amp 그룹에 비해 높은 생존율을 보였으며 나머지 실험구에서는 유의적인 차이가 없었다. 이상의 세가지 사양실험 결과, Exp-Ⅰ에서는 바이오플락의 사료 내 첨가가 성장, 사료효율, 비특이적면역력과 질병저항성을 개선시킬 수 있음을 확인 할 수 있었다. 하지만 Exp-Ⅱ, Ⅲ에서는 바이오플락의 사료 내 첨가가 성장과 사료효율에는 아무런 영향을 미치지 않았다. 따라서, 흰다리새우용 배합사료에 있어서의 바이오플락 이용성은 바이오플락의 수거방법, 유기물함량, 유용균주의 양 및 종류 등에 따라 달라질 수 있기 때문에 이에 관한 보다 다양한 연구가 부가적으로 필요할 것으로 사료된다.

ABSTRACT

Three feeding trials were carried out to examine the effects of biofloc powder on growth performance, innate immunity and disease resistance of white shrimp *Litopenaeus vannamei*. In experiment Ⅰ, seven experimental diets were prepared with supplementation of graded levels of dried biofloc powder by 0, 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0% (designated as Con, BF0.5, BF1, BF2, BF4, BF6 and BF8, respectively). Triplicate groups of shrimp (1.01 ± 0.01 g) were hand-fed with one of the diets four times a day for 8 weeks. At the end of the feeding trial, significantly ($P < 0.05$) higher growth performance and feed utilization were obtained at BF4 groups compared to those fed the Con diet. Significantly lower whole-body protein was recorded at 2.0 and 4.0% biofloc levels and lipid content decreased in shrimp fed BF4 and BF6 diets. Non-specific immune responses of shrimp were improved by dietary supplementation of biofloc. Significantly lower respiratory burst activity was observed in shrimp fed dietary biofloc levels of 0.5 and 4.0% compared to the shrimp offered the Con diet. Dietary inclusion of biofloc at the level of 4.0% significantly increased disease resistance of shrimp against *Vibrio harveyi*. The results indicates that biofloc can be used as a dietary supplement for growth performance, innate immunity and disease resistance of juvenile Pacific white shrimp and that its optimum inclusion level would be approximately 4.0%.

In experiment Ⅱ, six experimental diets were prepared with supplementation of graded levels of dried biofloc powder by 0, 0.25, 0.5, 1.0, 2.0 and 4.0% (designated as, Con, BF0.25, BF0.5, BF1, BF2 and BF4, respectively). Another experimental diet was prepared by supplementing 0.5% oxytetracyline (antibiotic, OTC) to the basal (control)

diet. Triplicate groups of shrimp (0.70 ± 0.00 g) were hand-fed with one of the diets four times a day for 6 weeks. The results indicates that dietary supplementation of biofloc has no significant effect on shrimp growth performance and feed utilization ($P > 0.05$). Nevertheless, innate immunity of shrimp were improved by dietary supplementation of biofloc. Significantly higher respiratory burst activity was observed in shrimp fed dietary biofloc levels of 1.0% compared to the shrimp offered BF2. Dietary inclusion of biofloc at level of 0.25% significantly enhanced total immunoglobulin level in shrimp compared to the Con diet. The diet containing OTC at a level of 0.5% was found to enhance the disease resistance of shrimp compared to BF0.5 group. The results show that biofloc enhances innate immunity of shrimp as evidenced by Ig level and NBT activity while dietary administration of OTC at 0.5% enhanced their resistance against *V. harveyi* infection.

In experiment Ⅲ, six experimental diets were prepared with supplementation of graded levels of dried biofloc powder by 0, 0.25, 0.5, 1.0, 2.0 and 4.0% (designated as, Con, BF0.25, BF0.5, BF1, BF2 and BF4, respectively). Two other diets were prepared by supplementing 0.5% of OTC or penicillin (antibiotic, Amp) to the basal diet. Triplicate groups of shrimp (1.0 ± 0.0 g) were hand-fed with one of the diets four times a day for 7 weeks. At the end of feeding trial, feed utilization of the shrimp was significantly affected by dietary supplementation of biofloc compared to that of shrimp fed the Con diet. Significantly higher feed intake was observed in shrimp fed the BF4 diet compared to that of shrimp fed the Con diet. Dietary inclusion of biofloc to the Con diet at level of 4.0% resulted in significant decrease of protein efficiency. Shrimp fed the BF1 diet had significantly higher immunoglobulin level than shrimp fed the Con

diet. At the end of the challenge test with the same pathogen, significantly higher survival rate was found in the Con and BF4 groups compared to the AMP group. In conclusion, the results from the three feeding trials indicate that bioflocs have some positive effects on the immune response in white shrimp leading to a higher resistance against *V. harvey* challenge. It seems clear that the potential of the biofloc availability as a dietary supplement is dependent upon the process method of the biofloc, quantity of organic compounds and beneficial microorganism. Further studies are necessary to elucidate possibility of the biofloc use in diets for Pacific white shrimp.

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1. INTRODUCTION

Shrimp aquaculture sector has been quickly expanding facing with major problems by bacterial diseases and use of large quantities of chemical and antibiotic products (Le et al., 2005; Tu et al., 2008). In aquaculture including shrimp culture, bacterial diseases have emerged as one of the most serious problems and represent the most important challenge to this industry (Morales, 2004; Holmström et al., 2003). In Korea many antibacterial substances including several sulfa drugs are used for treatment of fish and/or shrimp diseases. The potential hazards of using antibiotics in fish or shrimp aquaculture are development of antibiotic-resistance microorganism, antibiotic residuals in aquaculture products, contamination of surrounding ecological systems and reduced efficiency of antibiotics against diseases caused by resistance pathogens (McPhearson et al., 1991; Hernández Serrano, 2005). The antibiotic residue accumulations in the edible tissues of fish or shrimp may cause several serious problems, such as alteration of human intestinal micro-flora and food poisoning or allergy (Ma et al., 2006). The antibiotics most frequently used in aquaculture industry to combat the bacterial diseases include oxytetracycline, florfenicol, sarafloxacin, and enrofloxacin (Roque et al., 2001; Soto-Rodríguez et al., 2006). Among these, oxytetracycline is the most commonly used antibiotics in fish and shrimp farms and hatcheries. However, OTC is very poorly absorbed through the intestinal tract of fish resulting in slow excretion of large amounts of this antibiotic (Austin and Al-Zahrani 1988; Navarrete et al., 2008). Therefore, the rise in bacterial antibiotic resistance and antibiotic residues has increased scientist's interest to solve this problem. Research needs have been necessary to develop

alternative therapies to reduce the rise or to stop the occurrence of the antibiotic resistance or residues in aquaculture production systems. In this regard, alternative strategies to vaccination and use of antibiotics represent applications of various immunostimulatory substances as dietary supplements. Although little is known about the mechanism of their action in fish or shrimp, some of them appear to enhance the innate immunity to kill the pathogenic microbes. Hence, the concept of functional feed is an emerging paradigm in aquaculture industry to develop diets of balanced nutrition supplemented with feed additives for improving the health and disease resistance of cultured fishes (Li and Gatlin 2004).

The application of biofloc technology (BFT) in shrimp aquaculture has gained great attention recently because it offers a practical solution to effectively control water quality under negligible water exchange and improves shrimp growth performance, thus achieving efficient and healthy culture of shrimp (Avnimelech, 2012; Crab et al., 2012; De Schryver et al., 2008; Stokstad, 2010; Xu and Pan, 2013). The supply of feed nutrition, mainly protein, is required for the normal physiological metabolism and growth of cultured shrimp (Kureshy and Davis, 2002). This implies that the shrimp should not only be fed with adequate dietary protein for superior growth but also be maintained in a healthy nutritional and physiological state. Previous study has shown that biofloc could enhance immune cellular response and antioxidant status of cultured shrimp probably because of its being rich in natural microbes and bioactive compounds (Ju et al., 2008a; Xu and Pan, 2013). This result was found when *L. vannamei* juveniles were fed a formulated diet with 35% crude protein in biofloc-based culture tanks for a period of 30 days.

The white shrimp *L. vannamei* (Boone) is a commercially important shrimp species currently being cultured in many countries of the world. Over the past decade, production of *L. vannamei* has been covered and achieved in biofloc-based intensive systems under high aeration and negligible water exchange (Avnimelech, 2012; Haslun et al., 2012; Taw, 2010). Recently, it has been demonstrated that dietary inclusion of biofloc enhanced the growth performance of *L. vannamei* (Bauer et al., 2012; Ju et al., 2008b; Kuhn et al., 2010). However, there is no information to support the biofloc roles in diets on growth and innate immunity in this species. Prevention of shrimp from bacterial diseases through the stimulation of non-specific immune response by dietary supplementation of natural compounds and/or bioflocs containing many beneficial microorganisms would be a potential solution for development of sustainable antibiotic-free shrimp culture systems.

Therefore, we conducted three experiments to evaluate the effects of dietary supplementation of biofloc on growth performance, non-specific immune response and susceptibility to bacterial infection caused by *V. harveyi* in *L. vannamei* and to examine its efficiency as a substitute for common antibiotics such as OTC or penicillin antibiotic, AMP.

[EXPERIMENT Ⅰ]

2.1. MATERIALS AND METHODS

2.1.1. Experimental diets and design

Biofloc powder tested in this study was provided by NeoEnBiz Company (Neo Environmental Business Co.), Dangjin, South Korea. Proximate composition of the biofloc powder is presented in Table 1-1.

Formulation and proximate composition of the experimental diets are shown in Table 1-2. Seven experimental diets were formulated to be isonitrogenous (400 g kg^{-1} crude protein) and isocaloric (16 MJ kg^{-1}) (Table 1-2). A basal FM-based diet was regarded as a control and supplemented with biofloc at the incremental levels of 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0% (designated as Con, B0.5, B1, B2, B4, B6 and B8, respectively) at the expense of soybean meal and wheat flour. All dry ingredients were thoroughly mixed in a feed mixer (NVM-16, Gyeonggido, South Korea), and after addition of squid liver oil and 10% distilled water pelleted through a pellet machine (SP-50; Gumgang Engineering, Daegu, Korea) (Fig. 1-1). Diets were dried at 25 ℃ for 24 h, crushed into desirable particle sizes, and stored at -24 ℃ until used.

Figure 1-1. Preparation of the experimental diets.

Table 1-1. Biofloc chemical composition (% dry matter).

Composition (%)	**Biofloc**
Moisture	1.80
Protein	28.7
Lipid	2.30
Ash	43.1

Table 1-2. Dietary formulation of seven experimental diets (% dry matter).

Ingredient	Diets						
	Con	**BF 0.5%**	**BF 1.0%**	**BF 2.0%**	**BF 4.0%**	**BF 6.0%**	**BF 8.0%**
Brown fishmeal	28.0	28.0	28.0	28.0	28.0	28.0	28.0
Soy bean meal	30.0	30.0	30.0	29.5	28.5	27.5	26.5
Squid liver meal	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Wheat flour	29.0	28.5	28.0	27.5	26.5	25.5	24.5
Starch	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Squid liver oil	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mineral mix[1]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin mix[2]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Choline chloride	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Lecithin	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Biofloc	**0.00**	**0.50**	**1.00**	**2.00**	**4.00**	**6.00**	**8.00**
Chemical composition (% dry matter)							
Dry matter	93.2	93.8	94.0	93.2	93.1	93.6	93.5
Protein	40.8	40.9	40.7	40.6	41.0	41.0	41.0
Lipid	6.70	7.50	7.10	7.00	7.60	7.30	7.50
Ash	8.00	8.40	8.30	8.60	9.30	10.0	10.8
Energy, MJ/kg diet	16.6	16.6	16.6	16.5	16.4	16.2	16.1

[1]Mineral premix (g/kg mixture): MgSO4.7H2O, 80.0; NaH2PO4.2H2O, 370.0; KCl, 130.0; Ferric citrate, 40.0; ZnSO4.7H2O, 20.0; Ca-lactate, 356.5; CuCl2, 0.2; AlCl3. 6H2O, 0.15; Na2Se2O3, 0.01; MnSO4.H2O, 2.0; CoCl2.6H2O, 1.0

[2]Vitamin premix (g/kg mixture): L-ascorbic acid, 121.2; DL- tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myo-inositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003

2.1.2. Shrimp and feeding trial

The feeding trial was conducted in indoor shrimp culture facilities at the Marine Science Institute of the Jeju National University (Jeju, South Korea). Juvenile *L. vannamei* obtained from NeoEnBiz shrimp farm (Dangjin, South Korea). Shrimps were fed a commercial diet (35% crud protein) twice daily for 10 days to be acclimated to the experimental conditions and facilities. At the end of the acclimation period, the shrimp (initial mean body weight, 1.01 ± 0.01 g) were randomly distributed into twenty one acryl aquaria of 96 L capacity (Fig. 1-2) at a density of 18 shrimp per aquarium filled with filtered seawater and aeration to maintain enough dissolved oxygen. Triplicate groups of shrimp were hand-fed with one of the test diets four times a day at 08:00, 12.00, 16.00 and 18:00 h for 8 weeks. The daily feeding rates were slowly reduced from 10% to 8% of wet body weight during the 8 weeks feeding trial, and the daily feed amount was adjusted to the biomass in the tanks. Water was exchanged every 3rd days while inside of the aquaria were cleaned by a sponge to prevent the growth of microflora. A 12:12 h light/dark regime (08:00–19:00 h, light period) was maintained by timed fluorescent lighting. The water temperature was maintained at 28 ± 1 ℃, pH fluctuated between 7.04 and 8.04 and dissolved oxygen was kept above 6.0 mg L^{-1} and total ammonia nitrogen and nitrite were kept < 0.1 and 0.005 mg L^{-1} respectively, during the feeding trial. Growth of shrimp was measured with 2-week intervals. Feeding was stopped 16 h prior to weighing or hemolymph sampling to minimize handling stress on shrimp.

Figure 1-2. Experimental rearing tanks.

2.1.3. Sample collection and analyses

At the end of the feeding trial, all shrimp in each tank were counted and bulk-weighed for calculation of growth parameters and survival. Four intact shrimp per tank were randomly selected and kept at -24 ℃ for whole-body proximate composition analyses. Five shrimp per tank (fifteen shrimp per dietary treatment) in inter-molt stage were randomly captured, anaesthetized with ice-cold water, and hemolymph samples (200 μl) were individually collected from the ventral sinus of each shrimp using a 1-ml insulin syringe fitted with a 25-gauge needle (Fig. 1-3), and filled with an equal volume of an anticoagulant solution (Alsever's solution, Sigma). The molt stage of was determined by the examination of uropoda in which partial retraction of the epidermis could be distinguished (Robsertson et al., 1987). The hemolymph-anticoagulant mixture (diluted hemolymph) was placed in five sterile eppendorf tubes containing equal volume of pre-cooled anticoagulant for the determination of the total hemocyte counts (THC) and respiratory burst activity. After the above-mentioned measurements with diluted hemolynph, the remain samples was centrifuged at 700 ×g for 10 min using a

high-speed refrigerated microcentrifuge (Micro 17 TR; HanilBioMed Inc., Gwangju, Korea) and stored at -70 ℃ for the determination of the phenoloxidase (PO) and superoxide dismutase (SOD) activities and total immunoglobulin (Ig) level.

Analyses of moisture and ash contents of Biofloc powder, diet and whole-body samples were performed by the standard procedures (AOAC, 1995). Crude protein was measured by using an automatic Kjeltec Analyzer Unit 2300 (Foss Tecator, Höganäs, Sweden), and crude lipid was determined using the Soxhlet method with extraction in diethyl ether (Soxhlet Extraction System C-SH6, Korea).

Figure 1-3. Taking hemolymph from experimental shrimp.

2.1.4. Monitoring of non-specific immune responses

Oxidative radical production by hemocytes during respiratory burst was measured through the nitro-blue-tetrazolium (NBT) assay described by Dantzler et al (2001) with some modifications. Briefly, fifty µL of the diluted hemolymph was incubated with 200 µl mHBSS solution (Sigma) for 30 min at 25 ℃, followed by additional incubation with 100 µL zymosan (0.1% in Hank's balanced salt solution) for 2 h at 37 ℃. The mixture was then incubated with 100 µL nitroblue tetrazolium (0.3%) for 2 h at 37 ℃. The suspension was fixed with 600 µL of absolute methanol, and subsequently centrifuged at 4000 ×g for 10 min. The formazan pellet was then rinsed with 70% methanol for three times and air-dried. Formazan was dissolved with the addition of 700 µL KOH (2 M) and 800 µL dimethylsulfoxide (DMSO). The optical density was measured at 620 nm using a microplate reader (UVM 340, Biochrom, Cambridge, UK) and respiratory burst was expressed as NBT-reduction in 10 µL of hemolymph.

PO activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA, Sigma) following the procedure of Hernández-López et al (1996) with some modification. 50 µl of sample was incubated for 30 min at 25 ℃ with 50 µl of trypsin (1 mg ml^{-1} in cacodylate buffer) which served as the elicitor, followed by adding 50 µL of $_{L}$-DOPA (3 mg ml^{-1} in cacodylate buffer) and incubation at 25 ℃ for 10 min. Finally, the optical density was read at 490 nm in a microplate reader (UVM 340, Biochrom, Cambridge, UK) and shrimp's phenoloxidase activity expressed as dopachrome formation in 50 µl of hemolymph.

SOD was measured by the percentage reaction inhibition rate of 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. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37 ℃. The percent inhibition was normalized by mg protein and presented as SOD activity units.

Plasma Ig level was determined according to the method described by Siwicki and Anderson (1994). Briefly, diluted hemolymph protein content was measured using a microprotein determination method (C-690; Sigma), prior to and after precipitating down the Ig molecules, using a 12% solution of polyethylene glycol (Sigma). The difference in protein content represents the Ig content.

Glutathione peroxidase activity (GPx) was assayed using a kit (Biovision, Inc., Milpitas, CA, USA). In this assay, cumene hydroperoxide was used as a peroxide substrate (ROOH), and glutathione reductase (GSSG-R) and NADPH (b-nicotinamide adenine denucleotide phosphate, reduced) were included in the reaction mixture. The change in 340 nm due to NADPH oxidation was monitored for GPX activity. Briefly, 50μL of sample was added to 40μL of the reaction mixture and incubated for 15 min and then 10μLof cumene hydroperoxide was added and OD_1 read at 340 nm. After 5 min of incubation, OD_2 was read in 340 nm by the microplate reader.

2.1.5. Challenge trial

At the end of the feeding trial, 12 shrimp from each tank (36 shrimp per treatment) were randomly selected and subjected to a bacterial challenge. *V. harveyi* was used as

the pathogenic agent (provided by the Marine Microbiology Laboratory of Jeju National University). The shrimp were injected intramuscularly with *V. harveyi* suspension containing 2×10^8 CFU mL^{-1}. The pathogenic dose of bacterium had previously been determined in a preliminary test using shrimp of a similar size. After injection, the shrimp were distributed into 21 120 L acryl tanks (Fig. 1-4), and their mortality was monitored and recorded for 20 days.

Figure 1-4. Experimental tanks used for challenge tests.

2.1.6. Statistical analysis

All dietary treatments were assigned by a completely randomized design. Data were subjected to one-way analysis of variance (ANOVA) in SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the difference in means was made with LSD multiple range tests. Statistical significance was determined at $P < 0.05$. Data are presented as mean± SD. Percentage data were arcsine transformed before analysis.

3.1. RESULTS

Growth performance and feed utilization of the shrimp were significantly affected by dietary supplementation of biofloc compared to those of fish fed the Con diet (Table 1-3). Final body weight, weight gain and specific growth rate of shrimp fed the BF4 diet showed significant increase compared to those fed the Con diet. Dietary inclusion of the biofloc to the Con diet at level of 4.0% resulted in significant enhancement of protein efficiency ratio (PER) while fish fed the same diet showed the lowest feed conversion ratio (FCR) compared to those of fish fed the Con diet. Significantly higher feed intake (FI) values were observed in fish fed the Con diet compared to those of shrimp fed the BF8 diet. However no significant differences were found among all the other experimental groups. The highest survival rate was obtained in BF0.5 group which was significantly different from other dietary groups ($P < 0.5$).

The results of whole-body composition analysis are shown in Table 1-4. Protein content significantly decreased as dietary biofloc level increased from 0.5 to 4% while shrimp fed dietary biofloc levels of ≥ 0.6% exhibited significantly higher protein contents than fish fed the BF2 and BF4 diets. Ash content was decreased by increment of biofloc level and the lowest significant value was observed in shrimp fed 4 to 6% level of biofloc product. However, whole body moisture and lipid level were not significantly influenced by the dietary supplementation of biofloc ($P > 0.5$).

Shrimp fed the BF0.5 and BF4 diets showed significantly higher NBT activity than the group of shrimp fed the Con diet. Significantly higher PO activity was found in groups of shrimp fed the BF6 diet compare to those fed the Con diet. Significant

enhancement in GPx activity was observed in BF6 fed groups in comparison to Con group. Even though, numerically higher values of other examined non-specific immune parameters were observed in shrimp fed the biofloc supplemented diets, the differences were not significant ($P > 0.5$; Table 1-5).

During the challenge test, the first dramatic mortality was observed on the 13th day after injection and the shrimp fed the Con diet showed the lowest disease resistance compared to all of the other groups (Fig. 1-5). At the end of the challenge test, significantly higher survival rate was found in BF4 group compared to the Con groups, however, no significant difference was found among biofloc supplemented groups.

Table 1-3. Growth performance of white shrimp (Initial BW: 1.01±0.01g) fed the seven experimental diets for 8 weeks.

Treatment	FBW[1] (g)	WG[2] (%)	SGR[3] (%)	FCR[4]	PER[5]	FI[6]	Survival (%)
Control	6.63±0.55^{a}	565±49.6^{a}	3.32±0.13^{a}	2.25±0.21^{b}	1.10±0.11^{a}	12.8±0.13^{b}	79.6±16.0^{a}
Biofloc 0.5%	7.12±0.44^{ab}	610±41.5^{ab}	3.44±0.10^{ab}	2.09±0.16^{ab}	1.15±0.09^{ab}	12.8±0.02^{ab}	98.1±3.21^{b}
Biofloc 1.0%	7.12±0.82^{ab}	608±70.2^{ab}	3.43±0.17^{ab}	2.10±0.25^{ab}	1.18±0.15^{ab}	12.8±0.01^{ab}	85.2±8.49^{a}
Biofloc 2.0%	7.34±0.71^{ab}	635±67.5^{ab}	3.50±0.16^{ab}	2.01±0.22^{ab}	1.24±0.13^{ab}	12.7±0.01^{ab}	88.9±0.00^{a}
Biofloc 4.0%	7.90±0.13^{b}	677±8.40^{b}	3.60±0.02^{b}	1.84±0.04^{a}	1.32±0.03^{b}	12.7±0.04^{ab}	85.2±3.21^{a}
Biofloc 6.0%	7.27±0.81^{ab}	627±81.4^{ab}	3.47±0.19^{ab}	2.05±0.27^{ab}	1.20±0.16^{ab}	12.7±0.04^{ab}	88.9±5.56^{a}
Biofloc 8.0%	7.56±0.38^{ab}	639±39.8^{ab}	3.51±0.10^{ab}	1.91±0.15^{ab}	1.28±0.10^{ab}	12.5±0.48^{a}	90.7±6.42^{a}

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different letters are significantly different (LSD; $P < 0.05$).

[1]FBW=Final body weight

[2]Weight gain = [(final body weight - initial body weight)/ initial body weight] x 100

[3]Specific growth rate = 100 x [ln(final body weight) – ln(initial body weight)]/days

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

[5]Protein efficiency ratio = fish weight gain (g)/protein

[6]Feed intake (g/fish) = dry feed consumed (g)/fish

Table 1-4. Whole-body composition of white shrimp fed the experimental diets for 8 weeks.

Diets	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
Control	74.2±0.7^{ab}	86.7±1.20^{bc}	1.38±0.20	6.16±0.40^{c}
BF 0.5%	74.8±0.6^{b}	86.1±0.36^{bc}	1.38±0.25	6.29±0.79^{bc}
BF 1.0%	73.8±0.5^{a}	84.9±1.47^{abc}	1.23±0.26	5.81±0.16^{abc}
BF 2.0%	74.1±0.8^{ab}	83.8±0.35^{ab}	1.45±0.31	5.88±0.11^{bc}
BF 4.0%	74.2±0.9^{ab}	82.9±0.10^{a}	1.34±0.18	5.25±0.25^{a}
BF 6.0%	74.2±1.2^{ab}	87.6±1.69^{c}	1.24±0.24	5.51±0.32^{ab}
BF 8.0%	74.5±0.3^{ab}	87.3±1.91^{c}	1.20±0.11	5.82±0.25^{abc}

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different letters are significantly different (LSD; $P < 0.05$).

Table 1-5. Non-specific immune responses of white shrimp fed the seven experimental diets for 8 weeks.

Treatment	THC[1] (g)	NBT[2] (%)	PO[3] (%)	SOD (%)[4]	Ig[5]	GPx
Control	0.33±0.01	0.27±0.04[a]	0.16±0.01[a]	238±18.6	31.2±12.7	107±37.7[a]
Biofloc 0.5%	1.95±1.30	0.32±0.00[b]	0.18±0.02[a]	240±3.56	44.9±13.6	133±32.4[ab]
Biofloc 1.0%	4.14±0.52	0.28±0.03[ab]	0.17±0.02[a]	235±30.8	30.6±4.12	181±25.4[ab]
Biofloc 2.0%	2.26±0.71	0.28±0.02[ab]	0.18±0.02[a]	254±15.0	38.1±6.97	157±45.3[ab]
Biofloc 4.0%	4.52±3.81	0.32±0.04[b]	0.16±0.03[a]	249±12.0	39.6±6.29	184±76.5[ab]
Biofloc 6.0%	3.12±1.46	0.29±0.02[ab]	0.24±0.04[b]	250±37.5	43.6±18.3	205±55.3[b]
Biofloc 8.0%	4.40±4.29	0.31±0.03[ab]	0.18±0.04[a]	246±9.13	37.4±2.69	159±40.9[ab]

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different letters are significantly different (LSD; $P < 0.05$).

[1]Total hemocyte count (10^3 cells/ml)

[2]Nitro blue tetrazolium activity (absorbance)

[3]Phenoloxidase activity (absorbance)

[4]Superoxide dismutase

[5]Immunoglobulin

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

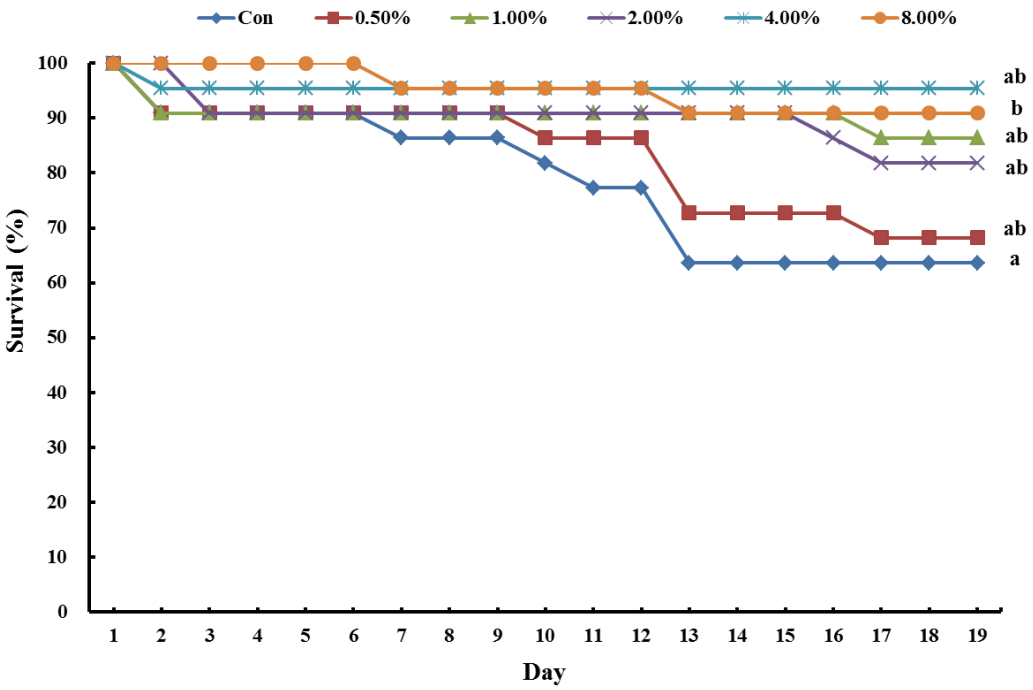

Figure 1-5. Survival of white shrimp fed the experimental diets after challenge with *Vibrio harveyi.*

[EXPERIMENT Ⅱ]

2.2. MATERIALS AND METHODS

2.2.1. Experimental diets and design

Biofloc powder tested in this study was provided by NeoEnBiz Company (Neo Environmental Business Co.), Dangjin, South Korea. Proximate composition of the biofloc powder is presented in Table 2-1.

Formulation and proximate composition of the experimental diets are shown in Table 2-2. Seven experimental diets were formulated to be isonitrogenous (410 g kg^{-1} crude protein) and isocaloric (16 MJ kg^{-1}) (Table 2-2). A basal FM-based diet was regarded as a control and supplemented with biofloc at the incremental levels of 0.25, 0.5, 1.0, 2.0 and 4.0% (designated as Con, BF0.25, BF0.5, BF1.0, BF2.0, and BF4.0, respectively) at the expense of soybean meal and wheat flour. The other experimental diet was prepared by supplementing 0.5% of Oxytetracycline (OTC) to the basal diet (Antibiotic). All dry ingredients were thoroughly mixed in a feed mixer (NVM-16, Gyeonggido, South Korea), and after addition of squid liver oil and 20% distilled water pelleted through a pellet machine (SP-50; Gumgang Engineering, Daegu, Korea). Diets were dried at 25 ℃ for 24 h, crushed into desirable particle sizes, and stored at -24 ℃ until used.

Table 2-1. Biofloc chemical composition (% dry matter)

Composition (%)	**Biofloc**
Moisture	3.03
Protein	48.0
Lipid	2.30
Ash	28.6

Table 2-2. Dietary formulation of seven experimental diets (% dry matter).

Ingredient	Diets						
	Con	**BF 0.25%**	**BF 0.50%**	**BF 1.00%**	**BF 2.00%**	**BF 4.00%**	**Antibiotic**
Brown fishmeal	28.0	28.0	28.0	28.0	28.0	28.0	28.0
Soy bean meal	26.0	25.9	25.8	25.5	25.5	25.0	26.0
Squid liver meal	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Wheat flour	28.0	27.9	27.8	27.5	26.5	25.0	27.5
Starch	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Squid liver oil	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mineral mix[1]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin mix[2]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Choline chloride	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Lecithin	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Biofloc	**0.00**	**0.25**	**0.50**	**1.00**	**2.00**	**4.00**	**0.00**
Antibiotic (OTC)	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.50**
Chemical composition (% dry matter)							
Dry matter	92.4	92.4	92.2	92.7	92.6	92.5	92.5
Protein	42.1	41.9	42.4	41.9	41.9	41.5	41.0
Lipid	5.88	5.62	5.76	5.73	5.77	5.50	5.93
Ash	8.26	8.38	8.49	8.65	9.06	9.86	8.78
Energy, MJ/kg diet	16.5	16.5	16.5	16.5	16.4	16.2	16.5

[1]Mineral premix (g/kg mixture): MgSO4.7H2O, 80.0; NaH2PO4.2H2O, 370.0; KCl, 130.0; Ferric citrate, 40.0; ZnSO4.7H2O, 20.0; Ca-lactate, 356.5; CuCl2, 0.2; AlCl3. 6H2O, 0.15; Na2Se2O3, 0.01; MnSO4.H2O, 2.0; CoCl2.6H2O, 1.0

[2]Vitamin premix (g/kg mixture): L-ascorbic acid, 121.2; DL- tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myo-inositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003

2.2.2. Shrimp and feeding trial

The feeding trial was conducted in indoor shrimp culture facilities at the Marine Science Institute of the Jeju National University (Jeju, South Korea). Juvenile *L. vannamei* obtained from NeoEnBiz shrimp farm (Dangjin, South Korea). Shrimps were fed a commercial diet (35% crud protein) twice daily for 10 days to be acclimated to the experimental conditions and facilities. At the end of the acclimation period, the shrimp (initial mean body weight, 0.70 ± 0.0 g) were randomly distributed into twenty one acryl aquaria of 120 L capacity (Fig. 2-1) at a density of 20 shrimp per aquarium filled with filtered seawater and aeration to maintain enough dissolved oxygen. Triplicate groups of shrimp were hand-fed with one of the test diets four times a day at 08:00, 12.00, 16.00 and 18:00 h for 6 weeks (Fig. 2-2). The daily feeding rates were slowly reduced from 13% to 8% of wet body weight during the 6 weeks feeding trial, and the daily feed amount was adjusted to the biomass in the tanks. Water was exchanged every 5th days while inside of the aquaria were cleaned by a sponge to prevent the growth of microflora. A 12:12 h light/dark regime (08:00–19:00 h, light period) was maintained by timed fluorescent lighting. The water temperature was maintained at 28 ± 2 ℃, pH fluctuated between 7.08 and 7.39 and dissolved oxygen was kept above 7.0 mg L^{-1}, and total ammonia nitrogen and nitrite were kept < 0.1 and 0.005 mg L^{-1} respectively, during the feeding trial. Growth of shrimp was measured with 3-week intervals (Fig. 2-3). Feeding was stopped 16 h prior to weighing or hemolymph sampling to minimize handling stress on shrimp.

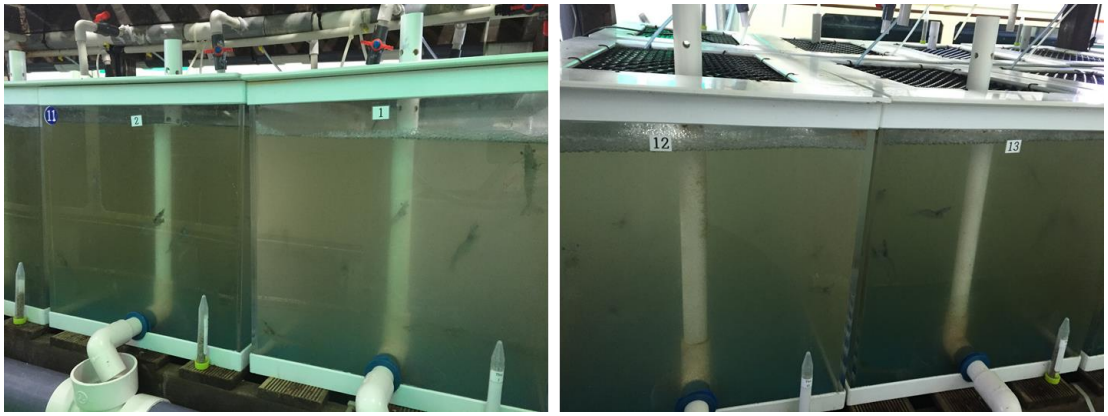

Figure 2-1. Experimental rearing tanks.

Figure 2-2. During the feeding trial.

Figure 2-3. Measurement of growth performance in every 3 weeks during 6 weeks feeding trial.

2.2.3. Sample collection and analyses

At the end of the feeding trial, all shrimp in each tank were counted and bulk-weighed for calculation of growth parameters and survival. Four intact shrimp per tank were randomly selected and kept at -24 ℃.

Three shrimp per tank (nine shrimp per dietary treatment) in inter-molt stage were randomly captured, anaesthetized with ice-cold water, and hemolymph samples (200 µl) were individually collected from the ventral sinus of each shrimp using a 1-ml insulin syringe fitted with a 25-gauge needle (Fig. 2-4), and filled with an equal volume of an anticoagulant solution (Alsever's solution, Sigma). The molt stage of was determined by the examination of uropoda in which partial retraction of the epidermis could be distinguished (Robsertson et al., 1987). The hemolymph-anticoagulant mixture (diluted hemolymph) was placed in five sterile eppendorf tubes containing equal volume of pre-cooled anticoagulant for the determination of the respiratory burst activity. After the above-mentioned measurements with diluted hemolymph, the remain samples was centrifuged at 700 ×g for 10 min using a high-speed refrigerated microcentrifuge (Micro 17 TR; HanilBioMed Inc., Gwangju, Korea) and stored at -70 ℃ for the determination of the phenoloxidase (PO) and superoxide dismutase (SOD) activities and total immunoglobulin (Ig) level.

Analyses of moisture and ash contents of Biofloc powder and diet samples were performed by the standard procedures (AOAC, 1995). Crude protein was measured by using an automatic Kjeltec Analyzer Unit 2300 (Foss Tecator, Höganäs, Sweden), and crude lipid was determined using the Soxhlet method with extraction in diethyl ether (Soxhlet Extraction System C-SH6, Korea).

Figure 2-4. Taking hemolymph from experimental shrimp.

2.2.4. Monitoring of non-specific immune responses

Oxidative radical production by hemocytes during respiratory burst was measured through the nitro-blue-tetrazolium (NBT) assay described by Dantzler et al (2001) with some modifications. Briefly, fifty µL of the diluted hemolymph was incubated with 200 µl mHBSS solution (Sigma) for 30 min at 25 ℃, followed by additional incubation with 100 µL zymosan (0.1% in Hank's balanced salt solution) for 2 h at 37 ℃. The mixture was then incubated with 100 µL nitroblue tetrazolium (0.3%) for 2 h at 37 ℃. The suspension was fixed with 600 µL of absolute methanol, and subsequently centrifuged at 4000 ×g for 10 min. The formazan pellet was then rinsed with 70% methanol for three times and air-dried. Formazan was dissolved with the addition of 700 µL KOH (2 M) and 800 µL dimethylsulfoxide (DMSO). The optical density was measured at 620 nm using a microplate reader (UVM 340, Biochrom, Cambridge, UK) and respiratory burst was expressed as NBT-reduction in 10 µL of hemolymph.

PO activity was measured spectrophotometrically by recording the formation of dopachrome produced from $_{L}$-dihydroxyphenylalanine ($_{L}$-DOPA, Sigma) following the procedure of Hernández-López et al (1996) with some modification. 50 µl of sample was

incubated for 30 min at 25 ℃ with 50 µl of trypsin (1 mg ml^{-1} in cacodylate buffer) which served as the elicitor, followed by adding 50 µL of $_L$-DOPA (3 mg ml^{-1} in cacodylate buffer) and incubation at 25 ℃ for 10 min. Finally, the optical density was read at 490 nm in a microplate reader (UVM 340, Biochrom, Cambridge, UK) and shrimp's phenoloxidase activity expressed as dopachrome formation in 50 µl of hemolymph.

SOD activity was measured by the percentage reaction inhibition rate of 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. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37 ℃. The percent inhibition was normalized by mg protein and presented as SOD activity units.

Plasma Ig level was determined according to the method described by Siwicki and Anderson (1994). Briefly, diluted hemolymph protein content was measured using a microprotein determination method (C-690; Sigma), prior to and after precipitating down the Ig molecules, using a 12% solution of polyethylene glycol (Sigma). The difference in protein content represents the Ig content.

GPX activity was assayed using a kit (Biovision, Inc., Milpitas, CA, USA). In this assay, cumene hydroperoxide was used as a peroxide substrate (ROOH), and glutathione reductase (GSSG-R) and NADPH (b-nicotinamide adenine denucleotide phosphate, reduced) were included in the reaction mixture. The change in 340 nm due to NADPH oxidation was monitored for GPX activity. Briefly, 50µL of sample was added to 40µL of the reaction mixture and incubated for 15 min and then 10µLof cumene hydroperoxide was added and OD_1 read at 340 nm. After 5 min of incubation, OD_2 was read in 340 nm by the microplate reader. Activity of GPX was calculated as nmol/min/mL.

2.2.5. Challenge trial

At the end of the feeding trial, 12 shrimp from each tank (36 shrimp per treatment) were randomly selected and subjected to a bacterial challenge. *V. harveyi* was used as the pathogenic agent (provided by the Marine Microbiology Laboratory of Jeju National University). The shrimp were injected intramuscularly with *V. harveyi* suspension containing 2×10^8 CFU mL^{-1}. The pathogenic dose of bacterium had previously been determined in a preliminary test using shrimp of a similar size. After injection, the shrimp were distributed into 21 120 L acryl tanks, and their mortality was monitored and recorded for 20 days.

2.2.6. Statistical analysis

All dietary treatments were assigned by a completely randomized design. Data were subjected to one-way analysis of variance (ANOVA) in SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the difference in means was made with LSD multiple range tests. Statistical significance was determined at $P < 0.05$. Data are presented as mean± SD. Percentage data were arcsine transformed before analysis.

3.2. RESULTS

At the end of 6 weeks, there were no differences in growth performance, feed utilization and survivals among all the dietary treatments (Table 2-3).

Shrimp fed the BF1 diet indicated significantly higher NBT activity than the group of shrimp fed the BF2 diet. Significantly higher immunoglobulin level was found in BF0.25 group compared to the Con group. Even though, numerically higher values of other examined non-specific immune parameters were observed in shrimp fed biofloc and OTC supplemented diets, the differences were not significant ($P > 0.5$; Table 2-4).

Results from the challenge test indicated that survival rate after 20 days in the BF0.5 group was significantly lower than that observed for the antibiotic group (Fig. 2-5). There were no significant difference in mortality between the OTC and other biofloc treatment groups.

Table 2-3. Growth performance of white shrimp (Initial BW: 0.70±0.00g) fed the seven experimental diets for 6 weeks.

Treatment	FBW[1] (g)	WG[2] (%)	SGR[3] (%)	FCR[4]	PER[5]	FI[6]	Survival (%)
Control	5.23±0.51	651±74.0	4.79±0.23	1.72±0.16	1.39±0.13	7.99±0.26	91.7±2.89
Biofloc 0.25%	5.12±0.15	631±24.0	4.74±0.08	1.79±0.04	1.34±0.03	8.14±0.23	81.7±7.64
Biofloc 0.50%	5.04±0.63	618±81.2	4.68±0.26	1.77±0.29	1.36±0.24	7.83±0.48	80.0±10.0
Biofloc 1.00%	5.06±0.20	618±31.8	4.69±0.10	1.77±0.13	1.35±0.10	8.04±0.21	85.0±5.00
Biofloc 2.00%	5.41±0.05	669±12.9	4.86±0.04	1.64±0.07	1.46±0.06	7.90±0.20	91.7±2.89
Biofloc 4.00%	5.26±0.44	651±62.3	4.80±0.20	1.68±0.19	1.45±0.17	7.84±0.26	88.3±5.77
Antibiotic (OTC)	4.98±0.51	610±72.9	4.66±0.25	1.82±0.27	1.36±0.19	7.90±0.15	90.0±5.00

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different letters are significantly different.

[1]FBW=Final body weight

[2]Weight gain = [(final body weight - initial body weight)/ initial body weight] x 100

[3]Specific growth rate = 100 x [ln(final body weight) – ln(initial body weight)]/days

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

[5]Protein efficiency ratio = fish weight gain (g)/protein

[6]Feed intake (g/fish) = dry feed consumed (g)/fish

Table 2-4. Non-specific immune responses of white shrimp fed the seven experimental diets for 6 weeks.

Treatment	NBT[1] (%)	PO[2] (%)	SOD[3] (%)	Ig[4]	GPx[5]
Control	0.194±0.02^{ab}	0.13±0.01	197±14.2	14.0±1.32^{a}	132±14.4
Biofloc 0.25%	0.199±0.02^{ab}	0.15±0.02	210±27.8	23.2±10.2^{b}	183±32.5
Biofloc 0.50%	0.203±0.04^{ab}	0.13±0.01	199±14.8	19.6±3.85^{ab}	168±43.4
Biofloc 1.00%	0.239±0.02^{b}	0.14±0.01	237±12.7	20.6±1.28^{ab}	154±39.8
Biofloc 2.00%	0.180±0.01^{a}	0.14±0.02	228±19.9	15.8±4.52^{ab}	205±45.4
Biofloc 4.00%	0.191±0.01^{b}	0.16±0.04	225±22.8	19.7±1.32^{ab}	204±43.4
Antibiotic (OTC)	0.207±0.04^{ab}	0.16±0.01	236±6.28	19.8±3.77^{ab}	167±29.3

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different letters are significantly different (LSD; $P < 0.05$).

[1]Nitro blue tetrazolium activity (absorbance)

[2]Phenoloxidase activity (absorbance)

[3]Superoxide dismutase

[4]Immunoglobulin

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

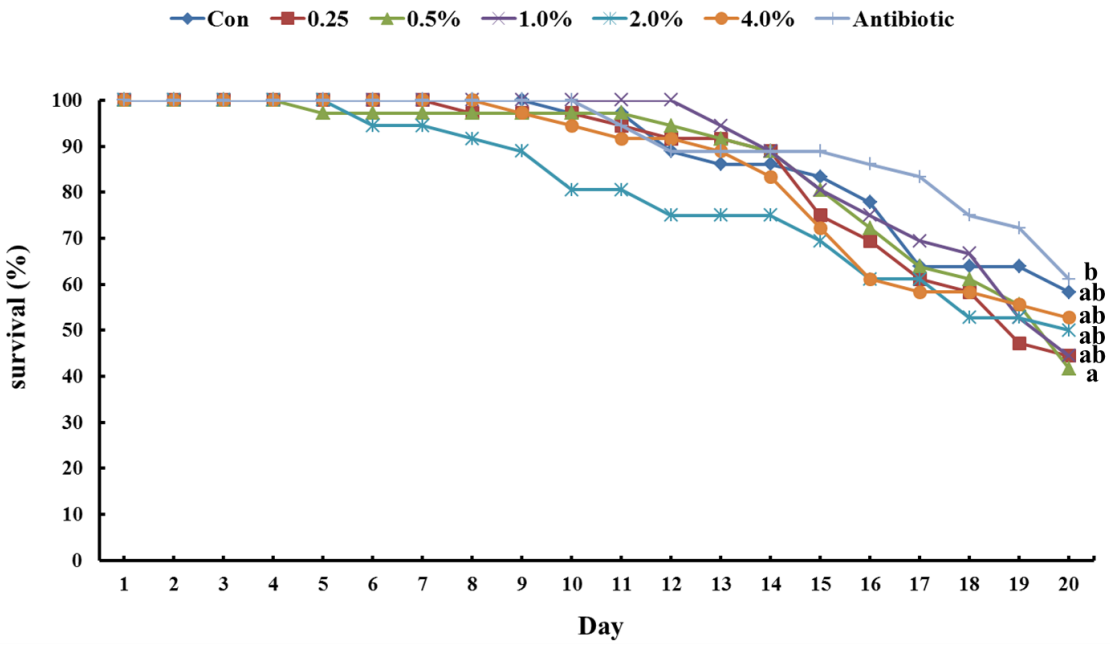

Figure 2-5. Survival of white shrimp fed the experimental diets after challenge with *Vibrio harveyi.*

[EXPERIMENT Ⅲ]

2.3. MATERIALS AND METHODS

2.3.1. Experimental diets and design

Biofloc powder tested in this study was provided by NeoEnBiz Company (Neo Environmental Business Co.), Dangjin, South Korea. Proximate composition of the biofloc powder is presented in Table 3-1.

Formulation and proximate composition of the experimental diets are shown in Table 3-2. Seven experimental diets were formulated to be isonitrogenous (400 g kg^{-1} crude protein) and isocaloric (16 MJ kg^{-1}) (Table 3-2). A basal FM-based diet was regarded as a control and supplemented with biofloc at the incremental levels of 0.25, 0.5, 1.0, 2.0 and 4.0% (designated as Con, BF0.25, BF0.5, BF1.0, BF2.0 and BF4.0, respectively) at the expense of soybean meal and wheat flour. The other two experimental diets were prepared by supplementing 0.5% of OTC and AMP to the basal diet (Antibiotic). All dry ingredients were thoroughly mixed in a feed mixer (NVM-16, Gyeonggido, South Korea), and after addition of squid liver oil and 20% distilled water pelleted through a pellet machine (SP-50; Gumgang Engineering, Daegu, Korea). Diets were dried at 25 ℃ for 24 h, crushed into desirable particle sizes, and stored at -24 ℃ until used.

Table 3-1. Biofloc chemical composition (% dry matter)

Composition (%)	**Biofloc**
Moisture	2.77
Protein	41.8
Lipid	2.28
Ash	29.7

Table 3-2. Dietary formulation of eight experimental diets (% dry matter).

Ingredient	Diets							
	Con	BF 0.25%	BF 0.50%	BF 1.00%	BF 2.00%	BF 4.00%	Antibiotic (OTC)	Antibiotic (Amp)
Brown fishmeal	28.0	28.0	28.0	28.0	28.0	28.0	28.0	28.0
Soy bean meal	26.0	25.75	25.5	25.0	24.0	22.0	26.0	26.0
Squid liver meal	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Wheat flour	28.0	28.0	28.0	28.0	28.0	28.0	27.5	27.5
Starch	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Squid liver oil	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mineral mix[1]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin mix[2]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Choline chloride	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Lecithin	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Biofloc	**0.00**	**0.25**	**0.50**	**1.00**	**2.00**	**4.00**	**0.00**	**0.00**
Antibiotic	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.50**	**0.50**
Chemical composition (% dry matter)								
Dry matter	91.7	91.7	91.6	91.6	91.6	91.9	92.1	92.0
Protein	41.7	41.6	41.7	41.7	40.9	41.2	41.6	41.7
Lipid	5.90	5.69	5.68	6.00	6.01	6.13	6.00	6.32
Ash	8.81	8.86	8.95	8.91	8.90	9.13	8.94	8.91
Energy, MJ/kg diet	16.5	16.5	16.5	16.5	16.5	16.4	16.5	16.5

[1]Mineral premix (g/kg mixture): MgSO4.7H2O, 80.0; NaH2PO4.2H2O, 370.0; KCl, 130.0; Ferric citrate, 40.0; ZnSO4.7H2O, 20.0; Ca-lactate, 356.5; CuCl2, 0.2; AlCl3. 6H2O, 0.15; Na2Se2O3, 0.01; MnSO4.H2O, 2.0; CoCl2.6H2O, 1.0

[2]Vitamin premix (g/kg mixture): L-ascorbic acid, 121.2; DL- tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myo-inositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003

2.3.2. Shrimp and feeding trial

The feeding trial was conducted in indoor shrimp culture facilities at the Marine Science Institute of the Jeju National University (Jeju, South Korea). Juvenile *L. vannamei* obtained from NeoEnBiz shrimp farm (Dangjin, South Korea). Shrimps were fed a commercial diet (35% crud protein) twice daily for 10 days to be acclimated to the experimental conditions and facilities. At the end of the acclimation period, the shrimp (initial mean body weight, 1.0 ± 0.0 g) were randomly distributed into twenty one acryl aquaria of 120 L capacity at a density of 15 shrimp per aquarium filled with filtered seawater and aeration to maintain enough dissolved oxygen. Triplicate groups of shrimp were hand-fed with one of the test diets four times a day at 09:00, 13:00, 17:00 and 19:30 h for 7 weeks. The daily feeding rates were slowly reduced from 13% to 8% of wet body weight during the 7 weeks feeding trial, and the daily feed amount was adjusted to the biomass in the tanks. Water was exchanged every 4th days while inside of the aquaria were cleaned by a sponge to prevent the growth of microflora. A 12:12 h light/dark regime (08:00–19:00 h, light period) was maintained by timed fluorescent lighting. The water temperature was maintained at 28 ± 2 ℃, pH fluctuated between 6.50 and 8.28 and dissolved oxygen was kept above 6.0 mg L^{-1}, and total ammonia nitrogen and nitrite were kept < 0.1 and 0.005 mg L^{-1} respectively, during the feeding trial. Growth of shrimp was measured with 3-week intervals (Fig. 3-1). Feeding was stopped 16 h prior to weighing or hemolymph sampling to minimize handling stress on shrimp.

Figure 3-1. Measurement of growth performance in every 3 weeks during 7 weeks feeding trial.

2.3.3. Sample collection and analyses

At the end of the feeding trial, all shrimp in each tank were counted and bulk-weighed for calculation of growth parameters and survival. Three shrimp per tank (nine shrimp per dietary treatment) in inter-molt stage were randomly captured, anaesthetized with ice-cold water, and hemolymph samples (200 µl) were individually collected from the ventral sinus of each shrimp using a 1-ml insulin syringe fitted with a 25-gauge needle (Fig. 3-2), and filled with an equal volume of an anticoagulant solution (Alsever's solution, Sigma). The molt stage of was determined by the examination of uropoda in which partial retraction of the epidermis could be distinguished (Robsertson et al., 1987). The hemolymph-anticoagulant mixture (diluted hemolymph) was placed in five sterile eppendorf tubes containing equal volume of pre-cooled anticoagulant for the determination of the respiratory burst activity. After the above-mentioned measurements with diluted hemolymph, the remain samples was centrifuged at 700 ×g for 10 min using a high-speed refrigerated microcentrifuge (Micro 17 TR; HanilBioMed Inc., Gwangju, Korea) and stored at -70 ℃ for the determination of the phenoloxidase (PO) and superoxide dismutase (SOD), glutathione peroxidase (GPx) activities and total immunoglobulin (Ig) level.

Analyses of moisture and ash contents of Biofloc powder and diet samples were performed by the standard procedures (AOAC, 1995). Crude protein was measured by using an automatic Kjeltec Analyzer Unit 2300 (Foss Tecator, Höganäs, Sweden), and crude lipid was determined using the Soxhlet method with extraction in diethyl ether (Soxhlet Extraction System C-SH6, Korea).

Figure 3-2. Taking hemolymph from experimental shrimp.

2.3.4. Monitoring of non-specific immune responses

Oxidative radical production by hemocytes during respiratory burst was measured through the nitro-blue-tetrazolium (NBT) assay described by Dantzler et al (2001) with some modifications. Briefly, fifty µL of the diluted hemolymph was incubated with 200 µl mHBSS solution (Sigma) for 30 min at 25 ℃, followed by additional incubation with 100 µL zymosan (0.1% in Hank's balanced salt solution) for 2 h at 37 ℃. The mixture was then incubated with 100 µL nitroblue tetrazolium (0.3%) for 2 h at 37 ℃. The suspension was fixed with 600 µL of absolute methanol, and subsequently centrifuged at 4000 ×g for 10 min. The formazan pellet was then rinsed with 70% methanol for three times and air-dried. Formazan was dissolved with the addition of 700 µL KOH (2 M) and 800 µL

dimethylsulfoxide (DMSO). The optical density was measured at 620 nm using a microplate reader (UVM 340, Biochrom, Cambridge, UK) and respiratory burst was expressed as NBT-reduction in 10 µL of hemolymph.

PO activity was measured spectrophotometrically by recording the formation of dopachrome produced from $_{L}$-dihydroxyphenylalanine ($_{L}$-DOPA, Sigma) following the procedure of Hernández-López et al (1996) with some modification. 50 µl of sample was incubated for 30 min at 25 ℃ with 50 µl of trypsin (1 mg ml^{-1} in cacodylate buffer) which served as the elicitor, followed by adding 50 µL of $_{L}$-DOPA (3 mg ml^{-1} in cacodylate buffer) and incubation at 25 ℃ for 10 min. Finally, the optical density was read at 490 nm in a microplate reader (UVM 340, Biochrom, Cambridge, UK) and shrimp's phenoloxidase activity expressed as dopachrome formation in 50 µl of hemolymph.

SOD activity was measured by the percentage reaction inhibition rate of 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. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37 ℃. The percent inhibition was normalized by mg protein and presented as SOD activity units.

Plasma Ig level was determined according to the method described by Siwicki and Anderson (1994). Briefly, diluted hemolymph protein content was measured using a microprotein determination method (C-690; Sigma), prior to and after precipitating down the Ig molecules, using a 12% solution of polyethylene glycol (Sigma). The difference in protein content represents the Ig content.

GPX activity was assayed using a kit (Biovision, Inc., Milpitas, CA, USA). In this assay, cumene hydroperoxide was used as a peroxide substrate (ROOH), and glutathione

reductase (GSSG-R) and NADPH (b-nicotinamide adenine denucleotide phosphate, reduced) were included in the reaction mixture. The change in 340 nm due to NADPH oxidation was monitored for GPX activity. Briefly, 50μL of sample was added to 40μL of the reaction mixture and incubated for 15 min and then 10μLof cumene hydroperoxide was added and OD_1 read at 340 nm. After 5 min of incubation, OD_2 was read in 340 nm by the microplate reader. Activity of GPX was calculated as nmol/min/mL.

2.3.5. Challenge trial

At the end of the feeding trial, 10 shrimp from each tank (20 shrimp per treatment) were randomly selected and subjected to a bacterial challenge. *V. harveyi* was used as the pathogenic agent (provided by the Marine Microbiology Laboratory of Jeju National University). The shrimp were injected intramuscularly with *V. harveyi* suspension containing 2×10^7 CFU mL^{-1}. The pathogenic dose of bacterium had previously been determined in a preliminary test using shrimp of a similar size. After injection, the shrimp were distributed into 16 120 L acryl tanks, and their mortality was monitored and recorded for 18 days.

2.3.6. Statistical analysis

All dietary treatments were assigned by a completely randomized design. Data were subjected to one-way analysis of variance (ANOVA) in SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the difference in means was made with Tukey's HSD multiple range tests. Statistical significance was determined at $P < 0.05$. Data are presented as mean± SD. Percentage data were arcsine transformed before analysis.

3.3. RESULTS

Feed utilization of the shrimp was significantly affected by dietary supplementation of biofloc compared to those of fish fed the Con diet (Table 3-3). However, final body weight, weight gain and specific growth rate of shrimp showed no significant difference among dietary treatments. Significantly higher feed intake (FI) was observed in shrimp fed the BF4 diet compared to those fed the Con diet; however no significant differences were found among all the other experimental groups. Dietary inclusion of the biofloc to the Con diet at the level of 4% resulted in significant decrease of protein efficiency ratio (PER) while shrimp fed the same diet showed the highest feed conversion ratio (FCR) compared to those of shrimp fed the Con and AMP diets. The best survival rate was obtained in AMP group which was significantly higher than that of the BF4 group ($P < 0.5$).

Shrimp fed the BF1 diet indicated significantly higher immunoglobulin level than the group of shrimp fed the Con diet. However, other examined non-specific immune parameters were not affected by dietary treatments ($P > 0.5$; Table 3-4).

During the challenge test, the first dramatic mortality was observed on the 13th day after injection where the shrimp fed the AMP diet showed the lowest disease resistance compared to all of the other groups (Fig. 3-1). At the end of the challenge test, significantly higher survival rate was found in Con and BF4 groups compared to the AMP group, however, no significant difference was found among all the other experimental groups.

Table 3-3. Growth performance of white shrimp (Initial BW: 1.00±0.00g) fed the eight experimental diets for 7 weeks.

Treatment	FBW[1] (g)	WG[2] (%)	SGR[3] (%)	FCR[4]	PER[5]	FI[6]	Survival (%)
Control	5.63±0.43	463±43.0	3.59±0.16	2.26±0.18^{a}	1.06±0.09^{b}	10.9±0.51^{a}	77.8±13.9^{ab}
Biofloc 0.25%	5.68±0.71	467±71.6	3.61±0.26	2.33±0.30^{a}	1.05±0.15^{ab}	11.1±0.19^{ab}	86.7±6.67^{ab}
Biofloc 0.50%	5.32±0.15	431±16.5	3.48±0.07	2.58±0.08^{ab}	0.93±0.03^{ab}	11.3±0.30^{ab}	80.0±6.67^{ab}
Biofloc 1.00%	5.46±0.42	446±45.7	3.53±0.17	2.56±0.17^{ab}	0.94±0.06^{ab}	11.0±0.22^{ab}	84.4±7.70^{ab}
Biofloc 2.00%	5.32±0.34	431±32.4	3.48±0.13	2.56±0.17^{ab}	0.96±0.06^{ab}	11.1±0.31^{ab}	84.4±3.85^{ab}
Biofloc 4.00%	4.88±0.46	390±46.7	3.30±0.20	3.27±0.50^{b}	0.76±0.12^{a}	13.2±1.93^{b}	53.3±17.6^{a}
Antibiotic (OTC)	5.32±0.97	430±97.1	3.45±0.40	2.64±0.32^{ab}	0.92±0.11^{ab}	11.9±0.86^{ab}	68.9±10.2^{ab}
Antibiotic (Amp)	5.99±0.63	497±62.2	3.75±0.22	2.20±0.31^{a}	1.10±0.14^{b}	11.0±0.24^{ab}	86.7±11.5^{b}

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different letters are significantly different (Tukey, HSD; $P < 0.05$).

[1]FBW=Final body weight

[2]Weight gain = [(final body weight - initial body weight)/ initial body weight] x 100

[3]Specific growth rate = 100 x [ln(final body weight) – ln(initial body weight)]/days

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

[5]Protein efficiency ratio = fish weight gain (g)/protein

[6]Feed intake (g/fish) = dry feed consumed (g)/fish

Table 3-4. Non-specific immune responses of white shrimp fed the eight experimental diets for 7 weeks.

Treatment	NBT[1] (%)	PO[2] (%)	SOD[3] (%)	Ig[4]	GPx[5]
Control	0.090±0.02	0.11±0.01	248.3±24.4	13.7±3.35[a]	40.7±3.69
Biofloc 0.25%	0.093±0.02	0.20±0.13	238.9±26.5	17.5±3.05[ab]	44.6±13.1
Biofloc 0.50%	0.097±0.01	0.12±0.01	255.7±20.3	17.4±1.12[ab]	45.0±5.73
Biofloc 1.00%	0.098±0.01	0.17±0.08	253.8±21.1	23.1±5.38[b]	42.1±5.67
Biofloc 2.00%	0.091±0.01	0.14±0.01	250.2±18.5	19.8±3.63[ab]	42.9±5.99
Biofloc 4.00%	0.101±0.01	0.16±0.05	272.1±10.0	16.2±1.49[ab]	35.4±7.51
Antibiotic (OTC)	0.103±0.01	0.23±0.15	256.8±33.5	18.5±2.00[ab]	39.9±10.0
Antibiotic (Amp)	0.091±0.00	0.15±0.03	244.4±14.6	19.4±1.22[ab]	41.3±8.42

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different letters are significantly different (Tukey, HSD; $P < 0.05$).

[1]Nitro blue tetrazolium activity (absorbance)

[2]Phenoloxidase activity (absorbance)

[3]Superoxide dismutase

[4]Immunoglobulin

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

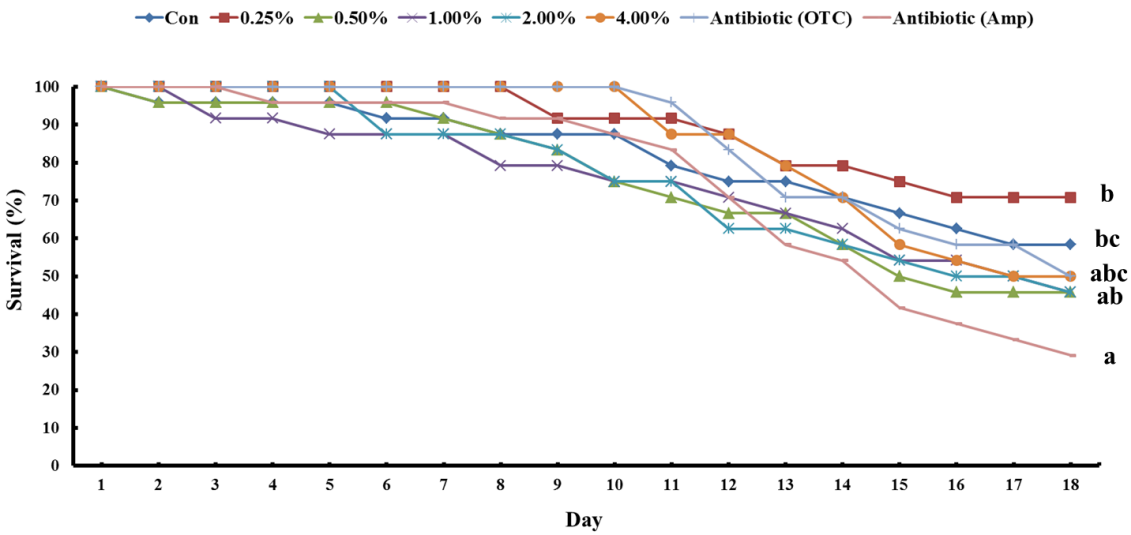

Figure 3-3. Survival of white shrimp fed the experimental diets after challenge with *Vibrio harveyi*.

4. DISCUSSION

Beneficial role of biofloc culture system in penaeid shrimp is well documented (Hari et al., 2006; Xu and Pan, 2012). Recently, it reported that use of biofloc as a dietary ingredient or supplement enhanced the growth rate of *L. vannamei* (Ju et al., 2008b; Kuhn et al., 2009, 2010). In experiment Ⅰ, dietary supplementation of biofloc at 4% level significantly enhanced the growth, PER and reduced the FCR in shrimp. It has been documented that bioflocs are the rich source of many bioactive compounds such as carotenoids, chlorophylls, phytosterols, bromophenols, amino sugars (Ju et al., 2008a) and anti-bacterial compounds (Crab et al., 2010). This suggests that microbial components, unknown growth factors or probiotic microorganisms like *Bacillus*, *Lactobacillus* present in the biofloc might have resulted in significantly higher growth rate and better FCR in shrimp fed with biofloc incorporated diets. On the other hand biofloc supplementation at 8% level (BF8) did not result in proportionate increase in growth rate or improvement of FCR compared to those of the Con diet. Kuhn et al. (2010) replaced fish meal with biofloc in *L. vannamei* diet and recorded significantly higher growth rate at 10 and 15%, and non-significant difference at 20 and 30% dietary inclusion level of biofloc. The present findings agree with those of Wang (2007) and Anand et al. (2013) who reported that increase in dietary supplementation of probiotic or periphytic algae in shrimp diets do not increase proportionately the growth performance of shrimp. Moreover, reduction in growth rate of fishes was recorded at higher level of microbial supplementation (Ajiboye et al., 2012; Kiessling and Askbrandit, 1993) as microbial products at higher level tend to reduce the feed palatability and digestibility (Kiessling and Askbrandit, 1993). Also, the higher ash level recorded in BF6 and BF8 diets compared to all other groups might have influenced

the digestibility and growth performance of shrimp. However, the growth performance of BF6 and BF8 were relatively higher compare to the Con diet suggesting that dietary supplementation of higher level of biofloc does not have growth retardation effects in shrimp juveniles. It has been demonstrated that the active and densed microorganisms together with suspended organic particles tend to form biofloc, which can be consumed constantly by cultured shrimp as a natural food source (Burford et al., 2004; Kent et al., 2011; Wasielesky et al., 2006). Like other crustaceans, shrimp lack specific or adaptive immune system that relies entirely on their innate immune mechanisms including both cellular and humoral responses for defense against pathogens (Vazquez et al., 2009). NBT activity increased in the shrimp fed BF0.5 and BF4 compared to those of the Con group, while significantly higher PO activity was observed in BF6 group compared to those fed the Con diet. It has been suggested that the biofloc ingestion by shrimp may release some useful substances into the gastrointestinal tract that could potently stimulate the innate immune response (especially phagocytosis) and release more hemocytes into the circulation (Zhao et al., 2012). It is possible that some beneficial bacteria such as *Bacillus* sp. in the ingested biofloc could facilitate the modification of physiological and immunological status of the host through the colonization in the gastrointestinal tract and the induction of changes in the endogenous microbiota (Johnson et al., 2008; Li et al., 2009). Moreover, Ju et al. (2008a) noted that both microbial components (*Bacillus* sp.) and bioactive compounds (e.g. polysaccharides and carotenoids) present in the biofloc could exert an immune-stimulating effect and this action would be continuous as long as the shrimp consumes the biofloc. However, the modes of action of biofloc on innate immune system of shrimp are complicated and still unknown up to date. Further study is required for more detailed information and knowledge. Research on antioxidants has been attracted

in aquaculture since fish or shrimp are susceptible to oxidative stress by pathogen pressure and environmental changes (Castex et al., 2010; Liu and Chen, 2004). Like other aerobic organisms, shrimp possess an integrated antioxidant system including enzymatic and non-enzymatic antioxidants to maintain normal oxidant status, especially to cope with natural or induced stressors (Castex et al., 2009; Parrilla-Taylor and Zenteno-Savín, 2011). Generally, the antioxidant capability of an organism under certain condition can reflect its health status. Increased GPx activity was observed in the groups of shrimp fed the BF6 diet compared to those fed the Con diet. This result indicates that the antioxidant system of the shrimp can be enhanced by unknown components present in biofloc in some way. Based on its composition characteristics, the biofloc may (i) play a role in antioxidant activity because it contains an appropriate amount of antioxidants such as carotenoids and fat-soluble vitamins (Ju et al., 2008a) and (ii) stimulate digestive enzyme activities and improve feed utilization (Xu and Pan, 2012; Xu et al., 2012) thereby increasing the assimilation of dietary antioxidants from the feed. Similarly, Xu and Pan (2013) reported an increase in total antioxidant capacity (T-AOC) and SOD activity of *L. vannamei* juveniles reared in biofloc-based tanks. At the end of the challenge test with *V. harveyi*, significantly higher survival rate was found in BF4 group compared to the Con group. Similar observations were obtained by Ekasari et al. (2014) where following a challenge test by injection with infectious myonecrosis virus (IMNV) the survival of the challenged shrimp from the experimental biofloc groups was significantly higher when compared to the challenged shrimp from the control treatment.

In experimental Ⅱ, shrimp growth performance and feed utilization was not significantly affected by dietary supplementation of either biofloc or OTC. Mean values of NBT activity increased in the shrimp of the two biofloc treatments including BF1 and BF4

as compared with the BF2 group, while significantly higher Ig level observed in BF0.25 group compared to those fed the Con diet. At the end of the challenge test with *V. harveyi*, significantly higher survival rate was found in groups of shrimp fed the OTC supplemented diet compared to those fed the BF0.25 diet. This study demonstrates that besides providing supplemental nutrition, like protein, lipid, mineral and vitamin, the biofloc is a source of abundant natural microbes and bioactive compounds that could exert a positive effect on the physiological health of cultured shrimp.

In experimental Ⅲ, dietary supplementation of biofloc at 4% level significantly enhanced the FI while showed the lowest PER and the highest FCR values compared to shrimp fed the Con and AMP diets. However, in the present study, the biofloc supplementation at the 4% level did not result in proportionate increase in growth rate or improvement of FCR compared with control. Mean value of Ig level increased in the shrimp fed the BF1 diet compared to those fed the Con diet. At the end of the challenge test with *V. harveyi*, significantly higher survival rate was found in BF0.25 and Con groups compared to the AMP group.

Results from the experiment Ⅰ indicate that dietary supplementation of the tested biofloc powder can improve growth performance and feed utilization of *L. vannamei*. These finding also showed that bioflocs have positive effects on the innate immune response of *L. vannamei* leading to an enhanced disease resistance against *V. harveyi* challenge. However, results from two other experiments did not show significant difference in growth performance by the bioflocs. This difference may be due to undetermined nutritional inconsistencies between biofloc variations or unknown factors. Therefore further studies are required to determine the amino acid profile, mineral profile and mechanisms of biofloc effects on the nutritional physiology of *L. vannamei*.

In conclusion, the findings from the three studies demonstrate that dietary supplementation of a good source of biofloc at 4% level had beneficial effects on growth performance and health status in *L. vannamei*. The concurrent increases of non-specific responses of the shrimp fed the biofloc supplemented diets may suggest that the antioxidant defense system and innate immune system could work synergistically to improve the physiological performance of the shrimp leading to higher disease resistance against bacterial challenge. These findings suggest that the biofloc could be practically used as a viable alternative dietary supplement. It seems clear that the potential of the biofloc availability as a dietary supplement is dependent upon the process method of the biofloc, quantity of organic compounds and beneficial microorganism. Future studies are required to determine the amino acid profile, mineral profile and non-protein contents of the biofloc in cases of several different collection methods for its usage in diets for shrimp.

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