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Effects of solid state fermented plant protein sources as fish meal replacers in whiteleg shrimp *Litopaeneus vannamei*

by

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흰다리새우에 있어 고체 발효처리한 식물 단백질원들의 어분대체효과

Advisor: Prof. Sungchul C. Bai

by

Ali Hamidoghli

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A dissertation

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Abstract

Two feeding trials were conducted to evaluate effects of different types of solid state fermented plant protein sources with *Bacillus* spp. as fish meal replacers in juvenile whiteleg shrimp, *Litopaeneus vannamei*. In the first trial, a control diet with no replacement for fish meal (CON) and seven other diets that replaced 30% of fish meal with fermented soybean meal with *Bacillus* spp. (FSM_B), sterilized FSM_B (FSM_{BS}), fermented soybean meal and corn gluten meal with *Bacillus* spp. (FSC_B), sterilized FSC_B (FSC_{BS}), fermented soy protein concentrate with *Bacillus* spp. (FSP_B), fermented soybean meal with lactic acid bacteria (FSM_L), and soy protein concentrate (SPC) were used. Results showed significantly higher weight gain and specific growth rate for shrimp fed the FSM_B and FSC_B diets comparing to those of shrimp fed the CON and FSM_L diets (P<0.05). Lysozyme activity of shrimp fed the FSC_B diet was higher than those of shrimp fed CON and FSM_L diets. After eight days of challenge test, shrimp fed the FSC_B diet showed higher survival compared to shrimp fed all the other diets (P<0.05). In the second trial, the effects of fermented plant proteins with or without bacterial water treatment was determined on water quality indices of whiteleg shrimp culture. Juvenile whiteleg shrimp were fed by the CON, FSM_B, FSC_B and FSP_B diets. All treatments were divided into two groups, one group with the addition of bacterial water treatment (AquaStar®) and one group without bacterial water treatment (assigned as O and X, respectively). Results for total ammonia nitrogen showed significantly lower values for FSC_B(O) and FSP_B(O) tanks compared to the CON(X) tanks (P<0.05). Same trend was observed for NO_2-N as CON(X) tanks had significantly higher values. Total *Bacillus* spp. counts, after 2 and 4 weeks, were significantly lower for both CON(O) and CON(X) tanks compared to all other tanks (P<0.05). Based on these results, replacement of fish meal with *Bacillus*-fermented soybean meal and corn gluten meal (FSC_B) can improve growth, feed utilization, immune response and disease resistance in whiteleg shrimp while improving the water quality indices when administered with bacterial water treatment.

First feeding trial:

Effects of solid state fermented plant protein sources as fish meal replacers on growth performance, intestinal bacterial count, immune responses and disease resistance in whiteleg shrimp *Litopaeneus vannamei*

An 8-week feeding trial was conducted to investigate fermented plant protein sources as fish meal replacers in juvenile whiteleg shrimp, *Litopaeneus vannamei*. Eight isonitrogenous and isoenergetic diets were used in this experiment. The control diet had no replacement for fish meal (CON), while other diets replaced 30% of fish meal with fermented soybean meal with *Bacillus* spp. (FSM_B), sterilized FSM_B (FSM_{BS}), fermented soybean meal and corn gluten meal with *Bacillus* spp. (FSC_B), sterilized FSC_B (FSC_{BS}), fermented soy protein concentrate with *Bacillus* spp. (FSP_B), fermented soybean meal with lactic acid bacteria (FSM_L), and soy protein concentrate (SPC). Three groups of 40 post-larvae whiteleg shrimp (0.5 ± 0.01g) were used in each tank (total 24 tanks) and fed the experimental diets. Results showed significantly higher weight gain and specific growth rate for shrimp fed the FSM_B and FSC_B diets comparing to those of shrimp fed the CON and FSM_L diets (P<0.05). Shrimp fed the FSC_B diet had significantly higher feed efficiency and protein efficiency ratio than those of shrimp fed the FSP_B diet (P<0.05). Intestinal *Bacillus* spp. count of shrimp fed diets FSM_B, FSC_B and FSP_B were significantly higher than those of shrimp fed the CON, FSM_{BS}, FSC_{BS}, FSM_L and SPC diets. Lysozyme activity of shrimp fed the FSC_B diet was higher than those of shrimp fed CON and FSM_L diets. After eight days of challenge test, shrimp fed the FSC_B diet showed higher survival compared to shrimp fed all the other diets (P<0.05). Therefore, replacement of fish meal with *Bacillus*-fermented soybean meal and corn gluten meal can improve growth, feed utilization, immune response and survival in whiteleg shrimp, *Litopaeneus vannamei*.

Second feeding trial:

Effects of solid state fermented plant proteins with or without bacterial water treatment on water quality in juvenile whiteleg shrimp *Litopaeneus vannamei*

A 4-week feeding trial was conducted to determine the effects of fermented plant proteins with or without bacterial water treatment on water quality indices of whiteleg shrimp culture. Juvenile whiteleg shrimp with initial weight of 4.08 ± 0.12 g (mean ± SD) were distributed in 24 aquaria with 50 L capacity (filled with 23 L of Sea water). Each tank contained ten shrimp and fed one of the four experimental diets: a diet without fish meal replacement was assigned as control (CON), and three other diets replaced 30% of fish meal with *Bacillus* spp.-fermented soybean meal (FSM_B), *Bacillus* spp.-fermented soybean meal and corn gluten meal (FSC_B) and *Bacillus* spp.-fermented soybean protein concentrate (FSP_B). All treatments were divided into two groups, one group with the addition of bacterial water treatment (AquaStar®: *Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp., *Thiobacillus* sp. and *Paracoccus* sp.) and one group without bacterial water treatment (assigned as O and X respectively). During the experiment, water was not exchanged in any of the tanks and only evaporated water was added. The pH significantly dropped after day 13 from 8 to 6.5 in all tanks. Results for total ammonia nitrogen showed significantly lower values for FSC_B(O) and FSP_B(O) tanks and the CON(X) tanks (P<0.05). Same trend was observed for NO_2-N as CON(X) tanks showed significantly higher values at days 15, 18, 21 and 24 compared to all other tanks. Whereas tanks fed the FSC_B(O) diet had the lowest NO_2-N compared to CON(X) and CON(O) tanks. Levels of NO_3-N increased throughout the experiment but with no significant differences at final days (P>0.05). Total bacterial counts, after four weeks, were significantly higher for tanks fed FSM_B(O), FSC_B(O) and FSP_B(O) compared to CON(X) tanks (P<0.05). Total *Bacillus* spp. counts, after 2 and 4 weeks, were significantly lower for both CON(O) and CON(X) tanks compared to all other tanks (P<0.05). Based on these results, replacement of fish meal with *Bacillus* spp.-fermented plant proteins along with bacterial water treatment could have positive effects on water quality in shrimp culture.

흰다리새우에 있어 고체 발효처리한 식물 단백질원들의 어분대체효과

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알리

요 약

본 연구는 치하기 흰다리새우 (*Litopaeneus vannamei*) 사료 내 식물 단백질원과 바실러스 균의 고체 발효공정처리를 통한 어분대체 효과를 평가하기 위하여 수행하였다. 실험 1에서는, 어분대체를 하지 않은 대조구 (Fishmeal, CON), 발효대두박과 바실러스균 (FSM_B), 멸균처리한 FSMB (FSM_{BS}), 발효대두박, 콘글루텐밀 및 바실러스균 (FSC_B), 멸균처리한 FSCB (FSC_{BS}), 발효 농축콩단백과 바실러스균 (FSP_B), 발효대두박과 유산균 (FSM_L), 농축대두단백 (SPC) 로 7개의 어분대체구를 설계하여 각각 30%의 어분대체를 실시하였다. 증체율 (weight gain)과 일간성장률 (specific growth rate)에 있어서 FSMB 와 FSC_B 구가 CON과 FSM_L 구에 비해 유의적으로 높은 값을 나타냈다 (P<0.05). Lysozyme 활성은 FSCB 구가 CON과 FSM_L 구에 비해 유의적으로 높은 값을 나타냈다 (P<0.05). 반면에, 공격실험 8일차에서는 FSCB 구가 타 실험구에 비해 유의적으로 높았다(P<0.05). 실험 2에서는, 수질개선 평가를 위해 실험 1에서 사용된 총 4개의 실험사료 (CON, FSM_B, FSC_B, FSP_B) 에 상업용 수질정화제 (AquaStar®) 를 사육수에 첨가 (O) 또는 무첨가 (X) 하여 무첨가구: CON (X), FSM_B (X), FSC_B (X) 및 FSP_B (X) 와 첨가구: CON (O), FSM_B (O), FSC_B (O) 및 FSP_B (O), 총 8개의 실험구로 설계하였다. 총 암모니아질소(TAN) 농도에서 FSC_B(O) 과 FSP_B(O) 구가 CON (X) 에 비해 유의적으로 낮은 값을 나타내었다. (P<0.05). Bacillus count (BSC) 분석결과 2주와 4주 후, CON(O) 과 CON(X) 구는 타 실험구에 비해 균 수가 유의적으로 낮은 결과를 나타났다 (P<0.05). 따라서, 본 연구결과를 통해 치어기 흰다리새우 사료 내 발효대두박, 콘글루텐밀 및 바실러스균 (FSC_B) 실험구는 어분대체재로써 성장 및 면역 상승효과를 나타낼 뿐만 아니라 수질개선에도 효과가 있다고 판단된다.

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CHAPTER I. Replacement of fish meal by alternative protein sources

1. Introduction

One of the world's greatest challenges is how to feed more than nine billion people by 2050 in a context of climate change, economic and financial uncertainty, and growing competition for natural resources. Food security is the term that is used to express the accessibility of people to sufficient, safe and nutritious food to maintain a healthy and active life. Food security is achieved when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life (WFC, 2010).Therefore, improved food security is a necessary precursor for global initiative towards reduction of hunger and poverty, and for economic development. One aim of the UN Millennium Development Goals is to reduce by half the proportion of people suffering from hunger. However, the continuing population and consumption growth will mean that the global demand for food will continue to increase. Presently, over 820 million people are affected by hunger in developing countries and these numbers are not decreasing rapidly enough, particularly in Africa and Southern Asia. Lack of quality food is still the main cause of under-nutrition among children, which is very common in developing areas (UN, 2010). Recent reports highlight the tremendous potential of the oceans and inland waters now, and even more so in the future, to contribute significantly to food security and adequate nutrition for a global population expected to reach 9.7 billion by 2050 (FAN 2018).

Fish has always been a source of rich food and played an important role in improving developing countries' food security and nutrition. Fish is also a major source of high-quality dietary protein, essential vitamins, minerals, and other micronutrients for about one billion people, many isolated in rural communities of developing and low income countries (Stephen et al., 2010). The nutritional benefits of fish and fish oil consumption on human health, including the prevention of cancer, diabetes and heart diseases, have been well established (Olsen 2003; Raatz & Bibus 2016). Nowadays, over 50 percent of all fish for human consumption globally is farmed. This has risen from only 28 percent in 1995 and the upward trend will continue as aquaculture plays an increasingly important role in providing fish for global consumers. In 2016, a total of 80 million tonnes of farmed food fish (US$231.6 billion) were produced in the world. Farmed food fish

include 54.1 million tonnes of finfish (US$138.5 billion), 17.1 million tonnes of molluscs (US$29.2 billion) and 7.9 million tonnes of crustaceans (US$57.1 billion) (FAO 2018). It seems that aquaculture is the only way to meet the increasing demands for aquatic foods. This increasing production of fisheries products follows closely the increasing demand of fisheries products due to the world population growth and the increasing per capita fish consumption. As fisheries captures have stagnated in the past decades, the additional demand of fisheries products will have to be fulfilled by aquaculture production (Klinger and Naylor, 2012).

The exponential growth of the aquaculture sector during the past two decades is a result of the progressive intensification of production systems. A major contributor to this intensive production system is the use of manufactured feeds formulated to meet the nutritional requirements of the targeted fish species. Feeds account for up to 70 % of the variable cost of a commercial aquaculture operation for many fish and shrimp species (Webster et al., 1999). Feed production costs are driven by the cost of feed ingredients. Fishmeal (FM) is considered the most adequate protein source for fish, as it has a high protein content, with adequate amino acid profile, high protein digestibility and high palatability; it is a rich source of taurine, minerals (including phosphorus) and vitamins (including choline); and it has no anti-nutritional factors. Therefore, it is not surprising that FM has been used as the main protein source in aquafeeds, particularly in diets for carnivorous fish (Hardy, 2010). The problem is that price of fishmeal has increased more than two fold in recent years influencing the price of aquafeed, production costs and fish price. Furthermore, the provisions of fishmeal are not adequate to sustain the current rate of growth of aquaculture in addition to the demand from other animal feed industries. Therefore, aquaculture researchers have focused on finding ways to replace or reduce the amount of FM in aquafeed since this was considered one of the main goals towards sustainability.

2. Replacement of fish meal

Most freshwater fish species are omnivorous or herbivorous and most diadromous and marine species being carnivorous. These differences in feeding habits are also reflected in the nutritional requirements of these fish. For instance, whereas omnivorous and herbivorous fish have relatively low protein requirements, ranging from 25% to 35% of the diet, carnivorous fish have high protein requirements, ranging from 40% to 55% of the diet. Also, whereas some species efficiently use

diets with carbohydrate levels up to 40-60%, other species do not tolerate more than 10-20% dietary carbohydrates (Wilson, 2002; NRC, 2011). Fishmeal is generally added to fish diets to cover the protein requirements, increase feed efficiency and growth and enhance feed palatability. As world capture fisheries are limited and have even decreased in past years, the world availability of FM is also limited. From an environmental standpoint, overfishing of wild stocks for production of FM is unsustainable (Nordahl, 2011). According to the International Fishmeal and Fish Oil Organisation (IFFO) it was estimated that in 2010 63% of FM production was used in aquaculture, which 25% of that went to salmonids and another 25% to marine fish (Tacon et al., 2011). Owing to the increase in aquaculture production and competition with other industries, enormous pressure is being put on the use of FM for inclusion in aquafeeds, with prices increasing accordingly (Tacon and Metian, 2008). For instance, the FM and soybean meal price ratio increased from 2:1 in the 1990s to 4:1 in 2010 (Shepherd and Jackson, 2013). Reducing aquafeed dependency on FM is of the utmost importance and it is recognized by stakeholders as a priority for the sustainable development of fed aquaculture. Dependency on FM is almost non-existent for most omnivorous fish, and nowadays practical diets for carp, tilapia or catfish are almost devoid of FM. Even during the initial growth phases, which are usually more exigent in terms of nutrient requirements, there is no apparent advantage of including animal protein in the diets of omnivorous species (Sink et al., 2010). Reduction of fisheries products use in aquafeeds is more challenging for carnivorous fish, namely marine fish and shrimp (Tacon, 2004). Even so, it is expected that substantial reductions in FM (40-50%) in aquafeeds will be achieved in the near future (Tacon et al., 2011). Overall, it can be assumed that replacing half of the FM in carnivorous fish diets with plant protein feedstuffs is relatively simple (Naylor et al., 2009). However, reaching low levels or complete elimination is more complicated without reducing growth performance and animal health. Thus, continued research efforts to overcome these problems and to further reduce the amount of FM used in aquafeeds are required if sustainable growth of global aquaculture is to be ensured. But in order to promote a performance similar to that obtained with FM-based aquafeeds, the alternative aquafeeds (low in FM) must ensure good fish health and welfare and a final product that is nutritionally adequate, safe to eat and well accepted by the consumer (New and Wijkstrom, 2002). There are several protein sources that have the potential of replacing fishmeal in aquaculture feeds without affecting the growth performance and health of fish. These alternative protein sources include plant proteins (fermented, non-fermented and by-products), animal proteins (animal by-

products and insect meals) and single cell proteins (algae, yeast and bacteria). When considering FM alternatives in aquafeeds aspects such as price, protein content, amino acid profile, digestibility, essential amino acid (EAA) deficiencies, antinutritional factors and palatability must be addressed (Gatlin et al., 2007; Hardy, 2010). Caution must be also taken to avoid unintended consequences in fish health, intestine homeostasis, immunological parameters and disease resistance. Therefore we aimed at reviewing the main protein sources that have the potential of replacing fish meal while sustaining feed quality.

3. Plant protein sources

Plant feedstuffs are the most abundant alternative protein sources to use in aquafeeds (Tacon et al., 2011). However, plant feedstuffs have a highly variable protein content and present several EAA inadequacies and anti-nutritional factors, and these characteristics impose some limitations to their use in diet formulations. Other than a few plant protein concentrates, such as soy protein concentrate or potato protein concentrate, most alternative protein sources have an EAA profile that presents imbalances in one or more EAA. Within the most used alternative protein sources in aquafeeds the first limiting EAAs are usually lysine and methionine. Tryptophan, threonine, arginine and histidine may also be limiting in several feedstuffs. Interestingly, within the potential alternative feedstuffs used in aquafeeds, those that have methionine as the first limiting EAA have an excess of lysine, and the opposite is true for feedstuffs deficient in lysine. This increases potential combinations of feedstuffs to include in aquafeeds, as they may complement one another to provide a balanced dietary EAA profile. Anti-nutritional factors in plant feedstuffs are highly abundant and diversified (Francis et al., 2001; Hendricks, 2002; Gatlin et al., 2007; Krogdahl et al., 2010) and various strategies are required to alleviate their negative nutritional impacts. These include technical treatments such as heat processing, solvent extraction and dehulling or the use of exogenous enzymes such as phytases (Jobling et al., 2001; Glencross et al., 2007; Krogdahl et al., 2010). Breeding new plant varieties with better amino acid (AA) profiles or low phytic acid is also a strategy for improving plant utilization in aquafeeds (Gatlin et al., 2007; Overturf et al., 2003). Dietary inclusion of exogenous enzymes that improve digestibility of nutrients, particularly of non-starch polysaccharides (NSPs) and phytic phosphorus, is gaining relevance in aquafeeds (Ai et al., 2007; Adeola and Cowieson, 2011; Dalsgaard et al., 2012). Care should be taken,

however, regarding the practical efficiency of adding the feed enzymes directly to the diet. Fish, particularly cold water species, are reared at water temperatures that are below the optimal activity of these enzymes, and therefore their efficacy is reduced. Alternatively, pre-treatment of plant feedstuffs at the optimal temperature for enzyme activity may be a better technological strategy and would provide a benefit across animal species.

Plant feedstuffs are the major dietary protein sources for omnivorous and herbivorous fish and are second to FM in diets for carnivorous species (Tacon et al., 2009). However, owing to the high dietary protein requirements of carnivorous fish, potential alternative protein sources are limited to a few feedstuffs with high protein content. These comprise mainly plant protein concentrates and oilseeds, animal by-products and unicellular organisms. Plant protein concentrates include corn gluten, wheat gluten and less abundant protein concentrates such as soy protein concentrate, pea protein concentrate, potato protein concentrate and rapeseed protein concentrate. Some of these protein concentrates are still expensive compared to FM, because of processing costs, and, with the exception of corn gluten, their use in aquafeeds is still limited. However, with the increasing price of FM the use of protein concentrates in diets for carnivorous fish species is expected to increase (Naylor et al., 2009; Tacon et al., 2011). Protein concentrates have great potential for use in aquafeeds because of their high protein content (between 60% and 80%) and because they are almost devoid of anti-nutritional factors. Lysine, threonine and methionine are the first limiting AAs in these feedstuffs. Plant protein concentrates may replace FM protein from almost 30% to 100% in experimental diets, corresponding to dietary incorporations of 10-60%. Corn gluten meal is currently used in feeds for carnivorous fish, with upper inclusion limits of 20e25% (Gatlin et al., 2007). Incorporation of other plant protein concentrates in carnivorous fish diets is usually lower than 15% (Tacon et al., 2011). It is worth noting that, when included in the diet at high concentrations, carotenoids present in corn gluten may confer undesirable colour to the flesh. For instance, in rainbow trout dietary inclusion of corn gluten above 10% will impart an undesirable yellow colour to the fillets (Gaylord et al., 2010). Wheat gluten is incorporated in diets at lower levels than corn gluten, partially because of its higher price, but also because of the binding proprieties of its protein, which have undesirable effects on pellet quality (Gatlin et al., 2007; Gaylord et al., 2010). Further, wheat gluten has very high levels of glutamic acid, which represents c. 30% of the protein, and although it may be of value as a nutraceutical if included at moderate levels in the diet, at high levels it may have undesirable effects. Oilseeds, such as soybean

meal, cottonseed meal, rapeseed meal or sunflower meal, have competitive prices and a protein content ranging from 38% to 52%. Soybean meal is the most available oilseed worldwide and it is also the most common plant feedstuff used in aquafeeds. Owing to the relatively low protein content, FM protein replacement by oilseeds is usually limited to 20-40%, corresponding to dietary incorporation of 15e30%. According to Tacon et al. (2009) the mean incorporation of oilseeds in practical diets for carnivorous fish is about 10e20%, except for sunflower meal, the inclusion level of which is usually lower (up to 10%) mainly owing to its high fiber content (Tacon et al., 2011). Oilseeds have some EAA deficiencies, the first limiting EAA being methionine in soybean meal and lysine in rapeseed, sunflower and cottonseed meals. Oilseeds also have several anti-nutritional factors, some of which are inactivated by heat processing or solvent extraction but others of which cannot be inactivated. Dehulling is routinely used in some oilseeds as an efficient technological treatment to reduce fiber and tannins and to increase protein content. An alternative to eliminating some resistant antinutrients is the selection of new cultivars. This has been successfully achieved for rapeseed/canola, in which levels of glucosinolates and erucic acid have been extremely reduced, and for cottonseed, with a variety almost free of gossypol. Oilseeds are not very palatable for fish and this may affect feed intake. In such cases, addition of feed stimulants or mixture with more palatable feedstuffs may reduce or overcome this inconvenience (Dias et al., 1997).

4. Animal protein sources

Animal by-products comprise meat meal, meat and bone meal, poultry by-product meal, feather meal and blood meal, among others, and have high potential as alternatives to FM in aquafeeds as they have acceptable protein content and competitive prices. However, in contrast to plant feedstuffs, which have relatively constant nutritional composition, animal feedstuff composition is highly variable, particularly that of poultry by-products and meat and bone meals, and therefore proximate composition must be closely checked. The use of animal by-products in aquaculture is highly variable depending on the region. For instance, in Australia the use of rendered animal products in aquafeeds is high, whereas in the European Union very strict regulations for their use, and also consumer concerns over the potential risk of disease transmission (due to bovine spongiform encephalopathy), virtually prevent their use in animal feeds (Klinger and Naylor, 2012). Protein content of animal by-products is usually high, ranging from 50% to 80% or even

more, as in blood meal or plasma hydrolysate, but AA deficiencies may occur, particularly for lysine, methionine and tryptophan. Depending on the source and nutritional quality, animal by-products may replace up to 20-40% of FM protein in experimental diets, corresponding to dietary incorporations of 15-60%. According to Tacon et al. (2009) the range of meat meal and poultry by-product incorporation in practical diets for carnivorous fish is 10-30%, whereas that of hydrolysed feather meal is limited to 5e20%, owing to its high methionine deficiency and very high cysteineemethionine imbalance (Tacon et al., 2009). Though having a very high protein content, blood meal is characterized by a severe leucineeisoleucine imbalance that limits its inclusion in aquafeeds to 1-8%, with a mean inclusion level of 2-4% (Tacon et al., 2009). Animal by-products have good palatability and present no anti-nutritional factors. However, poultry by-products, meat meal and meat and bone meal have high ash contents and high saturated fat levels and this limits their use in aquafeeds. Ash is rich in phosphorus, and high incorporation of animal by-products in aquafeeds may result in excess dietary phosphorus, with the concomitant environmental problems associated with phosphorus loss to the water bodies. Also, saturated fats tend to deposit in carcasses, thus affecting the nutritional and organoleptic quality of fish fillets. Even though, animal origin ingredients such as poultry by-product meal and meat and bone meal are considered among the most suitable protein sources for shrimp feeds. In spite of their importance, a considerable reduction in the use of these animal origin ingredients is expected in coming years. Limited availability, variable supply and safety issues are primary concerns. Given the growing demand by animal production industries for fish meal and its limited supply, prices are likely to continue to increase, therefore, restraining future use as the main protein source in shrimp feeds. Likewise, emerging environmental and safety issues associated to the use of potentially contaminated animal by-products in animal feeds and the effect of fish meal production from natural fish stocks have also been viewed negatively. It has been suggested that one way to address all these issues is through the development of all plant-protein feeds. Such a tactic could also provide an economical opportunity for shrimp producers, as some segments of the market would pay a higher price for a premium shrimp fed and produced under environmentally sound conditions (Davis et al., 2004; Samocha et al., 2004).

5. Other protein sources

Single-cell proteins (SCPs), such as bacteria or brewer's yeast, are rich protein sources (>50%), though they also contain high levels of nucleotides (12-20% of total N), are very palatable and are devoid of anti-nutritional factors. SCPs may have nutraceutical proprieties and are rich sources of B vitamins (Rana and Hasan, 2009; Oliva-Teles, 2012). Methionine is a potential limiting AA in these feedstuffs, which may replace up to c. 50% of FM protein in experimental diets, corresponding to a dietary incorporation of 30-55%. In practical diets, mean incorporation values are, however, limited to 2-4% (Tacon et al., 2009). Microalgae are a novel source of SCPs, with potential for incorporation in fish diets. However, they are still scarce and their price is very high. Moreover, their protein content is highly variable according to source and processing. SCPs are mostly included in diets as potential probiotics (Irianto and Austin, 2002; Balcazar et al., 2006; Nakano, 2007; Nayak, 2010). Probiotics are live organisms that may colonize the intestinal tract and contribute to improving health condition, disease resistance, microbiota balance and gut physiology (Irianto and Austin, 2002; Merrifield et al., 2010).

6. Conclusions

There is potential for significant sparing of FM in fish and shrimp diets during the grow-out phases without affecting overall performance. However, more studies are required evaluating the effects of simultaneous replacement of FM in the diet. It is highly suggested that future studies focus on practical alternatives for FM. Animal protein sources could be good FM replacers but high ash content and phosphorus and relatively high price makes them impractical. Also, single cell proteins cannot be compared with other FM replacers because of their high price. As mentioned before, plant protein sources contain anti-nutritional factors and lack some of the essential amino acids. But, plant proteins such as soybean meal could be a more sustainable and practical alternative for FM at this point. Anti-nutritional factors could be eliminated by fermentation processes and lack of amino acids could be covered as long as prices stay at the acceptable range.

7. References

Adeola O., Cowieson A.J. 2011. Opportunities and challenges in using exogenous enzymes to improve nonruminant animal production. J. Anim. Sci. 89: 3189-3218.

Ai Q.H., Mai K.S., Zhang W.B., Xu W., Tan B.P., Zhang C.X., Li H.T. 2007. Effects of exogenous enzymes (phytase, non-starch polysaccharide enzyme) in diets on growth, feed utilization,

nitrogen and phosphorus excretion of Japanese seabass, *Lateolabrax japonicus*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 147: 502-508.

Balcazar J.L., de Blas I., Ruiz-Zarzuela I., Cunningham D., Vendrell D., Muzquiz J.L. 2006. The role of probiotics in aquaculture. Vet. Microbiol. 114: 173-186.

Davis D.A., Samocha T.M., Bullis R.A., Patnaik S., Browdy C., Stokes A., Atwood H. 2004. Practical diets for *Litopenaeus vannamei*, (Boone. 1931): working towards organic and/or all plant production diets. Avances en Nutricion Acuicola VII. Memorias del VII Simposium Internacional de Nutricion Acuicola. 16–19 Noviembre, 2004. Hermosillo, Sonora, Mexico.

Dalsgaard J., Verlhac V., Hjermitslev N.H., Ekmann K.S., Fischer M., Klausen M., Pedersen P.B. 2012. Effects of exogenous enzymes on apparent nutrient digestibility in rainbow trout (*Oncorhynchus mykiss*) fed diets with high inclusion of plant-based protein. Anim. Feed Sci. Technol. 171: 181-191.

Dias J., Gomes E.F., Kaushik S.J. 1997. Improvement of feed intake through supplementation with an attractant mix in European seabass fed plant-protein rich diets. Aquat. Living Resour. 10: 385-389.

FAN 2018. FAO Aquaculture Newsletter. Food and Agricilture Organization of the United Nations. No. 58.

Francis G., Makkar H.P.S., Becker K. 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture 199: 197-227.

Gatlin D.M., Barrows F.T., Brown P., Dabrowski K., Gaylord T.G., Hardy R.W., Herman E., Hu G.S., Krogdahl A., Nelson R., Overturf K., Rust M., Sealey W., Skonberg D., Souza E.J., Stone D., Wilson R., Wurtele E. 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquacult. Res. 38: 551-579.

Glencross B.D., Booth M., Allan G.L. 2007. A feed is only as good as its ingredients e a review of ingredient evaluation strategies for aquaculture feeds. Aquacult. Nutr. 13: 17-34.

Gaylord G.T., Barrows F.T., Overturf K.E., Liu K., Hu G. 2010. An overview of progress toward developing an all plant-based diet for rainbow trout. Bull. Fish. Res. Agency 31: 9-14.

Hardy R.W. 2010. Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. Aquacult. Res. 41: 770-776.

Hendricks J.D. 2002. Adventitious toxins. In: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition, third ed. Elsevier, pp. 601-649.

Irianto A., Austin B. 2002. Probiotics in aquaculture. J. Fish Dis. 25, 633.

Jobling M., Gomes E., Dias J. 2001. Feed types, manufacture and ingredients. In: Houlihan, D., Boujard, T., Jobling, M. (Eds.), Food Intake in Fish. Wiley-Blackwell, pp. 25-48.

Klinger D., Naylor R. 2012. Searching for solutions in aquaculture: charting a sustainable course. Annu. Rev. Environ. Resour. 37: 247-276.

Krogdahl A., Penn M., Thorsen J., Refstie S., Bakke A.M. 2010. Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. Aquacult. Res. 41: 333-344.

Merrifield D.L., Dimitroglou A., Foey A., Davies S.J., Baker R.T.M., Bogwald J., Castex M., Ringo E. 2010. The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture 302: 1-18.

Nakano T. 2007. Microorganisms. In: Nakagawa H., Sato M., Gatlin D.M. (Eds.), Dietary Supplements for the Health and Quality of Cultured Fish. CAB International, pp. 86-108.

Nayak S.K. 2010. Probiotics and immunity: a fish perspective. Fish Shellfish Immunol. 29, 2-14.

Naylor R.L., Hardy R.W., Bureau D.P., Chiu A., Elliott M., Farrell A.P., Forster I., Gatlin D.M., Goldburg R.J., Hua K., Nichols P.D. 2009. Feeding aquaculture in an era of finite resources. Proc. Natl. Acad. Sci. 106: 15103-15110.

New M.B., Wijkstrom U.N. 2002. Use of Fishmeal and Fish Oil in Aquafeeds: Further Thoughts on the Fishmeal Trap. FAO, Rome.

Nordahl G. 2011. Is the Aquaculture Industry Caught in a Fishmeal Trap? Master Thesis in Economic Analysis. Norwegian School of Economics and Business Administration, p. 109.

NRC 2011. Nutrient Requirements of Fish and Shrimp. The National Academy Press, Washinghton, DC.

Oliva-Teles, A., 2012. Nutrition and health of aquaculture fish. J. Fish Dis. 35, 83-108.

Olsen S.O. 2003. Understanding the relationship between age and seafood consumption: The mediating role of attitude, health involvement and convenience. Food Qual. Prefer 14: 199-209.

Overturf K., Raboy V., Cheng Z.J., Hardy R.W. 2003. Mineral availability from barley low phytic acid grains in rainbow trout (*Oncorhynchus mykiss*) diets. Aquacult. Nutr. 9: 239-246.

Raatz S., Bibus D. 2016. Fish and Fish Oil in Health and Disease Prevention. Academic Press, London, UK.

Rana K.J., Hasan M.R. 2009. Impact of Rising Feed Ingredient Prices on Aquafeeds an Aquaculture Production. FAO.

Samocha T., Davis D.A., Saoud I.P., DeBault K. 2004. Substitution of fish meal by co-extruded soybean poultry by-product meal in practical diets for the Pacific white shrimp, *Litopenaeus vannamei*. Aquaculture 231: 197-203.

Shepherd C.J., Jackson A.J. 2013. Global fishmeal and fish-oil supply: inputs, outputs and markets. J. Fish Biol. 83: 1046-1066.

Sink T.D., Lochmann R.T., Kinsey N.R. 2010. Growth and survival of channel catfish, *Ictalurus punctatus*, fry fed diets with 36 or 45% total protein and all plant or animal protein sources. J. World Aquacult. Soc. 41: 124-129.

Stephen H.J., Dugan P., Allison E.H., Andrew N.L. 2010.The End of the Line: Who is Most at Risk from the Crisis in Global Fisheries? 24 February 2010. Royal Swedish Academy of Sciences 2010.

Tacon A.G.J. 2004. Use of fish meal and fish oil in aquaculture: a global perspective. Aquatic Resources. Cult. Dev. 1: 3-14.

Tacon A.G.J., Hasan M.R., Metian M. 2011. Demand and supply of feed ingredients for farmed fish and crustaceans. Trends and prospects. In: FAO Fisheries and Aquaculture Technical Paper.

Tacon A.G.J., Metian M. 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: trends and future prospects. Aquaculture 285: 146-158.

Tacon A., Metian M., Hasan M.R. 2009. Feed Ingredients and Fertilizers for Farmed Aquatic Animals. FAO.

United Nations 2010. MDG Report 2010 En 20100604 r14 Final. indd Sec 2:6

Webster C.D., Tiu L.G., Morgan A.M. 1999. Effect of Partial and Total Replacement of Fish Meal on Growth and Body Composition of Sunshine Bass *Morone Chrysops* x *M. saxatilis* Fed Practical Diets. J World Aquacult. Soc. 30: 443-453.

Wilson R.P. 2002. Amino acids and proteins. In: Halver J.E., Hardy R.W. (Eds.), Fish Nutrition, third ed. Academic Press, pp. 143-179.

World fish center 2010. Food security and poverty alleviation through improved valuation and governance of river fisheries in Africa-scale inland fisheries in sub- Sahara Africa. Lessons from a Research Project in Lake Chad Basin and Zambezi River Basin. February 2010.

CHAPTER II. Effects of solid state fermented plant protein sources as fish meal replacers on growth performance, intestinal bacterial count, immune responses and disease resistance in whiteleg shrimp *Litopaeneus vannamei*

1. Introduction

Aquaculture continues to develop rapidly, especially through its growth in Asia. World aquaculture production is increasing much more rapidly than animal husbandry and capture fisheries, the other two sources of animal protein for the world's population. There is widespread recognition that seafood production from capture fisheries is at or near its peak, and that aquaculture will become increasingly important as a source of seafood production, and ultimately the main source (FAO, 2018). Therefore, there is widespread public interest in aquaculture. The aquaculture feed industry is responsible for converting raw materials of agricultural origin into feeds. These feeds are not only important in terms of cost but also in terms of nutrition, as some of these feeds are the primary source of animal and plant protein required by cultivated aquaculture species for normal development. In addition, this is a broad industry employing people with a variety of skills, including process engineers, economists, marketing experts, shellfish and fish scientists, regulatory experts, quality control technicians, and transportation and distribution specialists (Tacon et al., 2011). Feed is the largest single cost item in aquaculture production and since it accounts for 50–60% of the total cost, any saving on feed, though small, may greatly reduce the total cost and increase returns. Formulating aquaculture feeds requires the use of combinations of several ingredients since most feedstuffs have been shown to have significant nutrient and functional limitations and cannot be used individually at very high levels in the diets of most aquaculture species. Adopting local ingredient alternatives for the formulation of an aquafeed mix is a logical step for aquaculture producers to remain profitable. Challenges and obstacles include material availability, farming, initial cost competitiveness, and handling and processing. Consequently, feed formulation is an important aspect of the aquaculture industry and accurate formulation must be overcome before alternative aquaculture feed formulas can be fully developed successfully (Nates 2016).

Protein is the single most important and expensive component in fish feeds, particularly for carnivorous and marine fish that tend to have higher dietary protein requirements than those of fresh water fish (Wilson, 2002). Fish meal (FM) has been the major dietary protein source for fish

and shrimp, comprising 20–60% of diets in general (Watanabe, 2002). Nevertheless, global fish meal and fish oil supplies are clearly inadequate to support the growing demand, and the cost of FM has increased more than two fold during the recent years. It is therefore necessary to find alternative protein sources as it may have a positive effect on the cost of fish feeds (Tidwell et al., 2005). Many studies have attempted to find alternative protein sources from plant origin, especially the grains, pulses and oilseeds (Gatlin et al., 2007). Among the plant-derived alternative protein sources for FM, protein sources from soybeans have received the most attention due to their reasonable price, relatively well-balanced amino acid profile, consistency and domestic availability. However, dietary utilization of soybean meal (SBM) in carnivorous species feeds has been limited by the presence of several antinutritional factors (ANFs) that may affect the digestion or absorption of nutrients, resulting in decreased growth performance and feed efficiency in finfish (Francis et al., 2001). In addition, protein sources from soybeans are deficient in essential amino acids (EAAs), methionine and lysine, which are essential for normal growth and health.

It has been reported that appropriate processing technologies can eliminate or deactivate several ANFs (Drew et al., 2007) and/or enhance the concentrations of the limiting EAAs (Gatlin et al., 2007). Various processing strategies can be used to reduce or eliminate ANFs in soy-derived feedstuffs, such as heat treatment, extraction and purification into protein concentrates and isolates. Although these products offer advantages in terms of ANF levels and nutrient digestibility, they are more costly than traditional SBM. Fermentation, by either bacterial or fungal organisms, is another lower-cost approach to increase protein concentration and decrease the levels of ANFs (Gatlin et al., 2007). Solid state fermentation is an economically viable processing technique adopted in recent years, which in part or totally eliminates the limitations related to plant protein sources (Shi et al., 2015). Traditionally, the quality of SBM was improved through fermentation using microorganisms, such as *Lactobacillus* spp., *Bacillus* spp., *Aspergillus* spp. and yeast (Rashad et al. 2011; Shi et al. 2013). Several studies have demonstrated that some properties (such as nutritional value, flavour and storage time of SBM can be improved by fermentation (Rashad et al., 2011; Vong et al. 2016; Yu et al. 2005). SBM processing by fermentation ameliorates the antinutritional factors and enhances protein utilization in animals. Previous studies with pompano *Trachinotus ovatus* (Lin et al., 2013), hybrid striped bass *Morone chrysops* × *M. saxatilis* (Rombenso et al., 2013), black seabream *Acanthopagrus schlegelii* (Azarm and Lee, 2014), rainbow trout *Oncorhynchus mykiss* (Barnes et al., 2015; Moniruzzaman et al. 2017) and olive

flounder *Paralichthys olivaceus* (Seong et al. 2018) suggested that fermentation may be equally or more effective than other, more costly processing strategies to improve the acceptance and utilization of soy protein products in feeds for carnivorous fish. Furthermore, information regarding the application of fermented soybean meal in shrimp feed is lacking.

Whiteleg shrimp or white shrimp, *Litopenaeus vannamei*, is a widely cultured shrimp species which is native to the Pacific coast from Northern Peru to Mexico. The annual aquaculture production of whiteleg shrimp has increased from 8286 MT in 1980 to 4,155,827 MT in 2016, accounting for approximately 80% of the global shrimp production. While limited success has been achieved in reducing the amount of FM in the diet of whiteleg shrimp with plant protein sources. The aim of the present study is to incorporate different types of fermented plant proteins in the diet of whiteleg shrimp as FM replacers and evaluate the effects on growth performance, intestinal bacterial count, cumulative survival against *Vibrio parahaemolyticus* and non-specific immune responses.

2. Materials and methods

Fermentation process of plant proteins

Different types of fermented plant protein sources that were used in this experiment were provided by the CJ CheilJedang Corporation (Seoul, Republic of Korea). Solid state fermentation (SSF) was performed as previously described by Moniruzzaman et al. (2017). Depositing solid substrates of plant protein sources (soybean meal and corn gluten meal) on flatbeds and seeding it with liquid cultures of *Bacillus* spp bacterium. Several days of preservation in temperature-controlled rooms allows the *Bacillus* spp to grow. In SSF method only a small amount of water is added to the substrate, therefore, less fermentor is needed per unit volume of substrate and there will be no effluent treatment required (Fig 2-1). The fermented plant protein ingredients used in this experiment were as follows:

FSM_B: Fermented soybean meal with *Bacillus* spp (Soytide®)

FSM_{BS}: Fermented soybean meal with *Bacillus* spp and sterilized

FSC_B: Fermented soybean meal and corn gluten meal with *Bacillus* spp (Aquatide®)

FSC_{BS}: Fermented soybean meal and corn gluten meal with *Bacillus* spp and sterilized

FSP_B: Fermented soy protein concentrate with *Bacillus* spp

FSM_L: Fermented soybean meal with lactic acid

Experimental diets

Formulation of the eight experimental diets is shown in Table 1-1. Fish meal and soybean meal were considered as the main protein sources and fish oil was the main lipid source. A diet without replacement of fish meal (FM) was considered as control (CON). The other seven diets were prepared by 30% replacement of FM in the CON diet with fermented soybean meal with *Bacillus* spp (FSM_B), sterilized fermented soybean meal with *Bacillus* spp (FSM_{BS}), fermented soybean meal and corn gluten meal with *Bacillus* spp (FSC_B), sterilized fermented soybean meal and corn gluten meal with *Bacillus* spp (FSC_{BS}), fermented soy protein concentrate with *Bacillus* spp (FSP_B), fermented soybean meal with lactic acid (FSM_L) and Soy protein concentrate (SPC). With some modifications, the procedures previously mentioned by Bai and Kim (1997) were used for preparing the ingredients, mixing, pelleting, drying and storing the diets. All fine-powered ingredients were mixed with an electric mixer (HYVM-1214, Hanyoung Food Machinery, Republic of Korea) according to the feed formulation. After through mixing of the ingredients, fish oil and water were added and a stiff dough was resulted. The dough was passed through a pelletizing machine (SFD-GT, Shinsung, Republic of Korea) with a flat die of approximately 0.2 cm in diameter. All diets were air dried for 48 hours until the moisture content of <10%, broken into desirable size and then stored at -20ºC until the initiation of feeding trial. According to Table 1-2, all diets were isonitrogenous (42 % crude protein) and isocaloric (4 kcal/g diet).

Shrimp husbandry and experimental condition

Post-larvae of whiteleg shrimp *Litopaeneus vannamei* were obtained from a local shrimp farm (Gyeongsang, Republic of Korea) and brought to the Department of Marine Bio-Materials & Aquaculture at Pukyong National University (Busan, Republic of Korea). Shrimp were stocked in 200 L plastic tanks and the health condition was checked. For about two weeks, shrimp were acclimated to the new environment and fed a commercial diet (Suhyup feed, Gyeongsangnam-do, Republic of Korea) with 45% protein until satiation. After this period, 40 shrimp with average weight of 0.5 ± 0.01 g (mean ± SD) were distributed in each of the 24 tanks with 50 L capacity. Three replicates were randomly considered for each experimental diet. A semi-recirculated system with only stone and sponge filtration was used to provide sea water with 1.2 L/min for all tanks.

Dark plastic covers were used on top of all the experimental tanks to reduce stress and prevent shrimp from jumping. During the eight weeks of the experiment, water temperature was maintained at 28±1ºC using electric heater. Aeration was performed using air stones attached to an air pump and pH was approximately 7.77±0.05. Shrimp were fed 4 times a day with the previously mentioned diets (Table 1-1) until satiation (7% of body weight). The amount of feed was adjusted according to the growth and mortalities. Siphoning was performed to collect uneaten feed and feces. All tanks were cleaned by scrubbing the walls and 20% of water was replaced every two days (Figure 2-1).

Sampling and analysis

Proximate composition analysis of experimental diets were performed according to Association of Official Analytical Chemists, AOAC (2005). Samples from the diets were frozen dried (Advantage 2.0, VirTis, New York, USA) and then grounded. Diet samples were dried to constant weight at 105 ºC for 24h and dry matter was determined. Nitrogen (N×6.25) was measured to determine protein content by acid digestion and Kjeldahl method (2300 Auto analyzer, Foss Tecator. AB, Hoganas, Sweden). Fat content were determined using Soxtec system 1046 (Tecator AB, Hoganas, Sweden) with ether extraction. Ash content was analyzed by combustion at 550°C in muffle furnace for 3h. Gross energy of diets were determined with a bomb calorimeter (Parr 1356, Moline, USA). According to Table 2-2, all diets were isonitrogenous (42 % crude protein) and isocaloric (4 kcal/g diet).

At the end of the eight weeks, feeding was stopped for a day to let the digestive tract empty. At the final biometry, number of shrimp in each tank and the total weight were measured to evaluate growth performance and nutrient utilization indices. Weight gain (WG), specific growth rate (SGR), feed efficiency (FE) and protein efficiency ratio (PER) were calculated according to the formulas described by Mohanty (1999):

WG = [final weight (g)–initial weight (g)] × 100/initial weight (g)

SGR = 100 × [ln final weight (g)–ln initial weight (g)]/days

FE = (final weight (g)–initial weight (g)) × 100/ feed ration (g)

PER = wet weight gain (g) /protein intake (g)

Survival (%) = (initial number shrimp – final number shrimp) ×100/initial number shrimp

Five samples of shrimp were taken from each tank for the analysis of biochemical parameters of hemolymph. 1-ml non-heparinized syringe (26-gauge hypodermic needle) was used to collect approximately 0.3 ml of hemolymph from the ventral sinus that is located in the initial abdominal segment. The hemolymph samples were kept at room temperature for approximately 30 min to colt; this was followed by 10 min of centrifugation at 5,000 g that separated the serum from the blood cells. Serum samples were kept in a freezer at -70°C until the analysis of non-specific immune response enzyme activities. Superoxide dismutase (SOD) activity was obtained by an inhibitory reaction against WST-1 (Water Soluble Tetrazolium dye) and xanthine oxidase (Kong et al. 2012). This was performed by a SOD kit (Sigma-Aldrich 19160) following the instructions of the product. The absorbance of the color product of WST-1 that reacted with superoxide after 20 min at 37°C was read at 450 nm wavelength. The inhibition percentage was assigned according to the amount of protein (mg) and was presented as a unit of SOD activity. Lysozyme activity experiment was conducted by adding 0.1 ml of test serum to a solution containing a sodium phosphate buffer (0.05 M) and 0.2 mg/ml of *Micrococcus lysodeikticus* with a pH of 6.2 (Mörsky 1983). A spectrophotometer (UV-1800 UV-VIS spectrophotometer, Shimadzu, Japan) was used to measure the absorbance (at 530 nm) of the reactions at 20°C between 0.5 and 4.5 min. The activity of lysozyme was considered the amount of produced enzyme that reduces the absorbance of 0.001/min.

Intestinal bacterial count

Total bacteria and *Bacillus* spp. count of shrimp intestine was performed at the end of eight weeks experiment using the methods previously described by Baumann and Schubert (1984). Two shrimp per tank (three shrimp for each diet), previously starved for 24 h, was anesthetized and opened aseptically. Then, 1-g sections of intestinal tissue were collected, cut into small pieces, and ground well. Half-gram quantities of ground tissue were diluted with 4.5 ml of phosphate-buffered saline (pH 7.0, 0.1 M). The suspension was held at room temperature for 10 min, vortexed, again held at room temperature for 7–8 min to allow the particles to settle. Then, 1-ml aliquots of supernatant were serially diluted at 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} and spread on lysogeny broth (LB) agar plates and incubated at 37 °C for 16 h. Intestinal bacterial count was performed by simply counting the number of colonies appeared on each plate. The amount of bacteria was calculated as colony forming unit (CFU) using the following the formula:

Total bacterial count (CFU per shrimp) = Number of bacterial colonies on plate × Dilute multiple × Volume of the homogenized liquid / Number of shrimp.

LB medium was used to growth *Bacillus* spp and incubation at 30 °C. Partial 16 S rDNA based high-throughput sequencing was used to identify *Bacillus* spp. All procedures were performed under sterile conditions at room temperature.

Challenge test

The pathogen *Vibrio parahaemolyticus* was provided by the CJ CheilJedang Corporation, Seoul, Republic of Korea. At first, bacteria were cultured in 10 ml brain heart infusion (BHI; Becton, Dickinson and Company, USA) broth and incubated for 24 h at 37 °C in a shaking incubator. Bacterial growth was observed by a spectrophotometer (Mecasys, Optizen, Republic of Korea) at OD_{600} nm (optical density), harvested by centrifugation and washed two times with phosphate-buffered saline (pH 7.0, 0.1 M). Contamination identification and cell number determination were confirmed on TSB broth (TSB; Sigma-Aldrich) plate by serial dilution method. Phosphate-buffered saline containing *Vibrio parahaemolyticus* cell was adjusted 2×10^9 CFU ml^{-1}. a dose capable of causing 100% mortality in shrimp.

At the end of the feeding trial, ten shrimp from each tank (30 shrimp per treatment) were randomly selected and subjected to the bacterial challenge. Each tank contained 5.5L of clean sea water and temperature was fixed at 30°C. 10 ml of *Vibrio parahaemolyticus* with 2×10^9 CFU ml^{-1} concentration was added to each tank, a dose capable of causing 100% mortality in shrimp. The pathogenic dose of bacterium had previously been determined in a preliminary test using shrimp of a similar size. Fish were starved during the challenge test period and water exchange was prohibited. Shrimp mortality was monitored and recorded for a week.

Statistical analysis

Completely randomized design was performed in this experiment all the data were analyzed by one-way ANOVA (SAS Version 9.1) to test the effects of the dietary treatments. When a significant treatment effect was observed, an LSD test was used to compare means. Treatment effects were considered at $P<0.05$ level of significance.

3. Results

Growth performances

The results for growth performance and feed utilization of *Litopenaeus vannamei* fed the eight experimental diets for eight weeks are shown in Table 1-3. According to these results, weight gain (WG) of shrimp fed the FSM_B and FSC_B diets were significantly higher than those of shrimp fed the CON and FSM_L diets ($P<0.05$). Although, there were no significant differences in WG of shrimp fed diets FSM_B, FSM_{BS}, FSC_B, FSC_{BS}, FSP_B and SPC. Therefore shrimp fed the FSM_L diet had the lowest WG with no significant differences with shrimp fed diets CON, FSM_{BS}, FSC_{BS}, FSP_B and SPC ($P>0.05$). Specific growth rate (SGR) of shrimp fed diets FSC_B and FSM_B were significantly ($P<0.05$) higher than shrimp fed the CON and FSM_L diets. Whereas, there were no significant differences in FSM_B, FSM_{BS}, FSC_B, FSC_{BS}, FSP_B and SPC groups ($P>0.05$). Shrimp fed diets FSC_B and FSC_{BS} had the highest feed efficiency (FE) significantly different with shrimp fed the FSP_B diet ($P<0.05$). FE of shrimp fed diets CON, FSM_B, FSM_{BS}, FSC_B, FSC_{BS}, FSM_L and SPC were not significantly different ($P>0.05$). Protein efficiency ratio (PER) of shrimp fed diets FSC_B and FSC_{BS} were significantly higher than those of shrimp fed the FSP_B diet ($P<0.05$). Although, there were no significant differences in PER of shrimp fed diets CON, FSM_B, FSM_{BS}, FSC_B, FSC_{BS}, FSM_L and SPC ($P>0.05$).

Intestinal bacterial count

Results for whiteleg shrimp intestinal total bacterial count (TBC) and *Bacillus* spp. count (BSC) fed the eight experimental diets for eight weeks are shown in Table 1-4. There were no significant differences in shrimp TBC ($\times 10^6$ CFU/g) of intestine fed all the experimental diets ($P>0.05$). Although no significant, intestine of shrimp fed the FSC_B diet showed a slightly higher TBC comparing to shrimp fed the other diets. On the other hand, BSC ($\times 10^4$ CFU/g) of whiteleg shrimp intestine were significantly higher when fed diets FSM_B, FSC_B and FSP_B comparing to those fed CON, FSM_{BS}, FSC_{BS}, FSM_L and SPC diets ($P<0.05$). Although, there were no significant differences between shrimp fed diets FSM_B, FSC_B and FSP_B ($P>0.05$).

Non-specific immune responses

The results for non-specific immune responses of shrimp hemolymph fed the eight experimental diets for eight weeks are shown in Table 1-5. Superoxide dismutase activity (SOD) did not show

any significant differences among shrimp fed all the experimental diets (P>0.05). Although, shrimp fed diet FSM_B showed a slightly higher SOD comparing to those of shrimp fed the other diets. Lysozyme activity of shrimp fed diets FSC_B and FSC_B were significantly higher than those fed diets CON and FSM_L (P<0.05). There were no significant differences in lysozyme activity between shrimp fed diets FSM_B, FSM_{BS}, FSC_B, FSC_{BS}, FSP_B and SPC (P>0.05). Also, differences in lysozyme activity of shrimp fed diets CON, FSM_{BS}, FSC_{BS}, FSP_B, FSM_L and SPC were not significant (P>0.05).

Challenge test

Results for challenge test of whiteleg shrimp with *Vibrio parahaemolyticus* for eight days after eight weeks of feeding trial are shown in Table 1-6 and Figure 1-2. The mortalities started from the first day of challenge test. Cumulative survival rate showed more significant differences between treatments after the third day. At day four, shrimp fed diets FSC_B and FSP_B had a significantly higher survival comparing to those of shrimp fed diets CON, FSM_B, FSM_{BS}, FSC_{BS}, FSM_L and SPC (P<0.05). The same trend was going on until day five with more mortalities happening in all treatments. At day six, shrimp fed diet FSC_B had the highest survival among all other groups, while there were not much significant differences among other groups. Finally, at day eight most of the shrimp from the experimental tanks were dead. At this day, shrimp fed diet FSC_B had the highest survival comparing to shrimp fed all the other diets (P<0.05). There were no significant differences in cumulative survival rate of shrimp fed diets CON, FSM_B, FSM_{BS}, FSC_{BS}, FSP_B, FSM_L and SPC (P>0.05).

4. Discussion

This study evaluated the viability of partially replacing FM (up to 30%), which is a prime, highly digestible source of protein with a high content of amino acids, vitamins, and minerals, in diets fed to young whiteleg shrimp (*Litopaeneus vannamei*) with different types of fermented soybean meal as alternatives. This was an attempt to reduce feed cost while not compromising growth performance, feed utilization or immunity. During the experiment, all detected water qualities (DO, salinity and temperature) were within acceptable ranges and were not significantly different among all groups. The feed formulation was done based on the nutritional requirements of whiteleg shrimp and the amount of protein (42%) was comparable to the commercial shrimp feed being

used in farms. All the experimental diets were well accepted by the shrimp during the 8-week experimental period.

Results for growth performance showed that replacement of FM with solid state Bacillus-fermented soybean meal could increase WG and SGR. The same results were observed in feed utilization parameters such as FE and PER. It has been previously shown that microbial fermentation can reduce the amount of trypsin inhibitors and remove glycinin, β-conglycinin, sugars and the majority of oligosaccharides larger than 20 kDa (Rojas & Stein 2013). This can reduce the digestibility and utilization of nutrients, thus reducing overall anti nutritional factors amount (Min et al. 2004; Yun et al. 2005). Van Nguyen et al. (2018) used *Bacillus*-fermented soybean meal in diets of whiteleg shrimp as a FM replacer. They found that apparent digestibility coefficients of crude protein in fermented soybean meal were high (888.4 g/kg) and equal in local fishmeal. But this amount was little lower than Chilean fishmeal while without any significant differences observed in apparent digestibility coefficients of crude lipid among the different test ingredients. After 75 days of growth trial, survival rates and feed conversion ratio were not significantly different among the experimental treatments. However, shrimp fed the diets with increasing inclusions of fermented soybean meal had a tendency to reduce weight gain and specific growth rate. Based on the correlation between weight gain and substituted fishmeal level analysed by broken-line regression, the optimum level of fishmeal replaced by fermented soybean meal in diet was about 30% without adverse effects on growth and feed utilization of whiteleg shrimp. In another study by Seong et al. (2018), were able to replace up to 27.9% of FM in the diet of olive flounder, *Paralichthys olivaceus* with fermented soybean and corn gluten meal by *Bacillus subtilis*. Soybean meal constitutes one of the largest volumes of both plant protein meals and feed ingredient resources available in the world. A range of processed soybean products (including protein concentrates and protein isolates) have been used by the aquaculture feed sector and have been evaluated in a range of species. Soybean meal is regarded as an economical and nutritious replacement for FM in aquafeed as it is relatively easy to produce, has high protein content, and has relatively high digestibility (Gatlin et al., 2007). To date, studies have demonstrated that SM can successfully replace 30% or more of the dietary protein for carnivorous species such as red sea bream (*Pagrus major*), turbot (*Scophthalmus maximus* L.), and European sea bass

(*Dicentrarchus labrax*) (Kader et al., 2012; Peng et al., 2013). However, the deficiency of some essential amino acids (EAA) in SM, its comparatively poor palatability, and the presence of heatstable plant anti-nutritional factors have limited its utilization in aquafeed. In comparison with soybean meal, fermented soybean meal was found to have eliminated trypsin inhibitor, and reduced peptide size (Hong et al. 2004), preventing various physiological abnormalities found in rainbow trout fed non-fishmeal diet (Yamamoto et al., 2010); higher nutrient digestibility and nutritive values in clawed crayfish (Safari et al. 2014); and improved growth of yellowtail fed FSBM plus taurine (Nguyen et al. 2013). Solid-state fermentation has a long history of production of traditional foods using different organisms, and it was reported that in solid-state fermentation, the production of metabolites, such as enzymes, antibiotics etc. are higher than that in submerged fermentation (Holker and Lenz 2005). Solid-state fermentation is used to produce traditional fermented soybean food, such as natto, a *Bacillus subtilis*-fermented soybean food with a venerable history in Japan. Natto contains isoflavones, dietary fibre, vitamins, linoleic acid and some minerals which originate from soybeans; in addition, it also contains some functional compounds such as enzymes, bioactive peptides, natto kinase, gamma-polyglutamic acid etc. which are produced by *B. subtilis* (El-Safey and Abdul- Raouf 2004; Ma et al. 2006) considered to be beneficial to human health (Hosoi and Kiuchi 2003).

The differences in growth performance could be related to presence of live *Bacillus* spp. in the digestive tract of shrimp as shown in Table 4. Manipulation of microbiota using probiotics have been reported as a worthy practice for aquaculture in order to control or inhibit the pathogen bacteria, improve the growth performances and digestive enzymes, and enhance the immune responses of the host against pathogens or physical stress (Balcazar et al. 2006; Perez et al. 2010). Zokaeifar et al. (2012) demonstrated that dietary administration of *B. subtilis* strains significantly improved final weight, WG, SGR and survival of whiteleg shrimp. Although several studies have demonstrated the beneficial effects of probiotics on the growth performance in shrimp the exact mechanism of action is not well understood. The first explanation could be related to the action of competitive exclusion, by which probiotics may create a hostile environment for pathogen colonization. This mechanism of action has been determined in this study because the treated shrimp with non-sterilized *Bacillus* spp. fermentation had significantly higher colonization of

Bacillus spp. in the intestine. Also previous studies demonstrated considerable reduction of *Vibrio* spp. populations in the digestive tract of shrimp fed *Bacillus* spp. diets which clearly showed the successful competitive exclusion of *B. subtilis* strains (Zokaeifar et al. 2012). Another possible explanation for the improvement of the shrimp growth factors by *B. subtilis* may be due to the induction of digestive enzymes, including protease and amylase, which consequently stimulate the natural digestive enzyme activity of the host (Liu et al. 2009; Wang 2007). In this study, probably higher level of digestive enzyme activities were present in shrimp fed *Bacillus* spp.-fermented diets where the better growth performances were observed compared to control. Similar results have been reported by Ziaei-Nejad et al. (2006) who observed a higher digestive enzyme activity in shrimp (*Fenneropenaeus indicus*) treated with *Bacillus* spp. than the controls.

In this study the non-specific immune responses as indicators of innate immunity were measured in the serum of whiteleg shrimp. Lysozyme is an endogenous enzyme with superior anti-pathogenic properties, which attacks the cell walls constituted by peptidoglycan and breaks the bond between N-acetylmuramic acid and acetylglucosamine (Samarakoon et al., 2013). SOD is another enzyme that acts as an antioxidant and prevents free radicals from damaging animal tissue by neutralization (McCord and Fridovich, 1969). These innate immune parameters are reliable evidences for the influence of nutritional treatments on the immunological status of fish. According to the results of the present study, replacement of FM with *Bacillus*-fermented soy bean meal which was not sterilized showed higher lysozyme activity comparing to all other diets. Previous studies have also shown that fermented soybean meal enhanced the absorption of phosphorus and non-specific immune responses in juvenile parrot fish (Kim et al., 2009). In line with our study, other studies reported improved non-specific immune responses in fish fed bioprocessed soybean meal, fermented soybean meal and soy protein concentrate based diets (Kader et al. 2012; Khosravi et al. 2015; Kokou et al. 2012). Results of these studies may be attributed to the better performance or immunity status. Shrimp, unlike vertebrates, are believed to lack adaptive immunity and completely depend on their innate immunity. Therefore, products which can enhance host immunity and disease resistance, such as immunostimulants and probiotics, are being used in shrimp disease prevention and have garnered much interest in recent years (Sakai 1999; Farzanfar 2006). Probiotics are harmless bacteria that promote the wellbeing of a host animal and contribute

to the direct and/or indirect protection of host animals against harmful bacteria (Decamp et al. 2008). The positive results observed in this study with lysozyme activity of shrimp fed unsterilized Bacillus-fermented soybean meal could be attributed to the benefits of probiotics.

In this experiment, shrimp were challenged with *Vibrio parahaemolyticus* for eight days using the immersion method. *V. parahaemolyticus*, a halophilic gram-negative bacterium, is commonly found in seawater. It is a common cause of food poisoning in people who consume raw or undercooked seafood. *V. parahaemolyticus* was first discovered in 1950 by Tsunesaburo Fujino at Osaka University. *V. parahaemolyticus* is currently a concern in Thailand because it is the bacterium that causes the Acute Hepatopancreatic Necrosis Disease (AHPND), which is also known as Early Mortality Syndrome (EMS). The EMS disease typically affects shrimp post-larvae and can cause up to 100% shrimp death within 20–30 days after stocking. It is also reported that the disease can also impact late-stage juvenile shrimp (Daniels et al. 2000; Shinoda 2011; Ananda Raja et al. 2017). Results for challenge test in our study showed that shrimp fed diets containing unsterilized *Bacillus*-fermented soybean meal and corn gluten meal had the highest survival among treatments. *Bacillus*-fermented soybean meal and corn gluten meal has previously shown to positively influence growth performance, haematological parameters, non-specific immune responses and distal intestinal morphology and reduce the antinutritional factors in feed ingredients (Moniruzzaman et al 2017). This has probably lead to higher survival of shrimp after challenge with *V. parahaemolyticus* for eight days.

The overall results of this experiment showed that bioprocessed soybean meal and corn gluten meal with *Bacillus* spp. bacteria can be a potential fish meal replacer in whiteleg shrimp. The results also showed that 30% replacement of fish meal with unsterilized *Bacillus*-fermented soybean meal and corn gluten meal can increase the growth performance, feed utilization, intestinal *Bacillus* spp. count, non-specific immune responses and survival against *V. parahaemolyticus* pathogen. Based on the findings of this experiment, designated price of shrimp feed could be reduced by replacing fish meal with sustainable plant protein sources.

5. References

Ananda Raja R., Sridhar R., Balachandran C., Palanisammi A., Ramesh S., Nagarajan K. 2017. Pathogenicity profile of *Vibrio parahaemolyticus* in farmed Pacific white shrimp, *Penaeus*

vannamei Fish. Shellfish Immunol. 67: 368-381

AOAC 2005. Official methods of analysis, 18th edn. Gaithersburg, MD: Association of Official Analytical Chemists.

Azarm H.M., Lee S.-M. 2014. Effects of partial substitution of dietary fish meal by fermented soybean meal on growth performance, amino acid and biochemical parameters of juvenile black seabream *Acanthopagrus schlegeli*. Aquac. Res. 45: 994-1003.

Bai S.C., Kim K.W. 1997. Effects of dietary animal protein sources on growth and body composition in Korean rockfish, *Sebastes schlegeli*. J. Aquacult., 10:77-85 (in Korean with English abstract).

Balcazar J.L., de Blas I., Ruiz-Zarzuela I., Cunningham D., Vendrell D., Muzquiz J.L. 2006. The role of probiotics in aquaculture. Vet Microbiol 114:173-186.

Barnes M.E., Brown M.L., Neiger R. 2015. Comparative performance of two rainbow trout strains fed fermented soybean meal. Aquac. Int. 23:1227–1238.

Baumann P., Schubert R.H.W. 1984. Vibrionaceae. N.A Krieg, J.G Holt (Eds.), Bergey's Manual of Systematic Bacteriology, vol. 1, William & Wilkins, Baltimore, pp. 516-549.

DanielN.A., MacKinnonL., R. Bishop, S. Altekruse, B. Ray, R.M. Hammond, S. Thompson, S. Wilson, N.H. Bean, P.M. Griffin, L. Slutsker. 2000. *Vibrio parahaemolyticus* infections in the United States, 1973-1998 J. Infect. Dis., 181: 1661-166

Decamp O., Moriarty D.J.W., Lavens P. 2008. Probiotics for shrimp larviculture: review of field data from Asia and Latin America. Aquaculture Res. 39:334-8.

Drew M.D., Borgeson T.L., Thiessen D.L. 2007. A review of processing of feed ingredients to enhance diet digestibility in finfish. Anim. Feed Sci. Technol. 138:118-136.

El-Safey E.M., Abdul-Raouf U.M. 2004. Production, purification, and characterization of protease enzyme from Bacillus subtilis. Proceedings for the International Conferences for Development and the Environment in the Arab Word, Assiut University, p. 14.

Farzanfar A. 2006. The use of probiotics in shrimp aquaculture. FEMS Immunol Med Microbiol 48:149-58.

Francis G., Makkar H.P.S., Becker K. 2001. Antinutritional factors present in plantderived alternate fish feed ingredients and their effects in fish. Aquaculture 199:197-227.

Gatlin D.M., Barrows F.T., Brown P., Dabrowski K., Gaylord T.G., Hardy R.W., Herman E., Hu G., Krogdahl A., Nelson R., Overturf K., Rust M., Sealey W., Skonberg D., Souza E.J., Stone

D., Wilson R., Wurtele E. 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquac. Res. 38:551–579.

Hasana M.T., Jang W.J., Kim H., Lee B., Kim K.W., Hur S.W., Lim S.G., Bai S.C., Kong I. 2018. Synergistic effects of dietary Bacillus sp. SJ-10 plus β-glucooligosaccharides as a synbiotic on growth performance, innate immunity and streptococcosis resistance in olive flounder (*Paralichthys olivaceus*). Fish and shellfish immunology 82:544-553.

Holker U., Lenz J. 2005. Solid-state fermentation-are there any biotechnological advantages? Current Opinion in Microbiology 8:301-306.

Hong K.J., Lee C.H., Kim S.W. 2004. *Aspergillus oryzae* 3.042GB-107 fermentation improves nutritional quality of food soybeans and feed soybean meals. Journal of Medicinal Food 7:430-434.

Hosoi T., Kiuchi K. 2003. Natto - A food made by fermenting cooked soybeans with Bacillus subtilis (natto). In: Handbook of Fermented Functional Foods, 2nd edn (ed. by E.R. Farnworth), pp. 227-250. CRC Press, Boca Raton, FL.

Kader M.A., Bulbul M., Koshio S., Ishikawa M., Yokoyama S., Nguyen B.T., Komilus C.F. 2012. Effect of complete replacement of fishmeal by dehulled soybean meal with crude attractants supplementation in diets for red sea bream, *Pagrus major*. Aquaculture 350-353: 109-116.

Khosravi S., Rahimnejad S., Herault M., Fournier V., Lee C., Bui H.T.D., Lee K. 2015. Effects of protein hydrolysates supplementation in low fish meal diets on growth performance, innate immunity and disease resistance of red sea bream *Pagrus major*. Fish & Shellfish Immunology, 45:858-868.

Kim S.S., Galaz G.B., Pham M.A., Jang J.W., Oh D.H., Yeo I.K., Lee K.J. 2009. Effects of dietary supplementation of meju, fermented soybean meal, and *Aspergillus oryzae* for juvenile parrot fish (*Oplegnathus fasciatus*). Asian Australian Journal of Animal Sciences, 22:849-856.

Kokou F., Rigos G., Kentouri M., Alexis M. 2016. Effects of DL-methionine- Supplemented dietary soy protein concentrate on growth performance and intestinal enzyme activity of gilthead sea bream (*Sparus aurata* L.). Aquaculture International, 24:257-271.

Kong W., Zhao Y., Liu F., He Y., Tian T., Zhou W. 2012. Fast Analysis of Superoxide Dismutase (SOD) Activity in Barley Leaves Using Visible and Near Infrared Spectroscopy. Sensors 12: 10871-10880.

Lin H., Chen X., Chen S., Zhuojia L., Huang Z., Niu J., Wu K., Lu X. 2013. Replacement of fish

meal with fermented soybean meal in practical diets for pompano *Trachinotus ovatus*. Aquac. Res. 44:151-156.

Liu C.H., Chiu C.S., Ho P.L., Wang S.W. 2009. Improvement in the growth performance of white shrimp, *Litopenaeus vannamei*, by a protease-producing probiotic, *Bacillus subtilis* E20, from natto. J App Microbiol 107:1031-1041.

Ma J., Zhang Z., Wang B., Kong X., Wang Y., Cao S., Feng Y. 2006. Overexpression and characterization of a lipase from *Bacillus subtilis*. Protein Expression and Purification 45:22-29.

McCord J.E., Fridovich I. 1969. Superoxide dismutase. An enzymatic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244:6049-6055.

Min B.J., Hong J.W., Kwon O.S., Lee W.B., Kim YC, Kim IH, et al. 2004. The effect of feeding processed soy protein on the growth performance and apparent ileal digestibility in weanling pigs. Asian-Australasian Journal of Animal Sciences 17:1271-1276.

Mohanty R.K. 1999. Growth performance of Penaeus monodon at different stocking densities. Journal of the Inland Fisheries Society of India 31:53-59.

Moniruzzaman M., Bae J.H., Won S.H., Cho S.J., Chang K.H., Bai S.C. 2017. Evaluation of solid-state fermented protein concentrates as a fish meal replacer in the diets of juvenile rainbow trout, *Oncorhynchus mykiss*. Aquaculture nutrition 24:1198-1212.

Mörsky P. 1983. Turbidimetric determination of lysozyme with *Micrococcus lysodeikticus* cells: Reexamination of reaction conditions. Analytical biochemistry 128:77-85.

Nates S. F. 2016. Aquafeed formulation. Elsevier, Amsterdam.

Nguyen H.P., Khaoian P., Fukada H., Suzuki N., Masumoto T. 2013. Feeding fermented soybean meal diet supplemented with taurine to yellowtail *Seriola quinqueradiata* affects growth performance and lipid digestion. Aquaculture Research 46:1101-110.

Peng M., Xu W., Ai Q., Mai K., Liufu Z., Zhang K. 2013. Effects of nucleotide supplementation on growth, immune responses and intestinal morphology in juvenile turbot fed diets with graded levels of soybean meal (*Scophthalmus maximus* L.). Aquaculture 392-395:51–58.

Perez T., Balcazar J.L., Ruiz-Zarzuela I., Halaihel N., Vendrell D., de Blas I., et al. 2010. Host-microbiota interactions within the fish intestinal ecosystem. Mucosal Immunol 3:355-360.

Rashad M.M., Mahmoud A.E., Abou H.M., Nooman M.U. 2011. Improvement of nutritional quality and antioxidant activities of yeast fermented soybean curd residue. African Journal of

Biotechnology, 10:5504-5513.

Rojas O.J., Stein H.H. 2013. Concentration of digestible, metabolizable, and net energy and digestibility of energy and nutrients in fermented soybean meal, conventional soybean meal and fish meal fed to weanling pigs. Journal of Animal Sciences 91:4397-4405.

Rombenso A., Crouse C., Trushenski J. 2013. Comparison of traditional and fermented soybean meals as alternatives to fish meal in hybrid striped bass feeds. N. Am. J. Aquac. 75:197-204.

Safari O., Shahsavani D., Paolucci M., AtashMehraban Sang M. 2014. Screening of selected feedstuffs by sub-adult narrow clawed crayfish, *Astacus leptodactylus leptodactylus* Eschscholtz, 1823. Aquaculture 420-421:211–218.

Sakai M. 1999. Current research status of fish immunostimulants in aquaculture. Aquaculture 172:63-92.

Samarakoon K.W., Cha S., Lee J., Jeon Y. 2013. The growth, innate immunity and protection against H2O2-induced oxidative damage of a chitosan-coated diet in the olive flounder *Paralichthys olivaceus*. Fish. Aquat. Sci. 16:149-158.

Seong M., Lee S., Lee S., Song Y., Bae J., Chang K., Bai S.C. 2018. The effects of different levels of dietary fermented plant-based protein concentrate on growth, hematology and non-specific immune responses in juvenile olive flounder, *Paralichthys olivaceus*. Aquaculture 483:196-202.

Shi C., He J., Yu J., Yu B., Huang Z., Mao X., Zheng P., Chen P. 2015. Solid state fermentation of rapeseed cake with *Aspergillus niger* for degrading glucosinolates and upgrading nutritional value. J. Anim. Sci. Biotechnol. 6:13-19.

Shi M., Yang Y., Guan D., Wang Y., Zhang Z. 2013. Evaluation of solid-state fermentation by *Ganoderma lucidum* using soybean curd residue. Food and Bioprocess Technology, 6:1856-1867.

Shinoda S. 2011. Sixty years from the discovery of *Vibrio parahaemolyticus* and some recollections Biocontrol. Sci., 16(4):129-137

Tacon A.G.J., Hasan M.R., Metian M. 2011. Demand and supply of feed ingredients for farmed fish and crustaceans: trends and prospects. FAO Fisheries and Aquaculture Technical Paper No. 564. FAO, 87 pp.

Tidwell J.H., Coyle S.D., Bright L.A., Yasharian D. 2005. Evaluation of plant and animal source proteins for replacement of fish meal in practical diets for the largemouth bass *Micropterus*

salmoides. J. World Aquacult. Soc. 36:454-463.

Van Nguyen N., Hoang L., Van Khanh T., Duy Hai P., Hung L.T. 2018. Utilization of fermented soybean meal for fishmeal substitution in diets of Pacific white shrimp (*Litopenaeus vannamei*) Aquaculture Nutrition. 24:1092-1100.

Vong W. C., Liu S.Q. 2016. Biovalorisation of okara (soybean residue) for food and nutrition. Trends in Food Science & Technology 52:139-147.

Wang YB. 2007. Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. Aquaculture 269:259-264.

Watanabe T. 2002. Strategies for further development of aquatic feeds. Fish. Sci. 68:242-252.

Wilson R.P. 2002. Amino acids and proteins. In: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition, third ed. Academic Press, San Diego, CA, USA, pp. 143-179.

Yamamoto T., Iwashita Y., Matsunari H., Sugita T., Furuita H., Akimoto A., Okamatsu K., Suzuki N. 2010. Influence of fermentation conditions for soybean meal in a non-fish meal diet on the growth performance and physiological condition of rainbow trout Oncorhynchus mykii. Aquaculture 309:173-180.

Yu Y., Deng Z., Li J., Liu W. 2005. The change of components in soybean residue fermented by *Monilia sitophila* Mont. Food Science 26(9):147-149.

Yun J.H., Kwon I.K., Lohakare J.D., Choi J.Y., Yong J.S., Zheng J. 2005. Comparative efficacy of plant and animal protein source on the growth performance, nutrient digestibility, morphology and caecal microbiology of early-weaned pigs. Asian-Australasian Journal of Animal Sciences 18:1285-1293.

Ziaei-Nejad S., Rezaei M.H., Takami G.A., Lovett D.L., Mirvaghefi A-R., Shakouri M. 2006. The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. Aquaculture 252:516-524.

Zokaeifar H., Balcázar J., C.R. Saad, M.S. Kamarudin, K. Sijam, A. Arshad, N. Nejat. 2012. Effects of Bacillus subtilis on the growth performance, digestive enzymes, immune gene expression and disease resistance of white shrimp, *Litopenaeus vannamei*. Fish & shellfish immunology 33:683-689.

Table 2-1. Composition of the eight experimental diets for whiteleg shrimp *Litopenaeus vannamei* (% of DM basis)[1]

Ingredients	CON	FSM_B	FSM_{BS}	FSC_B	FSC_{BS}	FSP_B	FSM_L	SPC
Fish meal[1]	30.0	21.0	21.0	21.0	21.0	21.0	21.0	21.0
Soytide[2]	-	10.97	-	-	-	-	-	-
Sterilized Soytide	-	-	10.81	-	-	-	-	-
Aquatide[3]	-	-	-	8.98	-	-	-	-
Sterilized Aquatide	-	-	-	-	8.89	-	-	-
FSPC[4]	-	-	-	-	-	9.24	-	-
FSML[5]	-	-	-	-	-	-	12.05	-
SPC[6]	-	-	-	-	-	-	-	10.06
Soybean meal	26.95	26.95	26.95	26.95	26.95	26.95	26.95	26.95
Wheat gluten	7.00	6.80	6.80	6.60	6.60	6.80	6.80	6.80
Squid liver powder	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Wheat flour	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0
Corn starch	10.0	7.05	7.10	9.01	9.07	8.47	5.99	7.82
Fish oil	4.00	4.60	4.70	4.30	4.30	4.70	4.60	4.60
Vitamin premix[7]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Mineral premix[8]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Lysine	0.00	0.19	0.17	0.37	0.37	0.14	0.18	0.13
Methionine	0.00	0.12	0.04	0.05	0.13	0.11	0.11	0.12
Cellulose	0.23	0.50	0.61	0.92	0.84	0.77	0.50	0.70
Others[9]	2.82	2.82	2.82	2.82	2.82	2.82	2.82	2.82
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

[1] Major protein sources: Soybean meal and Fish meal; Major lipid source: Fish oil
CON: a basal diet without fish meal replacement
FSM_B: 30% replacement of fish meal with Soytide®
FSM_{BS}: 30% replacement of fish meal with sterilized Soytide®
FSC_B: 30% replacement of fish meal with Aquatide®
FSC_{BS}: 30% replacement of fish meal with sterilized Aquatide®

FSP_B: 30% replacement of fish meal with fermented soy protein concentrate
FSM_L: 30% replacement of fish meal with lactic acid
SPC: 30% replacement of fish meal with SPC
[2]Fish meal (Chile): CJ CheilJedang Corporation, Seoul, Republic of Korea
[2] Soytide®: *Bacillus* sp.-fermented soybean meal, CJ CheilJedang Corporation, Seoul, Republic of Korea
[3] Aquatide®: *Bacillus* sp.-fermented soybean meal and corn gluten meal, CJ CheilJedang Corporation, Seoul, Republic of Korea
[4] FSPC: Fermented soy protein concentrate
[5] FSML: Fermented soybean meal with lactic acid
[6] SPC: Soy protein concentrate
[7] Vitamin premix (as mg/kg premix): A, 1000000 IU; D, 200000 IU; E, 10000; B1, 2000; B6, 1500; B12, 10; C, 10000; Calcium pantotenic acid, 5000; Nicotinic acid 4500; B-Biotin 10; Choline chloride, 30000; Inositol, 5000.
[8] Mineral premix (as g/kg premix): Ferrous fumarate, 12.50; Manganese sulfate, 11.25; Dried ferrous sulfate, 20.0; Dried cupric sulfate, 1.25; Cobaltous sulfate, 0.75; Zinc sulfate KVP, 13.75; Cancium iodate, 0.75; Magnesium sulfate, 80.20; Aluminum Hydroxide, 0.75.
[9] Others: Carboxymethyl cellulose (1.00%), soy lecithin (1.00%), calcium phosphate (0.30%), cholesterol (0.02%) and chromium oxide (0.50%)

Table 2-2. Proximate composition of the eight experimental diets (% of DM basis) [1]

	CON	FSM_B	FSM_{BS}	FSC_B	FSC_{BS}	FSP_B	FSM_L	SPC	Pooled SEM[2]
Protein	42.7	42.9	42.9	42.2	42.8	42.9	42.8	42.2	0.46
Fat	9.72	10.0	9.81	9.79	9.75	9.93	9.97	10.07	0.23
Ash	8.15	7.62	7.58	7.22	7.24	7.36	7.60	7.36	0.10
Moisture	9.60	9.64	9.37	9.77	9.32	9.55	9.57	9.37	0.10
Energy (kcal/g)	4.55	4.59	4.60	4.57	4.60	4.59	4.59	4.56	0.01

[1] For diet composition information refer to Table 2-1
[2] Pooled SEM: Standard deviation (SD)/√n

Table 2-3. Growth performance of juvenile whiteleg shrimp fed eight experimental diets for 8 weeks[1]

	CON	FSM_B	FSM_{BS}	FSC_B	FSC_{BS}	FSP_B	FSM_L	SPC	Pooled SEM[2]
WG [3]	1439^{b}	1790^{a}	1480^{ab}	1756^{a}	1560^{ab}	1617^{ab}	1425^{b}	1619^{ab}	48.6
SGR [4]	4.71^{b}	5.05^{a}	4.76^{ab}	5.03^{a}	4.84^{ab}	4.90^{ab}	4.70^{b}	4.89^{ab}	0.05
FE [5]	50.9^{ab}	48.7^{ab}	48.6^{ab}	54.4^{a}	54.7^{a}	40.1^{b}	47.3^{ab}	50.4^{ab}	1.63
PER[6]	1.18^{ab}	1.12^{ab}	1.12^{ab}	1.29^{a}	1.30^{a}	0.90^{b}	1.11^{ab}	1.18^{ab}	0.04

[1]Values are means from triplicate groups of shrimp where the values in each row with different superscripts are significantly different (P<0.05); for diet composition information refer to Table 2-1

[2] Pooled SEM: Standard deviation (SD)/√n

[3]Weight gain (WG, %) = (final wt. - initial wt.) × 100 / initial wt

[4]Specific growth rate (SGR, %/day) = (loge final wt. - loge initial wt.) × 100 / days

[5]Feed Efficiency (FE, %) = (wet weight gain / dry feed intake) × 100

[6]Protein efficiency ratio (PER) = (wet weight gain / protein intake)

*Survival was not significantly different between all groups

Table 2-4. Total bacteria count (TBC, 1×10^6 CFU/g) and *Bacillus* sp. count (BSC, 1×10^4 CFU/g) in intestines of the whiteleg shrimp fed eight experimental diets for 8 weeks[1]

	CON	FSM_B	FSM_{BS}	FSC_B	FSC_{BS}	FSP_B	FSM_L	SPC	Pooled SEM[2]
TBC	3.74^{ns}	4.48	3.97	4.57	4.06	4.23	4.16	3.77	0.11
BSC	12.7^{b}	24.4^{a}	15.5^{b}	25.6^{a}	14.2^{b}	23.1^{a}	14.1^{b}	13.0^{b}	1.95

[1]Values are means from triplicate groups of shrimp where the values in each row with different superscripts are significantly different ($P<0.05$); for diet composition information refer to Table 2-1

[2] Pooled SEM: Standard deviation (SD)/√n

Table 2-5. Non-specific immune responses of the whiteleg shrimp fed eight experimental diets for 8 weeks[1]

	CON	FSM_B	FSM_{BS}	FSC_B	FSC_{BS}	FSP_B	FSM_L	SPC	Pooled SEM[2]
SOD[3]	99.5^{ns}	100	97.2	99.5	99.2	99.4	98.8	97.6	0.54
LyZ[4]	0.14^{b}	0.25^{a}	0.18^{ab}	0.25^{a}	0.24^{ab}	0.20^{ab}	0.08^{b}	0.16^{ab}	0.01

[1]Values are means from triplicate groups of shrimp where the values in each row with different superscripts are significantly different (P<0.05); for diet composition information refer to Table 2-1

[2] Pooled SEM: Standard deviation (SD)/√n

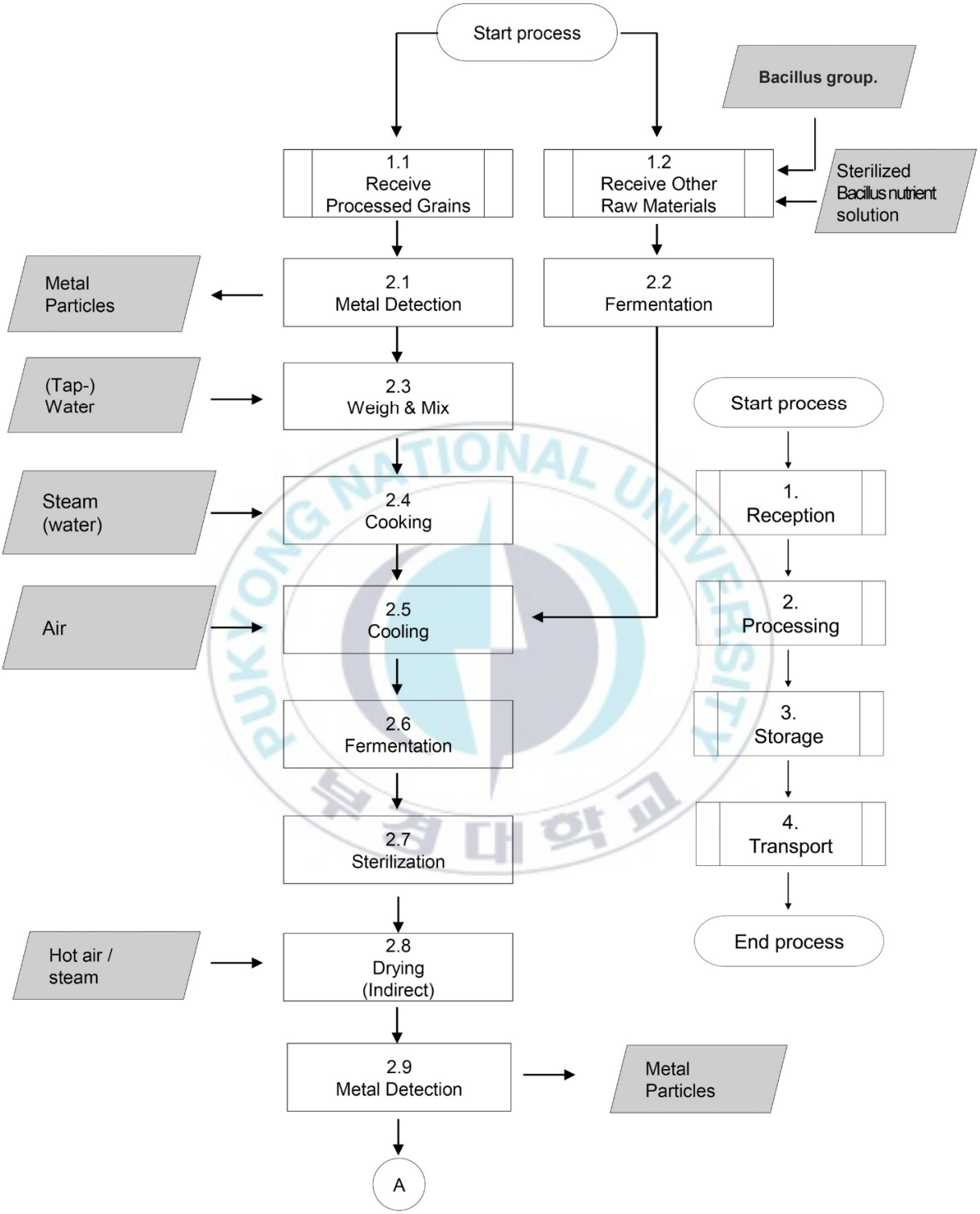
[3]Superoxide dismutase activity (% inhibition)

[4]Lysozyme activity (U/ml)

Table 2-6. Survival rate (%) of shrimp for eight days post-challenge with *Vibrio parahaemolyticus* after 8 weeks of feeding with the experimental diets.

Day[1]	CON	FSM_B	FSM_{BS}	FSC_B	FSC_{BS}	FSP_B	FSM_L	SPC	Pooled SEM
1	93.3^{b}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	2.76
2	80.0^{ab}	93.3^{ab}	86.7^{ab}	100^{a}	83.3^{ab}	100^{a}	76.7^{b}	96.7^{ab}	12.7
3	70.0^{bc}	90.0^{ab}	80.0^{ab}	93.3^{a}	56.7^{c}	93.3^{a}	73.3^{abc}	83.3^{ab}	15.3
4	56.7^{bc}	66.7^{b}	56.7^{bc}	90.0^{a}	43.3^{c}	90.0^{a}	50.0^{bc}	60.0^{bc}	18.9
5	43.3^{b}	53.3^{ab}	43.3^{b}	83.3^{a}	36.7^{b}	66.7^{ab}	40.0^{b}	50.0^{b}	20.2
6	20.0^{c}	30.0^{bc}	30.0^{bc}	70.0^{a}	23.3^{c}	50.0^{ab}	20.0^{c}	33.3^{bc}	19.6
7	6.67^{b}	20.0^{b}	16.7^{b}	53.3^{a}	20.0^{b}	20.0^{b}	10.0^{b}	30.0^{b}	16.8
8	0.00^{b}	10.0^{b}	6.67^{b}	26.7^{a}	6.67^{b}	13.3^{b}	0.00^{b}	20.0^{b}	10.8

[1]Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different ($P<0.05$); for diet composition information refer to Table 2-1



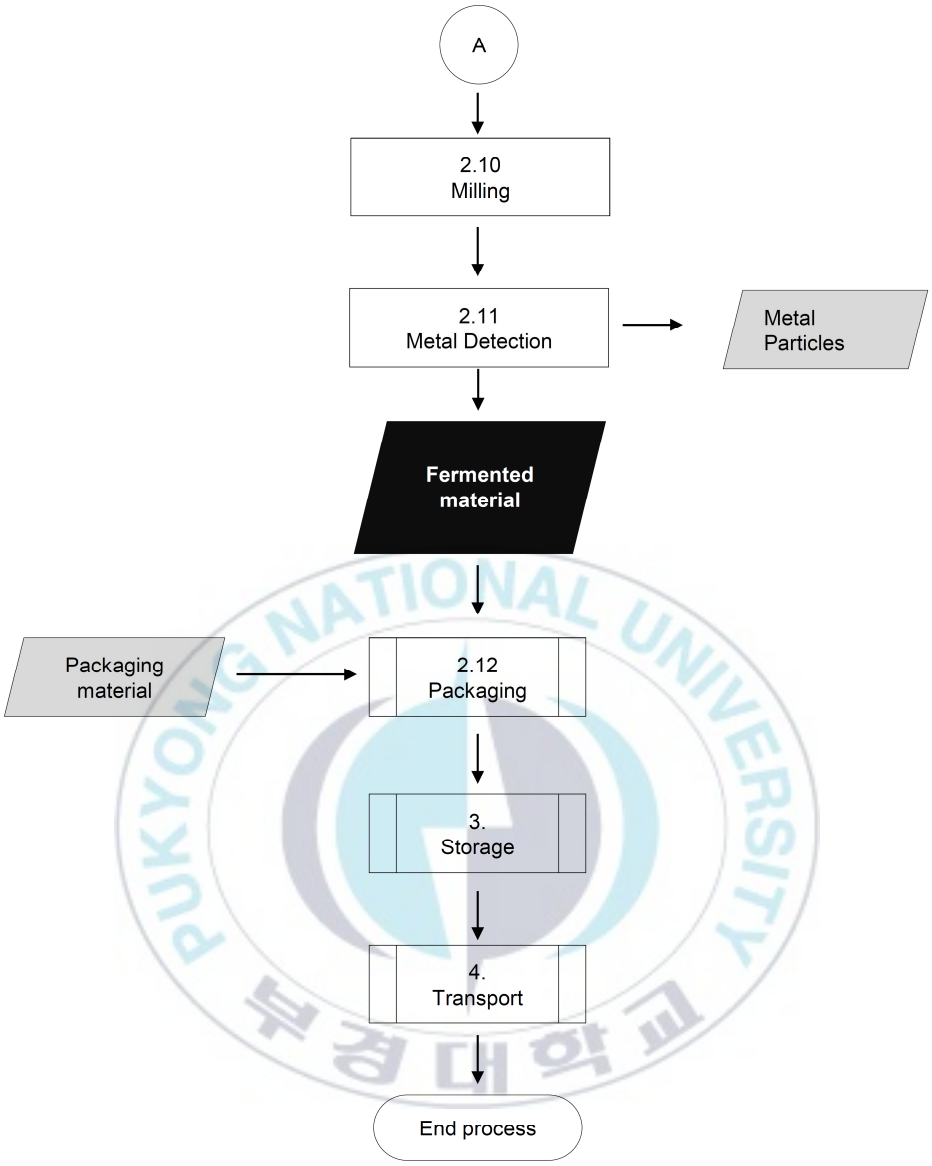
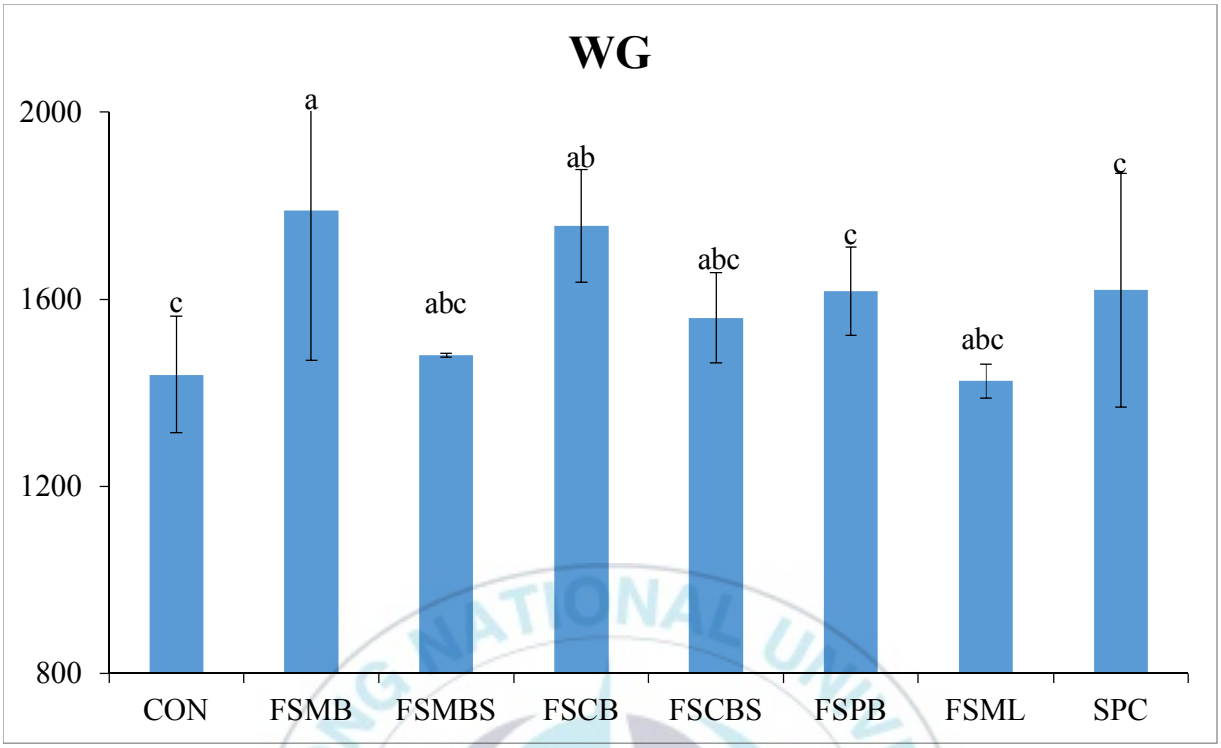
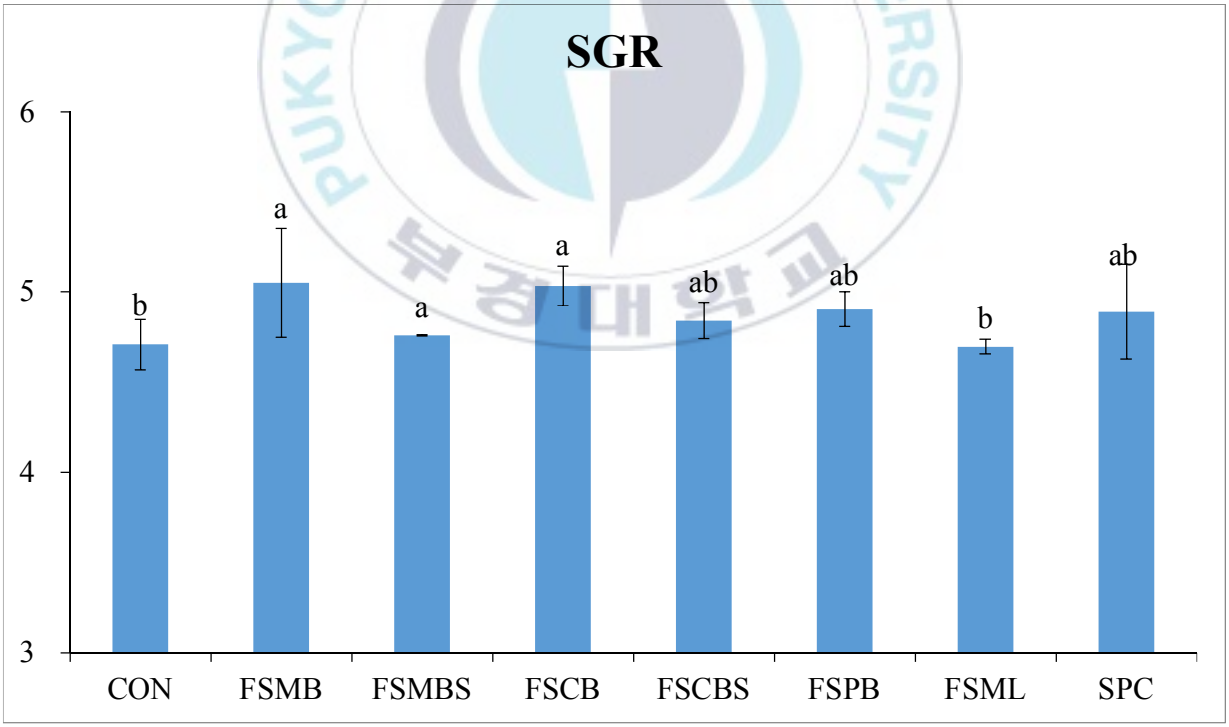
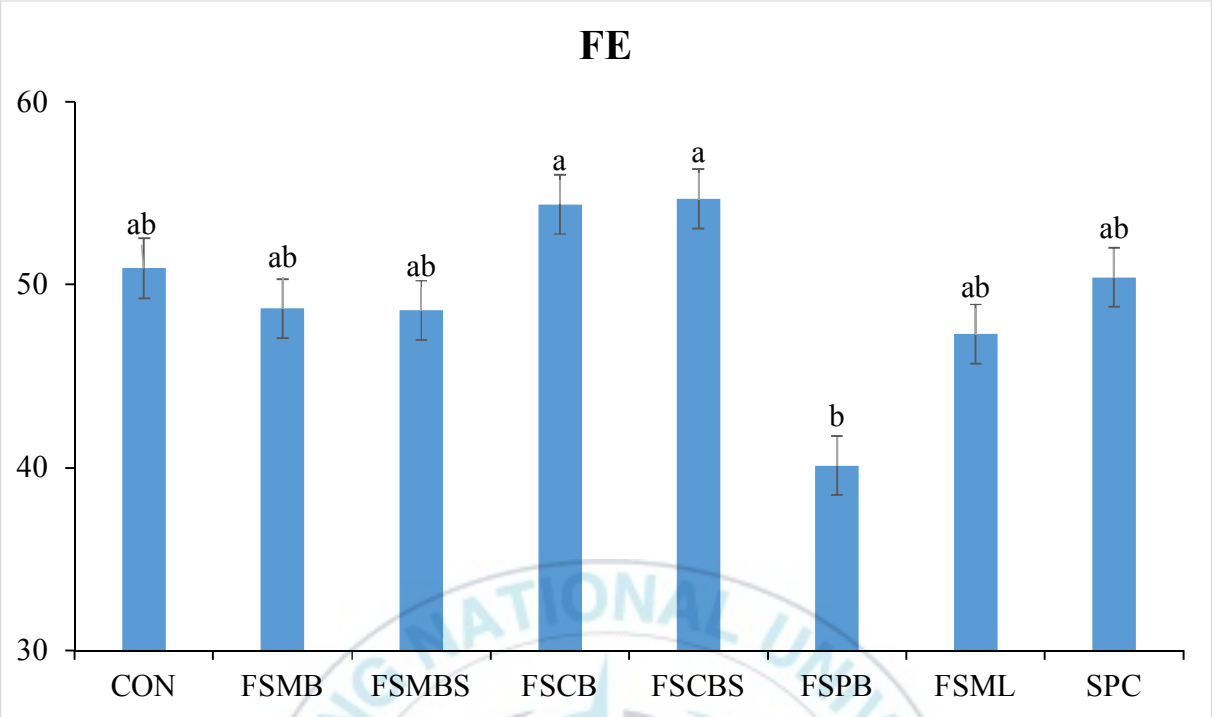


Figure 2-1. Solid state fermentation (SSF) of procedure plant protein ingredients (soybean meal and corn gluten meal) performed by CJ CheilJedang Corporation (Seoul, Republic of Korea).

Figure 2-2. The experimental tanks (22×47×32 cm) used for rearing of whiteleg shrimp for eight weeks, fed by the eight experimental diets.







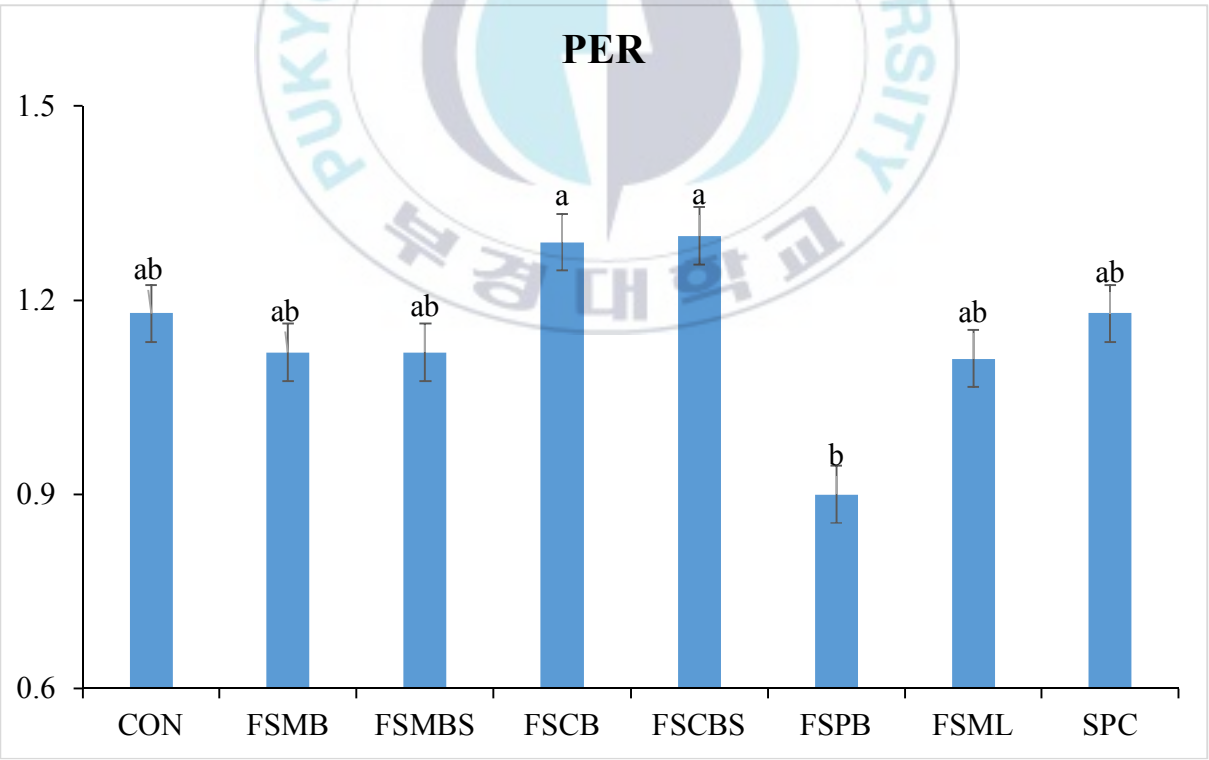
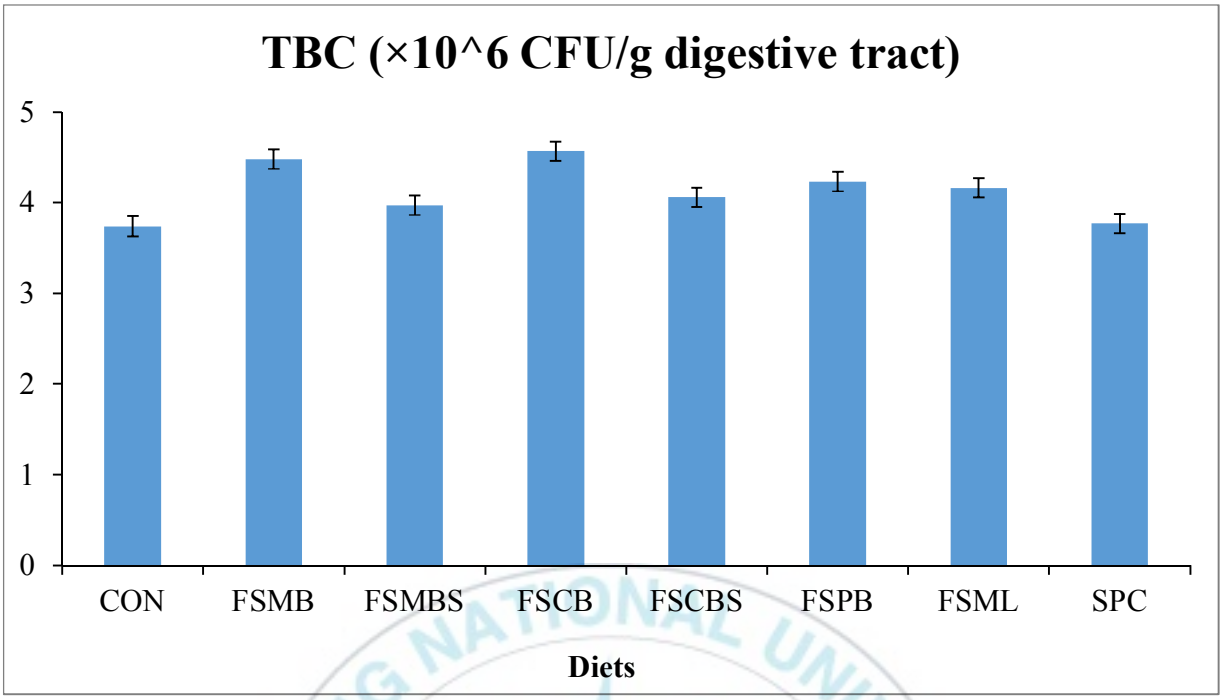


Figure 2-3. Growth performance of juvenile whiteleg shrimp fed eight experimental diets for 8 weeks; for diet composition information refer to Table 2-1



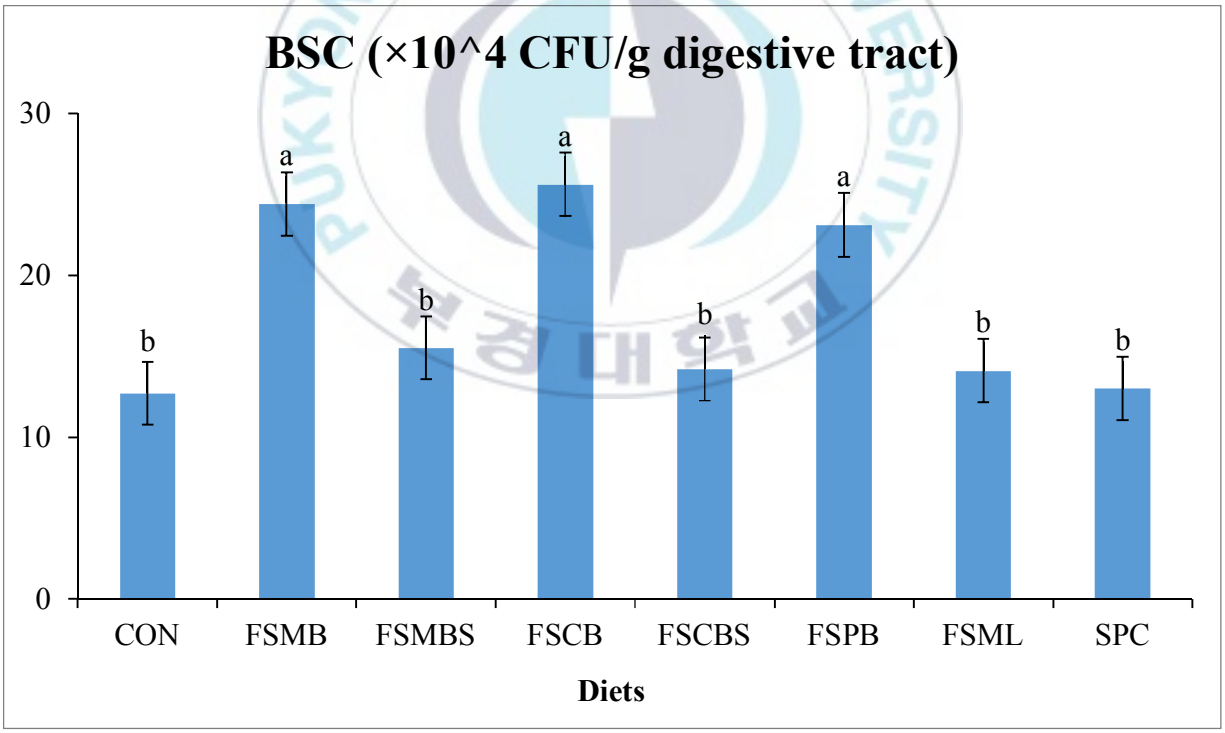
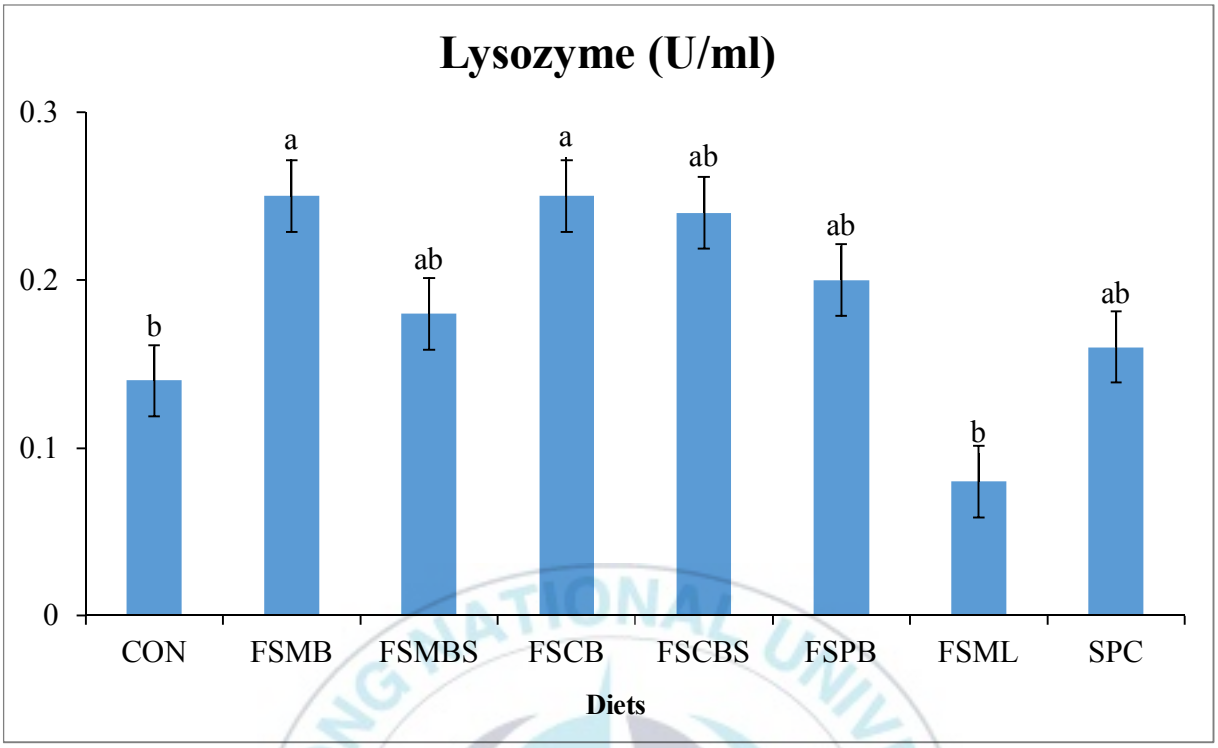


Figure 2-4. Total bacteria count (TBC) and *Bacillus* spp. count (BSC) in intestines of the whiteleg shrimp fed eight experimental diets for 8 weeks; for diet composition information refer to Table 2-1



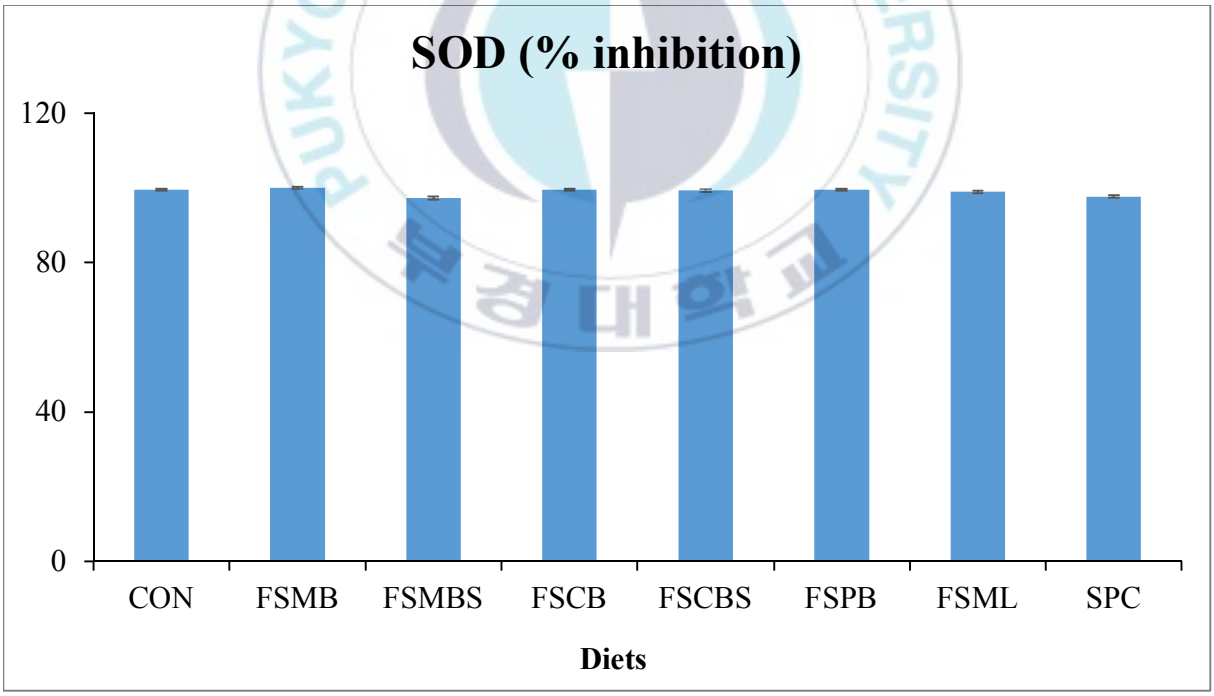


Figure 2-5. Lysozyme activity (U/ml) and superoxide dismutase (SOD) % inhibition of the whiteleg shrimp fed eight experimental diets for 8 weeks; for diet composition information refer to Table 2-1

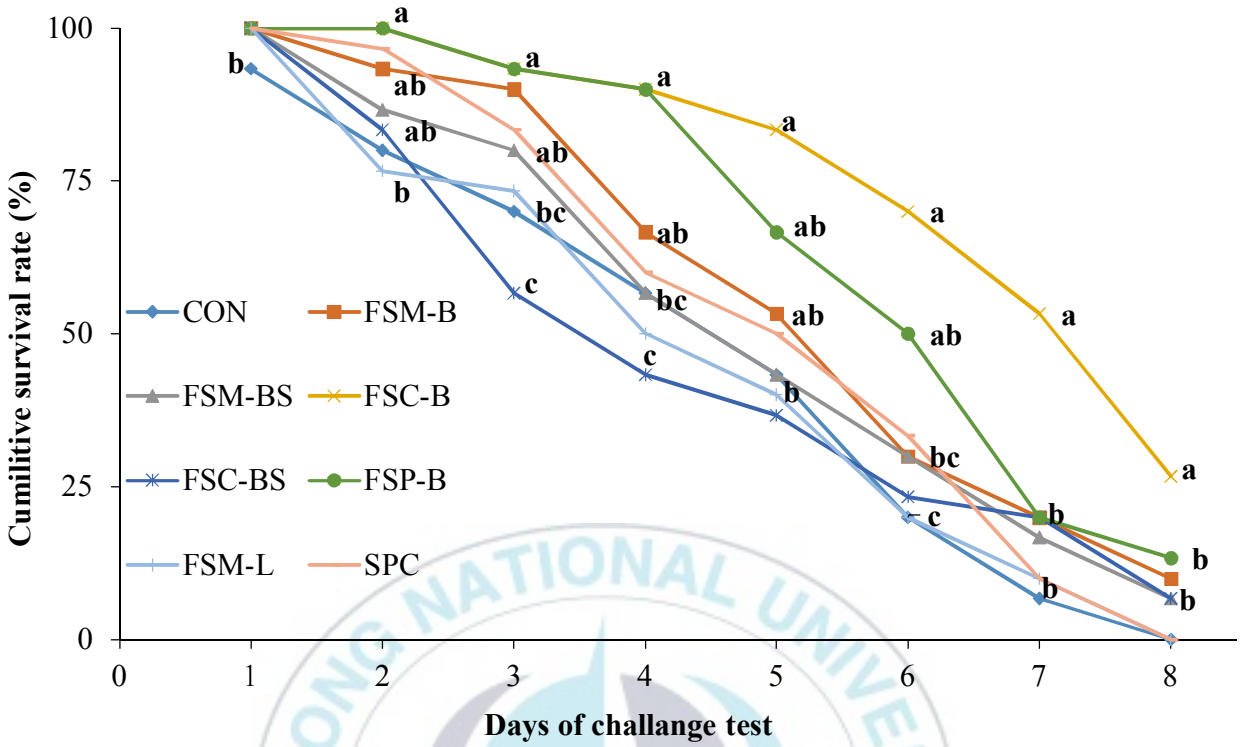


Figure 2-6. Cumulative survival rate (%) of shrimp for eight days post-challenge with *Vibrio parahaemolyticus* after 8 weeks of feeding with the experimental diets; for diet composition information refer to Table 2-1.

CHAPTER III. Effects of solid state fermented plant proteins with or without bacterial water treatment on water quality in juvenile whiteleg shrimp *Litopaeneus vannamei*

1. Introduction

Fish meal (FM) is a good source of high-quality protein and highly digestible essential amino acids (Anderson et al., 2016). However, in recent years many studies on alternatives of FM protein in aquafeeds were conducted due to its high pricing and the imbalance in demand and supply of FM as well as its sustainability issue in aquaculture (Alam et al. 2012; Tacon et al. 2011). In particular, the replacement of FM with vegetable proteins represents an opportunity; however, all plant proteins are not suitable as aquafeed ingredients in their unprocessed forms, as many of them contain antinutrients, which are detrimental in terms of fish nutrition (Francis et al. 2001). As for example, soy as recognized as the most promising plant protein source due to its sufficient supply, low price, balanced amino acids and highly digestible protein content (Gatlin et al., 2007; Hardy, 1999). However, due to the presence of antinutritional factors (ANFs) in soy proteins, such as protease inhibitors, lectins, phytates, glucosinolates, saponins and tannins have limited its inclusion in fish diets (Francis et al., 2001). In addition, soy proteins are limiting in sulphur-amino acids (Amerio et al. 1998). Some studies have reported that replacement of FM with soy proteins at high levels resulted in a decline of growth and alterations of intestinal morphology in fish (Kikuchi & Furuta, 2009; Wang et al. 2006), which may ultimately cause enteritis (Mosberian-Tanha et al. 2017).

Soybean-based protein concentrate has a high-quality refined crude protein (650–670 g/kg) among the terrestrial plant protein sources. However, this protein concentrate may contain higher levels of saponins than soybean meal when it was produced by extraction with water alone (Ireland et al. 1986). It has been reported that saponins in high plant protein-replaced diets may decrease growth performance of fish, although other researchers claimed that saponins do not affect the growth performance in European sea bass (Couto et al., 2015). Many studies have reported that FM replacement with soy proteins has adverse effects on carnivorous marine fish species in terms of growth performance (Lim et al., 2011), feed efficiency (Silva-Carrillo et al. 2012) and health condition (Ye et al. 2011). Gatlin et al. (2007) reported that technical removal of ANFs could increase the FM replacement level by soy proteins in fish diets. Numerous processing techniques

have been proposed to remove or inactivate soy ANFs such as heating, soaking, cooking, gamma-irradiation, alcohol extraction or fermentation technology (Drew et al. 2007; Zhang et al. 2014). On the other hand, corn gluten meal (CGM) is a widely used feedstuff in aquaculture. It contains at least 630 g/kg protein and low fat (NRC, 2011). CGM as a substitution for FM in fish has been reported in a number of studies including rainbow trout *Oncorhynchus mykiss* (Morales et al. 1994) and gilthead sea bream *Sparus aurata* (Robaina et al., 1997). However, CGM also contains some ANFs and limiting amino acids as a plant protein source (Pereira & Oliva-Teles, 2003). It has been reported that mixture of soy proteins and CGM could increase the FM replacement level in fish (Lunger, 2006).

Fermentation of plant proteins is a good choice in replacing fish meal in the diet of fish. In this regard, it has been reported that fermentation or bioprocessing of soy proteins can increase the nutritive value and decrease the ANFs of soy proteins (Tibaldi et al., 2006). Fermentation is a useful bioprocess technique for drying wet products with minimal nutrient loss. Teng et al. (2012) reported that protein content in soybean meal can increase up to 8.37% by solid-state fermentation with Bacillus subtilis. Kokou et al. (2012) reported that bioprocessed SBM can replace up to 40% of FM in gilthead sea bream without altering the health status. In another report, the same researchers found that the FM replacement level could be >40% in the case of soy protein concentrate inclusion in the diet of the same fish species (Kokou et al., 2016). However, Zhang et al. (2014) reported that gamma-irradiated SBM can replace about 16% of FM in the diets of Japanese sea bass. Yaghoubi et al. (2016) postulated the FM replaced by soy products (mixture of SBM and isolated soy protein) ranged between 16.5% and 27.3% in juvenile silvery-black porgy. It has been reported that supplementation of crystalline amino acids in plant protein-based diets could also improve the FM replacement level in carnivorous fish species without affecting growth and feed efficiency (Kader et al., 2012; Silva-Carrillo et al., 2012; Zhang et al., 2014).

Water quality parameters such as acidity, alkalinity, dissolved oxygen (DO), carbon-di oxide, nitrate, phosphorus, ammonia and hardness play vital role in fish production in aquaculture industries. Low water quality (lowDO, high ammonia, high nitrate etc.) enhances the percentage of diseases susceptibility in fish farming industries. Probiotic bacteria not only trigger the defense system in fish, but also improve the water quality. Several researchers have stated that probiotics can be used as an eco-friendly bio-control or bioremediation agents for sustainable development in aquaculture (Shariff et al., 2001; Dimitroglou et al., 2011; Iribarren et al., 2012). Padmavathi et

al. (2012) have investigated the effects of two probiotic bacterial candidates namely *Nitrosomonas* sp. and *Nitrobacter* sp., and reported that the uses of such beneficial bacteria decrease the pathogenic load in culture pond. However, ammonia and nitrite concentration in probiotics treated ponds have decreased dramatically. Furthermore, Melgar Valdes et al. (2013) have proved the potentiality of several probiotic candidates (*Rhodopseudomonas palustris*, *Lactobacillus plantarum*, *Lactobacillus casei* and *Saccharomyces cerevisiae*) in maintaining water quality. Phototropic bacteria are very important in marine ecosystem, which enhance the water quality in term of phosphorous availability, nitrate reduction and salt enhancement. In an experiment, Li et al. (1997) have stated that addition of probiotic bacteria in shrimp culture pond enhance the water quality and reduce the toxic material concentration. Several industries directly release their waste into water bodies without any prior processing, which cause pollution, kill the natural habitats and unbalance the ecosystem. Addition of beneficial or probiotic bacteria reduce the pollutant load (heavy metal like Pb, Cd, Hg, Ni, etc.) and maintain a healthy condition for aquatic animals (Banerjee et al., 2016).

The whiteleg shrimp, *Litopenaeus vannamei*, is a native species of the western South American coast and the eastern Pacific Ocean. It is the most cultured shrimp species in the world with a global production of 154,515 metric tons in 2000 that rose to 3,668,682 metric tons in 2014 with more than 17 million dollars increase in value (FAO, 2016). Whiteleg shrimp is mostly valued because of its favorable taste and suitability for aquaculture by exhibiting high growth performance, survival rate and disease resistance (Cuzon et al. 2004). This shrimp species can also tolerate a wide range of physicochemical parameters such as temperature, salinity, and oxygen (Felix-Portilloa et al., 2016; Lightner et al. 2009; Moss et al. 2007). According to what was mentioned, this study aims at replacing fish meal with sustainable *Bucillus* spp.-fermented plant protein sources and investigate the effects on water quality indices and bacterial count.

2. Materials and methods

Fermentation process of plant proteins

Different types of fermented plant protein sources that were used in this experiment were provided by the CJ CheilJedang Corporation (Seoul, Republic of Korea). Solid state fermentation (SSF) was performed by depositing solid substrates of plant protein sources (soybean meal and corn gluten

meal) on flatbeds and seeding it with liquid cultures of *Bacillus* spp. bacterium. Several days of preservation in temperature-controlled rooms allows the *Bacillus* spp. to grow. In SSF method only a small amount of water is added to the substrate, therefore, less fermenter is needed per unit volume of substrate and there will be no effluent treatment required (Fig 3-1). The fermented plant protein ingredients used in this experiment were as follows:

FSM_B: Fermented soybean meal with *Bacillus* spp. (Soytide®)

FSC_B: Fermented soybean meal and corn gluten meal with *Bacillus* spp. (Aquatide®)

FSP_B: Fermented soy protein concentrate with *Bacillus* spp.

Experimental diets

Formulation of the four experimental diets is shown in Table 3-1. Fish meal and soybean meal were considered as the main protein sources and fish oil was the main lipid source. A diet without replacement of fish meal (FM) was considered as control (CON). The other seven diets were prepared by 30% replacement of FM in the CON diet with fermented soybean meal with *Bacillus* spp (FSM_B), fermented soybean meal and corn gluten meal with *Bacillus* spp (FSC_B) and fermented soy protein concentrate with *Bacillus* spp (FSP_B). With some modifications, the procedures previously mentioned by Bai and Kim (1997) were used for preparing the ingredients, mixing, pelleting, drying and storing the diets. All fine-powered ingredients were mixed with an electric mixer (HYVM-1214, Hanyoung Food Machinery, Republic of Korea) according to the feed formulation. After through mixing of the ingredients, fish oil and water were added and a stiff dough was resulted. The dough was passed through a pelletizing machine (SFD-GT, Shinsung, Republic of Korea) with a flat die of approximately 0.2 cm in diameter. All diets were air dried for 48 hours until the moisture content of <10%, broken into desirable size and then stored at -20ºC until the initiation of feeding trial. According to Table 3-2, all diets were isonitrogenous (42 % crude protein) and isocaloric (4 kcal/g diet).

Shrimp husbandry and experimental condition

Juvenile whiteleg shrimp *Litopaeneus vannamei* weighing 4.08 ± 0.12 g (mean ± SD) were obtained from a local shrimp farm (Gyeongsang, Republic of Korea) and brought to the Department of Marine Bio-Materials & Aquaculture at Pukyong National University (Busan, Republic of Korea). Shrimp were stocked in 200L plastic tanks and the health condition was

checked. For about a week, shrimp were acclimated to the new environment and fed a commercial diet (Suhyup feed, Gyeongsangnam-do, Republic of Korea) with 45% protein until satiation. After this period, 10 shrimp were distributed in each of the 24 tanks with 50 L capacity that were filled up to 23L with clean sea water. Six replicates were randomly considered for each of the four experimental diets which were divided into two groups (three replicates per group). One group (three replicates) was treated with bacterial remediation (AquaStar® Pond, Biomin, Austria) and one group (three replicates) was not treated. All the tanks with bacterial treatment were assigned as O and those without bacterial treatment were assigned as X. The bacterial remediation contains *Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp., *Thiobacillus* sp. and *Paracoccus* sp. bacterium. Dark plastic covers were used on top of all the experimental tanks to reduce stress and prevent shrimp from jumping. During the four weeks of the experiment, water temperature was maintained at 27±2ºC using electric heater in each tank. Aeration was performed using air stones attached to an air pump and pH and dissolved oxygen was measured daily. Water was not recirculated during the experiment and only evaporation was recovered by adding water. Shrimp were fed 2 times a day with the previously mentioned diets (Table 3-1) until satiation (4% of body weight).

Sampling and analysis

Everyday dissolved oxygen and pH were measured for all the experimental tanks using YSI 85 DO meter (Yellow Springs Instrument, OH, USA) and digital LCD pocket pen type pH meter (SAFESEED, China), respectively. Also, the total ammonia nitrogen (TAN), nitrite nitrogen (NO2–N) and nitrate nitrogen ($NO3^-$-N) were measured every three days. TAN and $NO2^-$N were measured spectrophotometrically using HS-3300 spectrophotometer (HUMAS, Daejeon, Republic of Korea) following Standard Methods for the Examination of Water and Wastewater (APHA 1995). Measurement of $NO3^-$N was performed by the cadmium reduction method (8171) using DR/890 colorimeter (HACH Korea, Seoul, Republic of Korea) according to the procedures manual (Hach 2007).

Three times sampling of water from each tank was performed, the first time was at the beginning of the experiment, the second time was at the end of 2nd week and the third time was at the end of 4th week for the total bacteria and *Bacillus* spp. count. The methods previously described by Baumann and Schubert (1984) were used in this study. Water samples were diluted with 4.5 ml of phosphate-buffered saline (pH 7.0, 0.1 M). The suspension was held at room temperature for

10 min, vortexed, again held at room temperature for 7–8 min to allow the particles to settle. Then, 1-ml aliquots of supernatant were serially diluted at 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} and spread on LB agar plates and incubated at 37 °C for 16 h. Intestinal bacterial count was performed by simply counting the number of colonies appeared on each plate. The amount of bacteria was calculated as colony forming unit (CFU). LB medium was used to growth *Bacillus* spp and incubation at 30 °C. Partial 16 S rDNA based high-throughput sequencing was used to identify *Bacillus* spp. All procedures were performed under sterile conditions at room temperature.

3. Results

Water quality indices

Temperature was mostly consistent during the experiment period fluctuating between 26-28 °C in all tanks (Figure 3-2). Results for pH measurements in all experimental tanks are shown in Figure 3-3. According to this graph, pH in all tanks was almost consistent. For the first week, pH showed a value between 8-8.5 but as the days went on it dropped until pH 7.8 and it remained consistent until day 14-15. After two weeks of experiment, pH in all tanks dropped significantly until about 6.5. From day17 until the end of the experiment the pH did not change significantly.

The results for total ammonia nitrogen (TAN) during the four weeks of experiment in all tanks are shown in Table 3-2 and Figure 3-4. The significant differences in TAN were observed between different treatments form day six. Both treated and non-treated CON tanks showed higher TAN compared to other tanks. At day 12 differences in TAN level were more significant, as tanks $FSC_B(X)$ and both CONs showed significantly higher TAN level than tanks $FSC_B(O)$, $FSM_B(X)$, $FSP_B(X)$ and $FSP_B(O)$ ($P<0.05$). At day 15, the differences remained without much changes and tanks $FSC_B(O)$, $FSM_B(X)$, $FSP_B(X)$ and $FSP_B(O)$ still showed lower TAN level. At day 18 the differences became for clear where both CON tanks showed the highest TAN level (about 2 mg/L) comparing to other tanks. Although, the CON(X) tank showed a significantly higher TAN level comparing to $FSP_B(O)$, $FSP_B(X)$ and $FSM_B(X)$ tanks ($P<0.05$). At day 21, differences became even more significant, with CON(X) tank having significantly higher TAN level (about 2.2 mg/L) comparing to $FSC_B(O)$, $FSC_B(X)$, $FSM_B(X)$, $FSM_B(O)$, $FSP_B(X)$ and $FSP_B(O)$ tanks ($P<0.05$). Although, there were no significant differences in TAN level between CON(X) and CON(O) tanks ($P>0.05$). At the final days of the experiment, tanks $FSP_B(O)$ and $FSC_B(O)$ showed significantly

lower TAN value comparing to the CON(X) tank (P<0.05). Although, there were no significant differences between the TAN level of tanks CON(O), FSC_B(O), FSC_B(X), FSM_B(O), FSM_B(X), FSP_B(O) and FSP_B(X) tanks (P>0.05).

The measurements for nitrite (NO_2-N) during the four weeks of experiment are shown in Table 3-3 and Figure 3-5. Slight differences were observed from day 6 of experiment and CON(X) tanks had significantly higher NO_2-N compared to FSM_B(O) tanks (P<0.05). Although, there were no significant differences for NO_2-N among tanks CON(O), CON(X), FSC_B(O), FSC_B(X), FSM_B(X), FSP_B(O) and FSP_B(X) and also among tanks CON(O), FSC_B(O), FSC_B(X), FSM_B(O), FSM_B(X), FSP_B(O) and FSP_B(X) (P<0.05). At day 9, a wider range of differences were observed in NO_2-N level between tanks. While the CON(X) tanks still showed significantly higher NO_2-N comparing to FSC_B(O), FSC_B(X) and FSM_B(O) tanks (P<0.05). At day 12, tanks CON(X) had the highest NO_2-N level comparing to FSC_B(O), FSC_B(X), FSM_B(O), FSM_B(X) and FSP_B(O) tanks. Although, there were no significant differences between CON(O), FSC_B(O), FSC_B(X), FSM_B(O), FSM_B(X), FSP_B(O) and FSP_B(X) tanks (P>0.05). At day 15, still there were significant differences between the NO_2-N level of CON(X) tank and FSM_B(O), FSM_B(X) and FSC_B(O) tanks (P<0.05). At day 18, 21 and 24, levels of NO_2-N were similar and non-treated CON tanks (CON(X)) showed the highest levels of nitrite comparing to all other tanks (P<0.05). At the last days of experiment, tanks CON(O) and CON(X) showed the highest NO_2-N level with significant differences with FSC_B(O), FSM_B(O), FSM_B(X), FSP_B(O) and FSP_B(X) tanks (P<0.05).

Results for nitrate (NO_3-N) of experimental tanks are shown at Table 3-4 and Figure 3-6. At day 3 there were no significant differences in NO_3-N level between all tanks. At day 6, the differences in NO_3-N levels were observed between CON(O) and FSP_B(O) and FSP_B(X) tanks. At day 12, tanks FSC_B(X) and FSP_B(X) showed significantly higher NO_3-N levels comparing to CON(O) tanks. Results for days 15, 18 and 21 were relatively consistent and tanks FSM_B(O) showed significantly lower NO_3-N levels compared to FSP_B(X), FSP_B(O) and FSC_B(O) tanks (P<0.05). There were no significant differences in NO_3-N levels between all experimental tanks at days 24 and 27 (P>0.05).

Water bacterial counts

Results for total bacterial counts (TBC CFU/ml) of all the experimental tanks are shown in table 3-5. There were no significant differences in TBC between all experimental tanks at first and

second weeks. After four weeks, CON(X) tanks showed significantly lower TBC comparing to FSC_B(O) and FSM_B(O) tanks (P<0.05). Although, there were no significant differences in TBC between tanks CON(O), FSC_B(X), FSC_B(O), FSM_B(X), FSM_B(O), FSP_B(X) and FSP_B(O) (P>0.05). Also, results for *Bacillus* spp. count (BSC CFU/ml) of experimental tanks are shown in Table 3-5 for four weeks of experiment. There were no significant differences in BSC between all experimental tanks for the first week of experiment (P>0.05). At week two, both treated and non-treated CON tanks showed significantly lower BSC compared to all other tanks (P<0.05). Although, there were no significant differences among all other tanks (P>0.05). These results were consistent until the last week of the experiment and tanks CON(X) and CON(O) represented the lowest BSC compared to all other experimental tanks (P<0.05).

4. Discussion

Production of fish, marine shrimp, and other species by aquaculture depends upon a supply of high-quality water. In cases of aquaculture projects sited where water quality is naturally impaired or polluted, the water quality limitations must be overcome or the aquaculture projects will fail. Water quality also declines in aquaculture systems because of waste accumulation and, thus, water quality management must be applied to avoid stress and mortality of aquaculture species, and to assure efficient production. Furthermore, the discharges from aquaculture facilities contain nutrients, organic matter, and suspended solids that pollute receiving water bodies. Many governments require aquaculture facilities to implement practices to minimize pollution (Boyd 2012). The purpose of this study was to compare different types of *Bacillus* spp.-fermented plant protein sources as FM replacers in whiteleg shrimp and evaluate the effects on water quality indices.

Water temperature was measured every day and it was mostly consistent during the experiment period fluctuating between 26-28 °C in all tanks. Results for pH measurements in all experimental tanks are shown consistency in all tanks. For the first week, pH showed a value between 8-8.5 but as the days went on it dropped until pH 7.8 and it remained consistent until day 14-15. After two weeks of experiment, pH in all tanks dropped significantly until about 6.5. The water was not changed in this experiment and only evaporated water was added to the tanks. The chemical reactions happening in the tanks due to respiration, ammonia excretion and microorganism activity

could have resulted in depressed pH. The removal of nitrogen by biological nitrification and denitrification is a two-step process. In the first step (nitrification), ammonia is converted aerobically to nitrate (NO_3^-). In the second step (denitrification), nitrates are converted to N_2O or nitrogen gas (N_2) under anoxic conditions.

$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+$

$2NO_2^- + O_2 \rightarrow 2NO_3^-$

$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$

Nitrification is an autotrophic process that means that the energy for bacterial growth is derived from the oxidation of nitrogen compounds, primarily ammonia. Both of these processes can produce H^+ in the water body and this will increase the pH level (Halling-Sorensen and Jorgensen. 1993).

Total ammonia nitrogen (TAN) of water was influenced by both the dietary treatments and presence/absence of bacterial treatment. After 27 days of experiment, tanks fed with fermented soybean meal with Bacillus spp. and received bacterial treatment had lower TAN level than those tanks fed with CON diet and not treated with bacterial treatment. It is important to provide shrimp with a healthy environment and probiotics has a great deal of potential (Gomez-Gil et al., 2000).Wang et al. (2005) investigated the effect of commercial probiotics on water quality in shrimp, *P. vannamei*, ponds and the results showed that probiotics could significantly reduce the concentrations of nitrogen and phosphorus in pond water compared with the control. In the present study, the amount of NO_2-N in the experimental tanks after 27 days was more influenced by diets compared to the bacterial treatment. The control tanks which were not treated had higher NO_2-N than all other tanks but with no differences with treated control tanks. This could be related to higher digestibility of the fermented plant protein sources. Water quality parameters such as acidity, alkalinity, dissolved oxygen (DO), nitrate and ammonia play vital role in fish production in aquaculture industries. Low water quality (lowDO, high ammonia, high nitrate etc.) enhances the percentage of diseases susceptibility in fish farming industries. Probiotic bacteria not only trigger the defense system in fish, but also improve the water quality (Table 5). Several researchers have stated that probiotics can be used as an eco-friendly bio-control or bioremediation agents for sustainable development in aquaculture (Shariff et al., 2001; Dimitroglou et al., 2011; Iribarren et al., 2012). Until now, the use of probiotic candidates in fish culture ponds are not so popular, but in near future it would be randomly used in aquaculture industries and local fish farming sectors.

5. References

Alam M.S., Watanabe W.O., Sullivan K.B., Rezek T.C., Seaton P.J. 2012. Replacement of menhaden fish meal protein by solvent-extracted soybean meal protein in the diet of juvenile black sea bass supplemented with or without squid meal, krill meal, methionine, and lysine. North American Journal of Aquaculture, 74:251-265.

Amerio M., Vignali C., Castelli L., Fiorentini L., Tibaldi E. 1998. Chemical and nutritional evaluation of vegetable protein sources as possible dietary ingredients for sea bream (*Sparus aurata*). 8th International Symposium on Nutrition and Feeding in Fish. Las Palmas De Gran Canaria, Spain. pp. 145.

Anderson A.D., Alam M.S., Watanabe W.O., Carroll P.M., Wedegaertner T.C., Dowd M.K. 2016. Full replacement of menhaden fish meal protein by low-gossypol cottonseed flour protein in the diet of juvenile black sea bass *Centropristis striata*. Aquaculture 464:618-628.

APHA 1995. WPCF, Standard Methods for the Examination of Water and Wastewater. American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA.

Bai S.C., Kim KW. 1997. Effects of dietary animal protein sources on growth and body composition in Korean rockfish, *Sebastes schlegeli*. J. Aquacult. 10:77-85 (in Korean with English abstract).

Banerjee G., Nandi A., Dan S.K., Ghosh P., Ray A.K. 2016b. Mode of association, enzyme producing ability and identification of autochthonous bacteria in the gastrointestinal tract of two Indian air-breathing fish, murrel (*Channa punctatus*) and stinging catfish (*Heteropneustes fossilis*). Proc. Zool. Soc.

Baumann P., Schubert R.H.W. 1984. Vibrionaceae. N.A Krieg, J.G Holt (Eds.), Bergey's Manual of Systematic Bacteriology, vol. 1, William & Wilkins, Baltimore, pp. 516-549.

Couto A., Kortner T., Penn M., Østby G., Bakke A., Krogdahl Å., Oliva-Teles A. 2015. Saponins and phytosterols in diets for European sea bass (*Dicentrarchus labrax*) juveniles: Effects on growth, intestinal morphology and physiology. Aquaculture Nutrition 21:180-193.

Cuzon G., Lawrence A., Gaxiola G., Rosas C., Guillaume J. 2004. Nutrition of *Litopenaeus vannamei* reared in tanks or in ponds. Aquaculture 235:513-551.

Dimitroglou A., Merrifield D.L., Carnevali O., Picchietti S., Avella M., Daniels C., Güroy

D., Davies S.J. 2011. Microbial manipulations to improve fish health and production-a Mediterranean perspective. Fish shellfish Immunol. 30:1–16.

Drew M., Borgeson T., Thiessen, D. 2007. A review of processing of feed ingredients to enhance diet digestibility in finfish. Animal Feed Science & Technology 138:118-136.

FAO 2016. FISHSTAT Plus, universal software for fishery statistical time series. Food and Agriculture Organization, United Nations, Rome. Electronic webpage.

Felix-Portilloa M., Martınez-Quintanab J.A., Arenas-Padillaa M., Mata-Haroa V., Gomez-Jimeneza S., Yepiz-Plascencia G. 2016. Hypoxia drives apoptosis independently of p53 and metallothionein transcript levels in hemocytes of the whiteleg shrimp *Litopenaeus vannamei*. Chemosphere, 161:454-462.

Francis G., Makkar H.P., Becker K. 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture 199:197-227.

Gatlin D.M., Barrows F.T., Brown P., Dabrowski K., Gaylord T.G., Hardy R.W., Nelson, R. 2007. Expanding the utilization of sustainable plant products in aquafeeds: A review. Aquaculture Research 38:551-579.

Hardy R. 1999. Aquaculture′s rapid growth requirements for alternate protein sources. Feed Management 50:25-28.

Hach 2007. Procedures Manual, DR2700 Spectrophotometer. Hach, Co, USA.

Ireland P.A., Dziedzic S.Z., Kearsley M.W. 1986. Saponin content of soya and some commercial soya products by means of high-performance liquid chromatography of the sapogenins. Journal of the Science of Food and Agriculture 37:694-698.

Iribarren D., Daga P., Moreira M.T., Feijoog 2012. Potential environmental effects of probiotics used in aquaculture. Aquacult. Int. 20:779-789.

Kader A., Koshio S., Ishikawa M., Yokoyama S., Bulbul M., Nguyen B.T., Laining A. 2012. Can fermented soybean meal and squid by-product blend be used as fishmeal replacements for Japanese flounder (*Paralichthys olivaceus*)? Aquaculture Research 43:1427-1438.

Kikuchi K., Furuta T. 2009. Use of defatted soybean meal and blue mussel meat as substitute for fish meal in the diet of tiger puffer, *Takifugu rubripes*. Journal of the World Aquaculture Society 40:472-482.

Kokou F., Rigos G., Henry M., Kentouri M., Alexis M. 2012. Growth performance, feed utilization and non-specific immune response of gilthead sea bream (*Sparus aurata* L.) fed graded levels of a bioprocessed soybean meal. Aquaculture 364:74-81.

Kokou F., Rigos G., Kentouri M., Alexis M. 2016. Effects of DL-methionine- supplemented dietary soy protein concentrate on growth performance and intestinal enzyme activity of gilthead sea bream (*Sparus aurata* L.). Aquaculture International 24:257-271.

Li Z., Zhang Q., Yang H. 1997. The effect of the probiotics to the shrimp ponds. Aquacult. China 5, 30–31 [in Chinese].

Lightner D.V., Redman R.M., Arce S., Moss S.M. 2009. Specific pathogen-free (SPF) shrimp stocks in shrimp farming facilities as a novel method for disease control in crustaceans. In S. Shumway & G. Rodrick (Eds.), Shellfish safety and quality. London, UK: Woodhead Publishers.

Lunger A.N. 2006. Evaluation of organically certifiable alternate protein sources for production of the marine carnivore, cobia (*Rachycentron canadum*). Masters Thesis, Virginia Polytechnic Institute and State University, VA, USA.

Melgar Valdes C.E., Barba Macías E., Alvarez-González C.A., Tovilla Hernández C., Sánchez, A.J. 2013. Microorganisms effect with probiotic potential in water quality and growth of the shrimp Litopenaeus vannamei (Decapoda: Penaeidae) in intensive culture. Rev. Biol. Trop. 61: 1215-1228.

Morales A.E., Cardenete G., De la Higuera M., Sanz A. 1994. Effect of dietary protein source on growth, feed conversion and energy utilization in rainbow trout, *Oncorhynhus mykiss*. Aquaculture 124:117-126.

Mosberian-Tanha P., Schrama J.W., Landsverk T., Mydland L.T., Øverland M. 2017. The effect of plant-based diet and suboptimal environmental conditions on digestive function and diet-induced enteropathy in rainbow trout (*Oncorhynchus mykiss*). Aquaculture Nutrition 1–11 (in press).

Moss D.R., Arce S.A., Otoshi C.A., Doyle R.W., Moss S.M. 2007. Effects of inbreeding on survival and growth of Pacific white shrimp, *Litopenaeus vannamei*. Aquaculture 272:S30–S37.

National Research council (NRC) 2011. Nutrient requirements of fish and shrimp. Washington, DC: The National Academic Press.

Padmavathi P., Sunitha K., Veeraiah K. 2012. Efficacy of probiotics in improving water quality and bacterial flora in fish ponds. Afr. J. Microbiol. Res. 6:7471-7478.

Pereira T.G., Oliva-Teles A. 2003. Evaluation of corn gluten meal as a protein source in diets for gilthead sea bream (*Sparus aurata* L.) juveniles. Aquaculture Research 34:1111-1117.

Robaina L., Moyano F.J., Izquierdo M.S., Socorro J., Vergara J.M., Montero D. 1997. Corn gluten and meat and bone meals as protein sources in diets for gilthead seabream (*Sparus aurata*): Nutritional and histological implications. Aquaculture 157:347-359.

Shariff M., Yusoff F.M., Devaraja T.N., SrinivasaRao P.S. 2001. The effectiveness of a commercial microbial product in poorly prepared tiger shrimp, *Penaeus monodon* (Fabricius) ponds. Aquacult. Res. 32:181-187.

Silva-Carrillo Y., Hernández C., Hardy R.W., González-Rodríguez B., Castillo-Vargasmachuca S. 2012. The effect of substituting fish meal with soybean meal on growth, feed efficiency, body composition and blood chemistry in juvenile spotted rose snapper *Lutjanus guttatus* (Steindachner, 1869). Aquaculture 364:180-185.

Tacon A.G.J., Hasan M.R., Metian M. 2011. Demand and supply of feed ingredients for farmed fish and crustaceans: Trends and prospects. FAO Fisheries and Aquaculture Technical Paper No. 564. pp. 87.

Teng D., Gao M., Yang Y., Liu B., Tian Z., Wang J. 2012. Bio-modification of soybean meal with Bacillus subtilis or Aspergillus oryzae. Biocatalysis and Agricultural Biotechnology 1:32-38.

Tibaldi E., Hakim Y., Uni Z., Tulli F., de Francesco M., Luzzana U., Harpaz S. 2006. Effects of the partial substitution of dietary fish meal by differently processed soybean meals on growth performance, nutrient digestibility and activity of intestinal brush border enzymes in the European sea bass (*Dicentrarchus labrax*). Aquaculture 261:182-193.

Wang Y., Kong L., Li C., Bureau D.P. 2006. Effect of replacing fish meal with soybean meal on growth, feed utilization and carcass composition of cuneate drum (*Nibea miichthioides*). Aquaculture 261:1307-1313.

Yaghoubi M., Mozanzadeh M.T., Marammazi J.G., Safari O., Gisbert E. 2016. Dietary replacement of fish meal by soy products (soybean meal and isolated soy protein) in silvery-black porgy juveniles (*Sparidentex hasta*). Aquaculture 464:50-59.

Ye J., Liu X., Wang Z., Wang K. 2011. Effect of partial fish meal replacement by soybean meal on the growth performance and biochemical indices of juvenile Japanese flounder *Paralichthys olivaceus*. Aquaculture International 19:143-153.

Zhang Y., Wu Y., Jiang D., Qin J., Wang Y. 2014. Gamma-irradiated soybean meal replaced more fish meal in the diets of Japanese seabass (*Lateolabrax japonicus*). Animal Feed Science & Technology, 197:155-163.

Table 3-1. Composition of five experimental diets in whiteleg shrimp (% of DM basis)[1]

Ingredients	CON	FSM_B	FSC_B	FSP_B
Fish meal (Chile)[2]	30.0	21.0	21.0	21.0
Soytide[3]	-	10.97	-	-
Aquatide[4]	-	-	8.98	-
FSPC[5]	-	-	-	9.24
Soybean meal	26.95	26.95	26.95	26.95
Wheat gluten	7.00	6.80	6.60	6.80
Squid liver powder	4.00	4.00	4.00	4.00
Wheat flour	10.0	10.0	10.0	10.0
Corn starch	10.0	7.05	9.01	8.47
Fish oil	4.00	4.60	4.30	4.70
Vitamin premix[6]	2.00	2.00	2.00	2.00
Mineral premix[7]	2.00	2.00	2.00	2.00
Lysine	0.00	0.19	0.37	0.14
Methionine	0.00	0.12	0.05	0.11
Cellulose	0.23	0.50	0.92	0.77
Others[8]	2.32	2.32	2.32	2.32
Total	100.0	100.0	100.0	100.0

[1] For diet composition information refer to Table 2-1
[2]Fish meal (Chile): CJ CheilJedang Corporation, Seoul, Republic of Korea
[3] Soytide®: *Bacillus* spp-fermented soybean meal, CJ CheilJedang Corporation, Seoul, Republic of Korea
[4] Aquatide®: *Bacillus* spp-fermented soybean meal and corn gluten meal, CJ CheilJedang Corporation, Seoul, Republic of Korea
[5] FSPC: *Bacillus* spp-fermented soy protein concentrate
[6] Vitamin premix (as mg/kg premix): A, 1000000 IU; D, 200000 IU; E, 10000; B1, 2000; B6, 1500; B12, 10; C, 10000; Calcium pantotenic acid, 5000; Nicotinic acid 4500; B-Biotin 10; Choline chloride, 30000; Inositol, 5000.
[7] Mineral premix (as g/kg premix): Ferrous fumarate, 12.50; Manganese sulfate, 11.25; Dried ferrous sulfate, 20.0; Dried cupric sulfate, 1.25; Cobaltous sulfate, 0.75; Zinc sulfate KVP, 13.75; Cancium iodate, 0.75; Magnesium sulfate, 80.20; Aluminum Hydroxide, 0.75
[8]Others: Carboxymethyl cellulose (1.00%), Soy lecithin (2.00%), Calcium phosphate (0.30%) and Cholesterol (0.02%)

Table 3-2. Proximate composition of the eight experimental diets (% of DM basis)[1]

	CON	FSM_B	FSC_B	FSP_B
Protein	42.7±0.24	42.9±0.59	42.2±0.98	42.9±0.51
Fat	9.72±0.05	10.0±0.12	9.79±0.03	9.93±0.11
Ash	8.15±0.05	7.62±0.14	7.22±0.37	7.36±0.07
Moisture	9.60±0.06	9.64±0.13	9.77±0.25	9.55±0.12
Energy (kcal/g)	4.55±0.01	4.59±0.01	4.57±0.01	4.59±0.01

[1]For diet composition information refer to Table 2-1

Table 3-3. Total ammonia nitrogen (TAN) of juvenile whiteleg shrimp culture tanks fed by the experimental diets for 4 weeks, with (O) or without (X) probiotic water treatment[1]

Day	CON(X)	CON(O)	FSC_B(X)	FSC_B(O)	FSM_B(X)	FSM_B(O)	FSP_B(X)	FSP_B(O)	Pooled SEM
3	0.46	0.55	0.43	0.49	0.48	0.46	0.45	0.44	0.01
6	1.59^{ab}	1.67^{a}	1.48^{bc}	1.44^{bc}	1.37^{c}	1.51^{abc}	1.48^{bc}	1.44^{bc}	0.03
9	2.19	2.14	2.10	2.06	2.10	2.06	2.13	2.11	0.15
12	2.13^{ab}	2.06^{abc}	2.18^{a}	1.87^{c}	1.91^{c}	1.92^{bc}	1.88^{c}	1.84^{c}	0.05
15	2.09^{ab}	2.02^{ab}	2.18^{a}	1.87^{b}	1.87^{b}	1.99^{ab}	1.88^{b}	1.91^{b}	0.04
18	2.14^{a}	2.02^{ab}	1.99^{ab}	1.87^{b}	1.87^{b}	1.99^{ab}	1.88^{b}	1.91^{ab}	0.03
21	2.20^{a}	2.02^{ab}	1.99^{b}	1.87^{b}	1.87^{b}	1.91^{b}	1.87^{b}	1.85^{b}	0.04
24	2.09	2.08	2.04	1.94	1.99	2.00	1.97	1.93	0.02
27	2.13^{a}	2.08^{ab}	2.04^{ab}	1.94^{b}	1.99^{ab}	1.96^{ab}	2.01^{ab}	1.93^{b}	0.03

[1]Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different (P<0.05); for diet composition information refer to Table 2-1

Table 3-4. NO_2-N of juvenile whiteleg shrimp culture tanks fed by the experimental diets for 4 weeks, with (O) or without (X) probiotic water treatment[1]

Day	CON(X)	CON(O)	FSC_B(X)	FSC_B(O)	FSM_B(X)	FSM_B(O)	FSP_B(X)	FSP_B(O)	Pooled SEM
3	0.05	0.05	0.04	0.07	0.04	0.08	0.06	0.04	0.01
6	3.03^{a}	2.60^{ab}	2.50^{ab}	2.49^{ab}	2.58^{ab}	2.44^{b}	2.77^{ab}	2.68^{ab}	0.07
9	6.60^{a}	6.23^{ab}	5.28^{bc}	5.04^{c}	6.01^{abc}	5.46^{bc}	5.55^{abc}	5.70^{abc}	0.18
12	10.1^{a}	8.89^{ab}	7.37^{b}	7.41^{b}	7.53^{b}	7.43^{b}	8.74^{ab}	8.27^{b}	0.34
15	12.7^{a}	9.71^{abc}	10.93^{ab}	6.86^{c}	7.20^{bc}	7.15^{c}	9.52^{abc}	9.63^{abc}	0.72
18	13.8^{a}	10.0^{b}	8.74^{bcd}	6.54^{d}	7.17^{cd}	7.15^{cd}	8.96^{bcd}	9.40^{bc}	0.81
21	13.3^{a}	9.19^{b}	8.46^{bc}	6.86^{c}	6.87^{c}	7.30^{c}	7.96^{bc}	7.42^{c}	0.75
24	12.3^{a}	9.13^{b}	8.65^{bc}	7.28^{d}	7.49^{cd}	7.27^{d}	8.34^{bcd}	7.32^{cd}	0.59
27	11.9^{a}	10.8^{ab}	9.10^{bc}	7.25^{c}	8.04^{c}	7.88^{c}	6.84^{c}	6.69^{c}	0.26

[1]Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different (P<0.05); for diet composition information refer to Table 2-1

Table 3-5. NO_3-N of juvenile whiteleg shrimp culture tanks fed by the experimental diets for 4 weeks, with (O) or without (X) probiotic water treatment[1]

Day	CON(X)	CON(O)	FSC_B(X)	FSC_B(O)	FSM_B(X)	FSM_B(O)	FSP_B(X)	FSP_B(O)	Pooled SEM
3	1.63^{ab}	2.10^{ab}	1.13^{ab}	1.77^{ab}	4.63^{a}	2.00^{ab}	1.37^{ab}	0.43^{b}	0.44
6	1.97^{abc}	3.30^{ab}	1.40^{bc}	1.93^{abc}	3.77^{a}	2.30^{abc}	1.23^{bc}	0.97^{c}	0.35
9	16.0^{a}	14.7^{ab}	10.0^{b}	11.0^{b}	14.0^{ab}	12.3^{ab}	13.0^{ab}	14.7^{ab}	0.72
12	32.7^{ab}	28.3^{b}	37.0^{ab}	38.3^{a}	33.7^{ab}	36.3^{ab}	38.0^{a}	33.7^{ab}	1.19
15	59.3^{abc}	56.3^{abc}	55.0^{bc}	61.3^{ab}	51.0^{bc}	41.7^{c}	74.0^{a}	68.0^{ab}	3.52
18	61.0^{ab}	54.3^{bc}	57.0^{abc}	63.3^{ab}	54.0^{bc}	45.7^{c}	69.7^{a}	67.3^{a}	2.79
21	60.7^{ab}	66.3^{a}	61.7^{ab}	67.7^{a}	59.3^{ab}	56.7^{b}	67.7^{a}	70.0^{a}	1.69
24	63.7	75.3	67.3	73.7	64.3	65.3	66.0	71.3	1.58
27	66.0	82.3	67.0	83.0	63.0	66.0	63.7	71.7	2.84

[1]Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different (P<0.05); for diet composition information refer to Table 2-1

Table 3-6. Total bacterial count (TBC, 1×10^5 CFU/g) and *Bacillus* sp. count (BSC, 1×10^4 CFU/g) in the culture water of juvenile whiteleg shrimp treated with (O) and without (X) multistrain probiotics for 4 weeks[1]

	Time (week)	CON(X)	CON(O)	FSC_B(X)	FSC_B(O)	FSM_B(X)	FSM_B(O)	FSP_B(X)	FSP_B(O)	Pooled SEM
TBC	0	5.52	5.56	5.52	5.56	5.52	5.56	5.52	5.56	0.01
	2	5.57	5.60	5.60	5.63	5.58	5.63	5.58	5.63	0.01
	4	5.75^{b}	5.81^{ab}	5.84^{ab}	5.90^{a}	5.85^{ab}	5.88^{a}	5.82^{ab}	5.87^{a}	0.02
BSC	0	2.24	2.27	2.24	2.27	2.24	2.27	2.24	2.27	0.01
	2	2.25^{b}	2.30^{b}	2.48^{a}	2.50^{a}	2.46^{a}	2.47^{a}	2.48^{a}	2.46^{a}	0.03
	4	2.25^{c}	2.32^{b}	2.61^{a}	2.62^{a}	2.58^{a}	2.62^{a}	2.58^{a}	2.61^{a}	0.05

[1]Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different (P<0.05); for diet composition information refer to Table 2-1

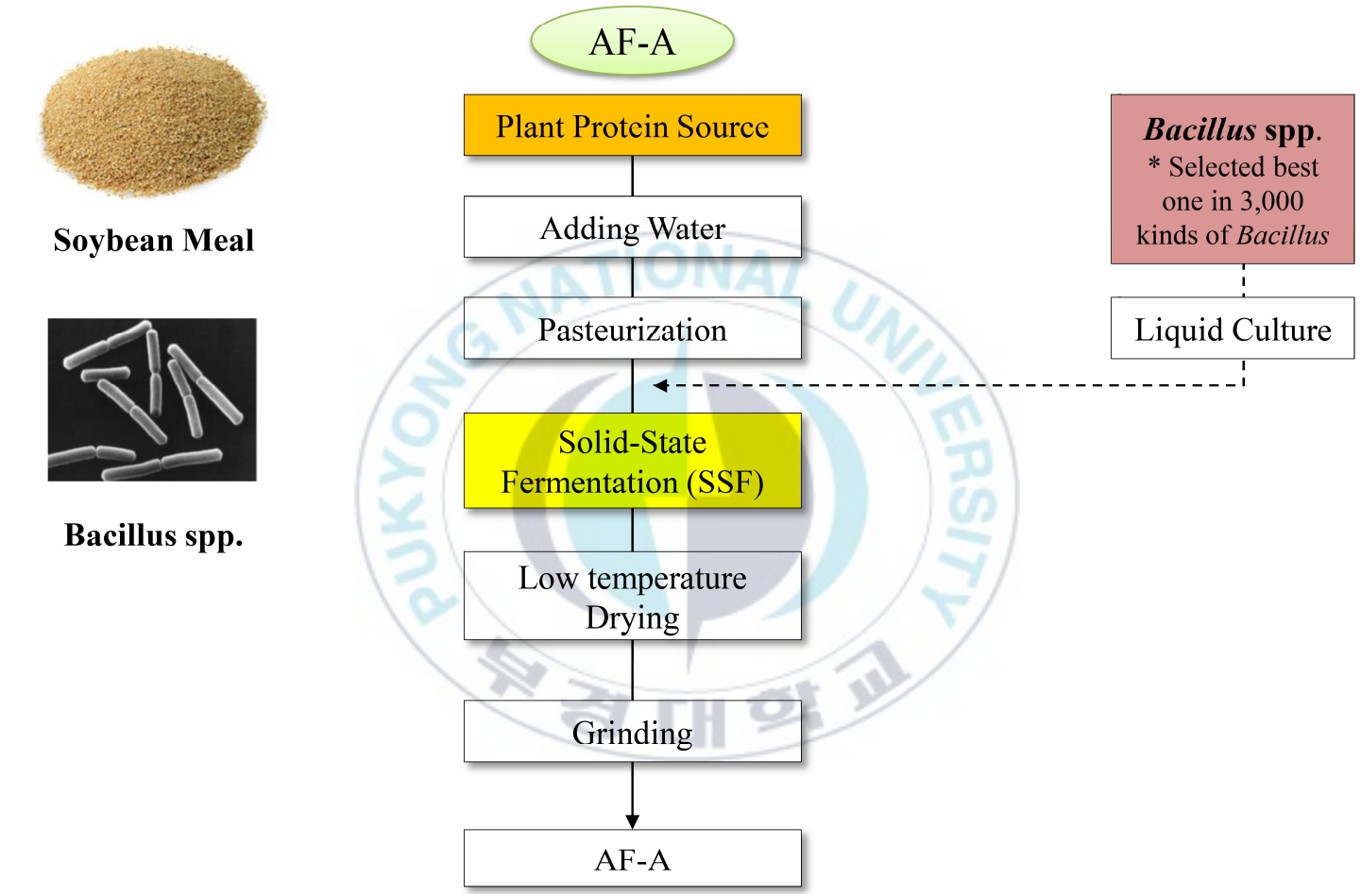


Figure 3-1. Solid state fermentation (SSF) of procedure plant protein ingredients (soybean meal and corn gluten meal) performed by CJ CheilJedang Corporation (Seoul, Republic of Korea).

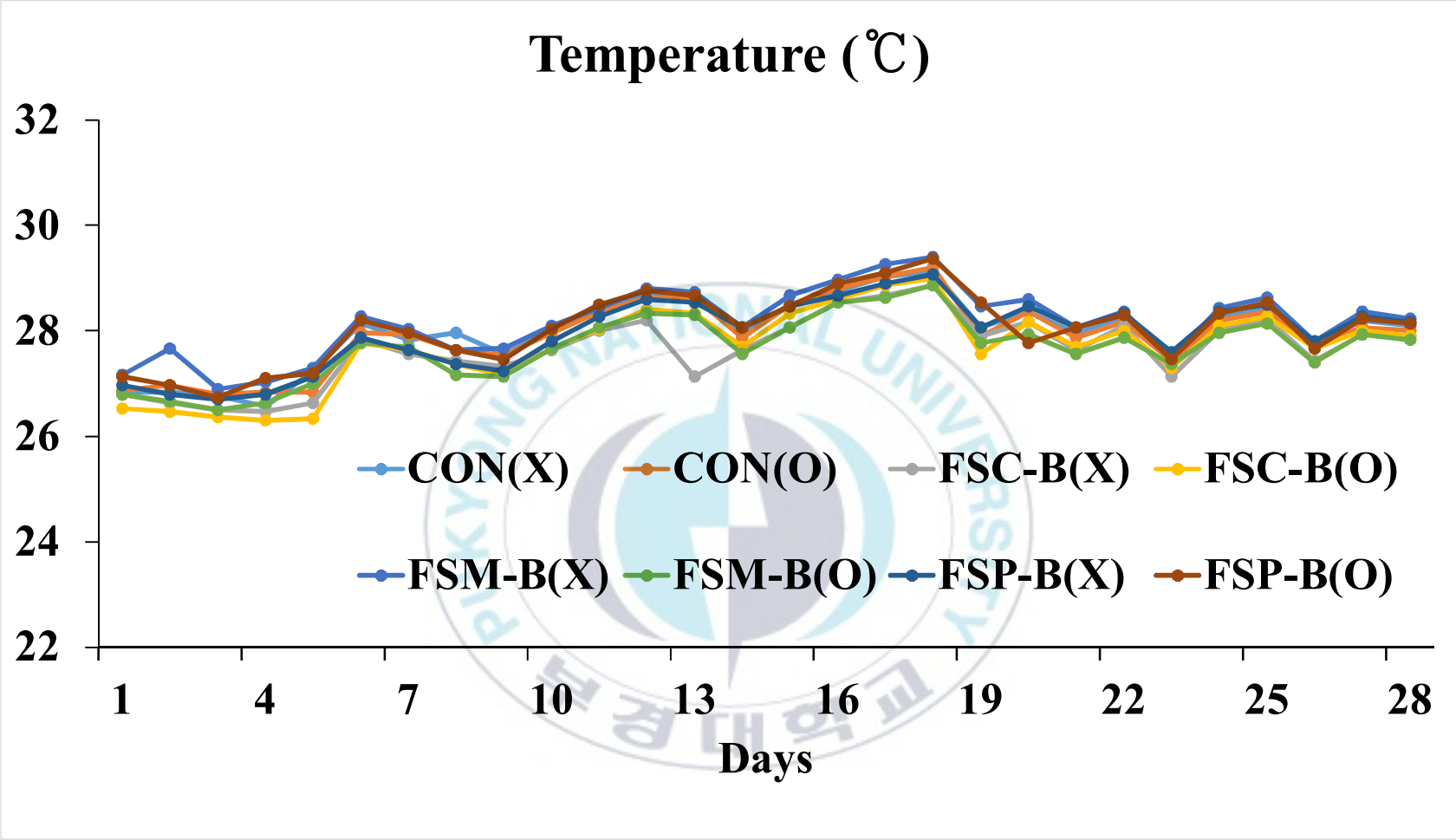


Figure 3-2. Water temperature (℃) of non-recirculating system in juvenile whiteleg shrimp fed experimental diets for 4 weeks; for diet composition information refer to Table 2-1

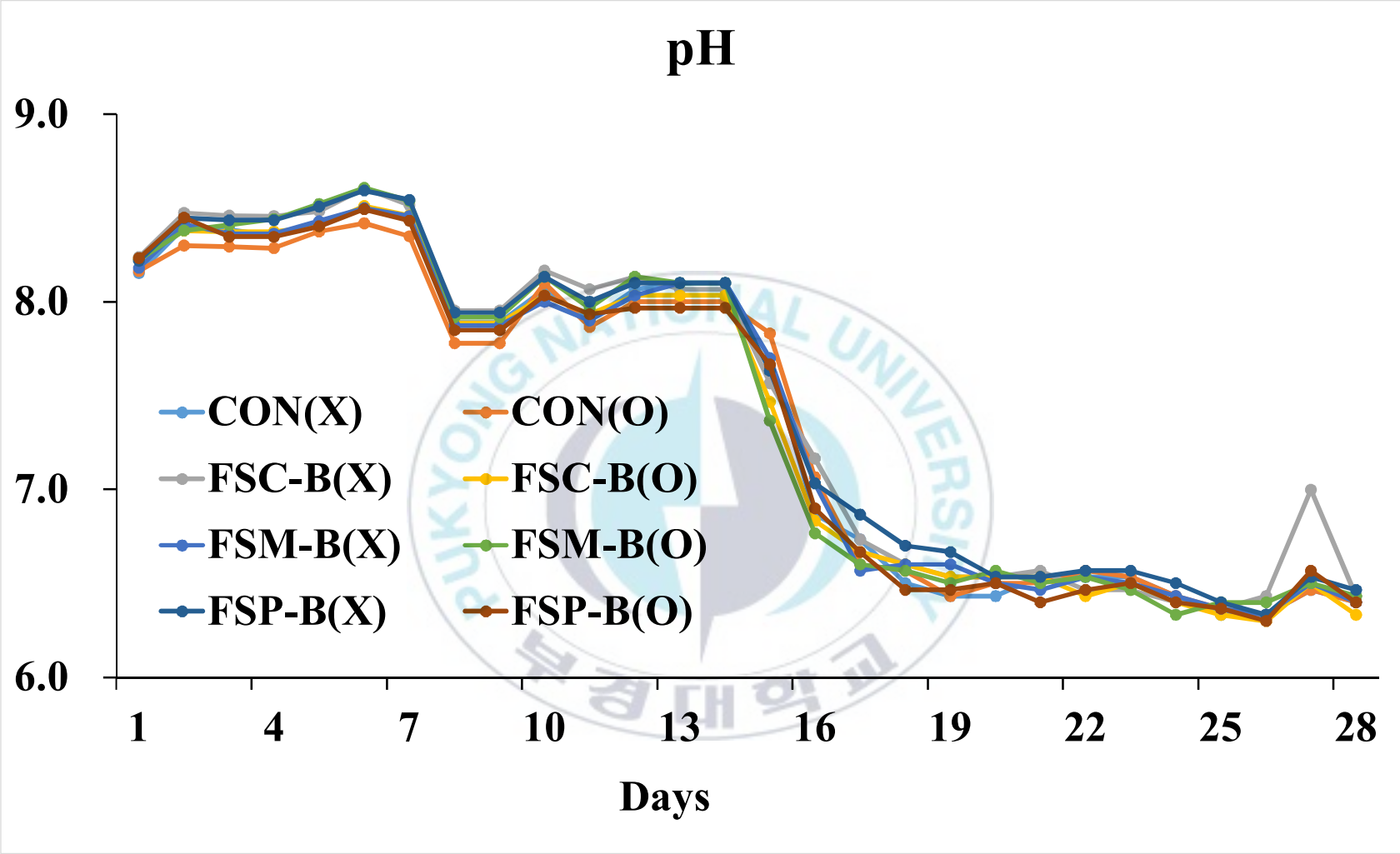


Figure 3-3. Water pH value of non-recirculating system in juvenile whiteleg shrimp fed experimental diets for 4 weeks; for diet composition information refer to Table 2-1

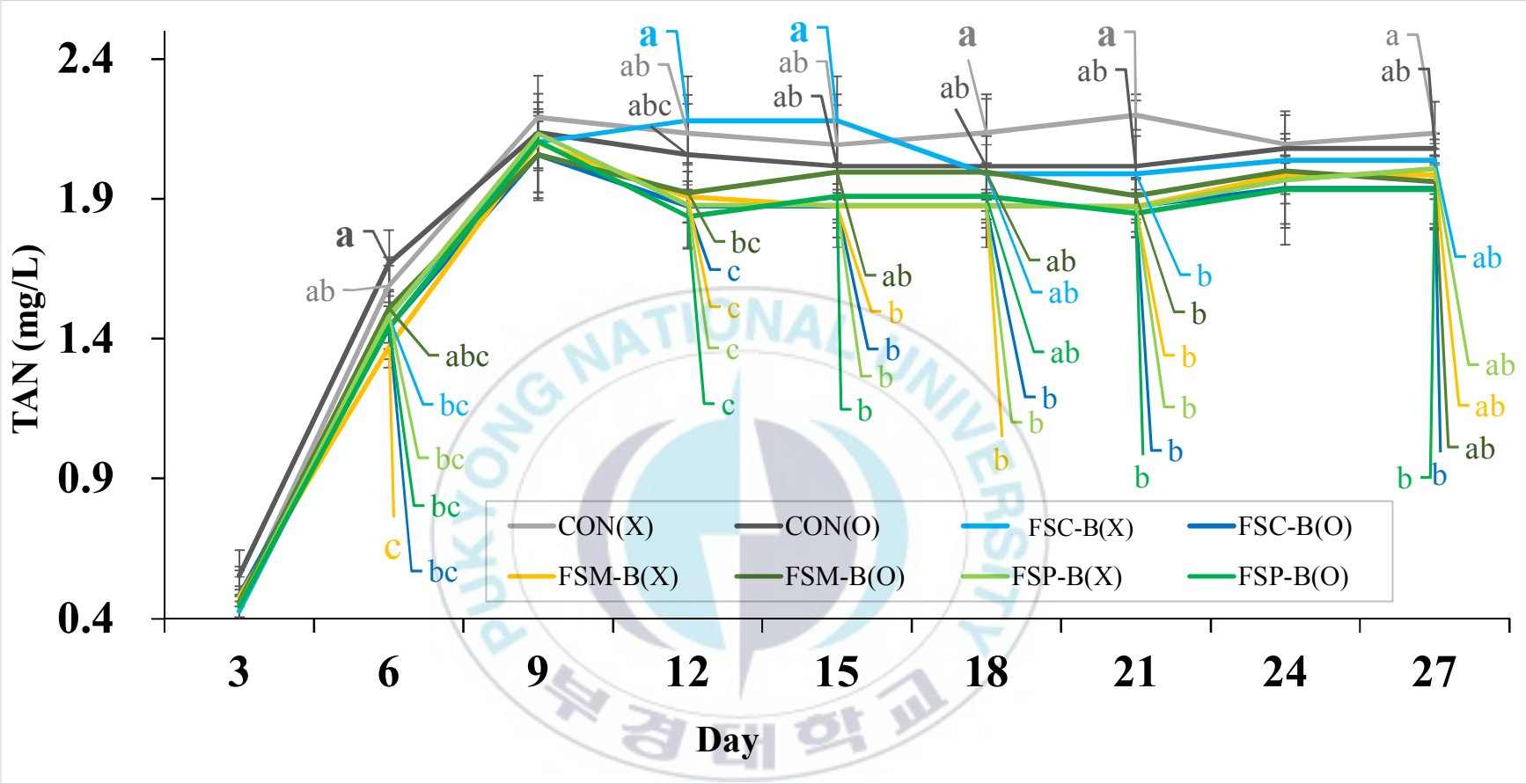


Figure 3-4. Water TAN value of non-recirculating system in juvenile whiteleg shrimp fed experimental diets for 4 weeks; for diet composition information refer to Table 2-1

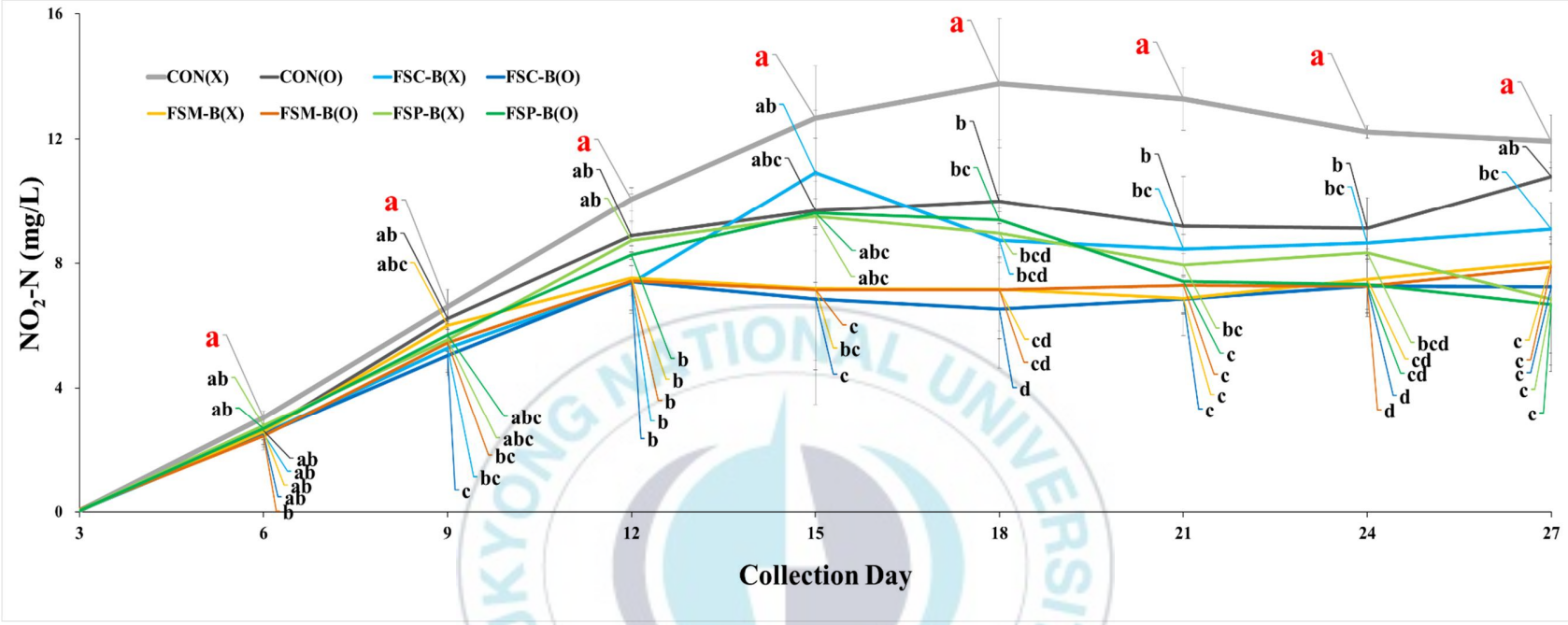


Figure 3-5. Water NO_2-N value of non-recirculating system in juvenile whiteleg shrimp fed experimental diets for 4 weeks; for diet composition information refer to Table 2-1

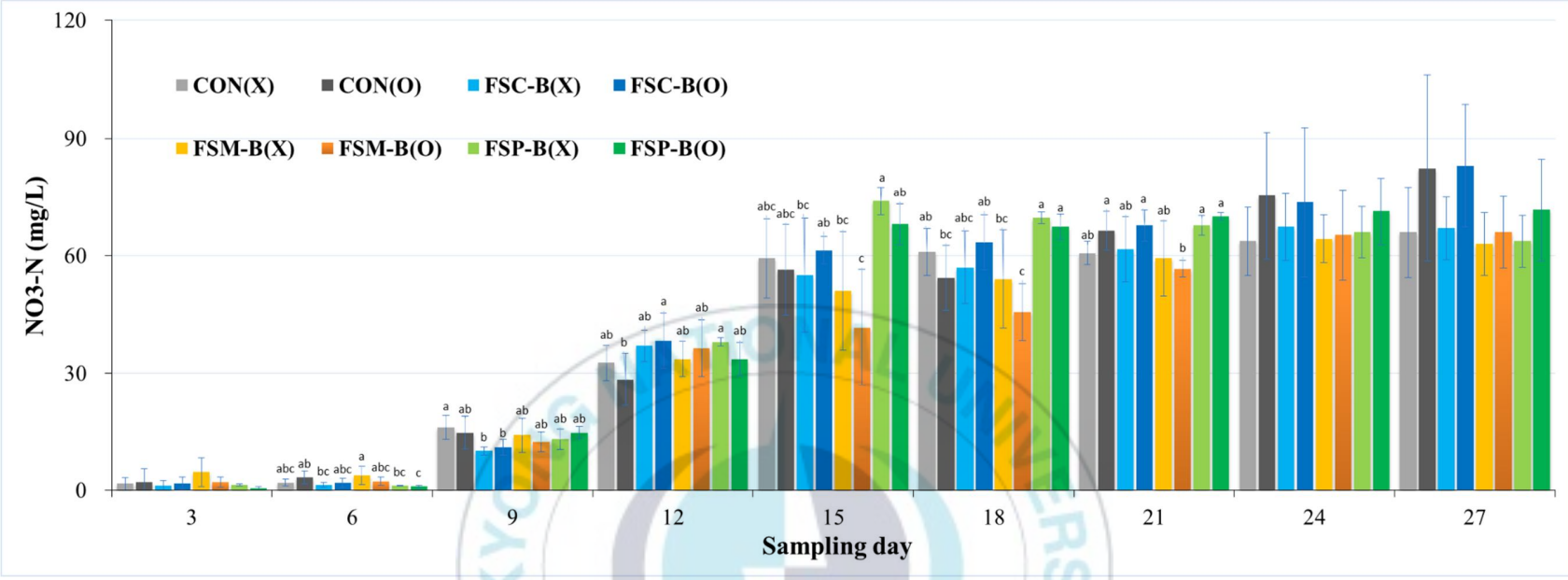


Figure 3-6. Water NO_3-N value of non-recirculating system in juvenile whiteleg shrimp fed experimental diets for 4 weeks; for diet composition information refer to Table 2-1

CHAPTER IV. General conclusions and future research directions

1. General discussions

The main goal of the present study was to replace an expensive and unsustainable ingredient such as fish meal (FM) with practical alternative plant protein sources. The potential plant protein ingredients should be able to cover the nutritional requirements of shrimp while reducing the end price of feed. A lot of feed ingredients have been tested to replace fish-meal in the diet of shrimp, such as poultry by-product meal, shrimp meal, meat and bone meal, blood meal, soybean meal, peanut meal, corn gluten meal and soy protein concentrate (SPC) (Amaya et al., 2007, Liu et al., 2013, Sookying et al., 2013, Yang et al., 2009). Plant proteins have been and will probably continue being the main choice due to the low price and large production. However plant proteins have several nutritional drawbacks compared to fish-meal: a relatively low content of proteins, vitamins and minerals, unbalanced AA composition, and the presence of anti-nutritional components (protease inhibitors, phytic acid, saponins tannins, lectins, non-starch polysaccharides, alkaloids etc.) which will reduce the digestion or absorption of nutrients, counteract the function of vitamins and may even induce toxicity (Francis et al., 2001, Gatlin et al., 2007, Krogdahl et al., 2010). Soybean meal is one of the most widely used plant protein ingredients in the feed of shrimp, four of the main anti-nutritional factors in it are trypsin inhibitors, saponins, non-starch polysaccharides and phytic acid (Francis et al., 2001). In a previous study by Moniruzzaman et al. (2017), obtained the protein profile of soybean meal, corn gluten meal and fermented soybean meal and corn gluten meal with *Bacillus* spp. (FSC_B) by bio-analyzing the urea extracts. The results showed several antinutritional factors for soybean meal such as β-conglycinin, glycinin and trypsin inhibitor. The three major bands at MW c.a. 80, 75 and 50 kDa correspond to α`-, α- and β-subunits of β-conglycinin. Moreover, three major bands at MW c.a. 35, 32 and 20 kDa and one minor band at MW c.a. 39 kDa correspond to acidic and basic polypeptides of glycinin. The main protein form of CGM corresponds to zein, which showed two major bands at

MW c. a. 19 and 22 kDa. In contrast, FSC_B had a different protein profile with several bands only below 15 kDa.

Several studies have evaluated the replacement of FM by plant proteins in the diet of whiteleg shrimp *Litopaeneus vannamei*. Xie et al. (2016), conducted an experiment to evaluate the effect of FM replacement on growth performance, whole body composition, apparent digestibility

coefficients and anti-oxidative ability of juvenile whiteleg shrimp. Results showed that soy protein concentrate and soybean meal based protein blend were able to replace 40% FM without adverse effects of growth, immunity or digestibility performance. In another study, 25% of FM was successfully replaced with *Lactobacillus* spp.-fermented soybean meal and no differences in growth performance, feed utilization and survival was observed with the control diet (Lin and Mui 2017). Van Nguyen et al. (2017), conducted an experiment with four isonitrogenous and isocaloric diets formulated to contain graded levels of dietary fishmeal (0 g/kg, 150 g/kg, 300 g/kg and 450 g/kg) substituted by *Bacillus* spp.-fermented soybean meal in whiteleg shrimp. They found no significant differences in survival and feed conversion ratio. But broken-line regression for weight gain showed the optimum level of fishmeal replaced by *Bacillus* spp.-fermented soybean meal in diet was about 25% without adverse effects on growth and feed utilization of whiteleg shrimp. In another study by Shiu et al. (2015), they were able to reduce the amount of FM in the diet of whiteleg shrimp from 56% to 33% using solid-state fermented soybean meal with *Bacillus subtilis* E20. It is known form previous studies that different types of prospected plant proteins can replace 25-40% of FM in the diet of whiteleg shrimp. Although this amount highly depends on shrimp size, culture condition and feed formulation and price. Based on these studies and the commercial shrimp diet produced in Republic of Korea, 30% of FM was replaced in the diet of whiteleg shrimp in the present study.

Our results for growth performance showed that 30% replacement of FM was possible by all types of plant protein sources in the diet of whiteleg shrimp. Weight gain and specific growth rate of whiteleg shrimp fed the control diet (FM diet) was similar to those of shrimp fed fermented soybean meal with *Bacillus* spp. and sterilized (FSM_{BS}), fermented soybean meal and corn gluten meal with *Bacillus* spp. and sterilized (FSC_{BS}), fermented soy protein concentrate with *Bacillus* spp. (FSP_B) and fermented soybean meal with lactic acid (FSM_L). Although, shrimp fed the diets containing fermented soybean meal with *Bacillus* spp. (FSM_B) and fermented soybean meal and corn gluten meal with *Bacillus* spp. (FSC_B) had even higher weight gain and specific growth rate than the control (FM) diet. These differences could be due to the presence of live *Bacillus* spp. in FSM_B and FSC_B diets. Application of *Bacillus* spp. as probiotic has brought very promising results for shrimp aquaculture. This bacterium is a nonpathogenic Gram positive spore-forming which has been used to improve the growth performance and also shrimp health and disease management (Balcazar et al. 2007; Keysami et al. 2011). In addition it is well documented that *Bacillus* species

are able to produce a wide range of extracellular substances and antimicrobial peptides against variety of microorganisms (Perez et al. 1993; Korenblum et al. 2005). Probiotics are viable cell preparations that have beneficial effects on the health of a host by improving its intestinal balance via improved feed value, enzymatic contribution to digestion, inhibition of pathogenic micro-organisms, anti-mutagenic and anti-carcinogenic activities, growth-promoting factors, and an increased immune response (Verschuere et al. 2000). A traditional Japanese foods, natto, is a fermented soybean product produced by a bacterium, *B. subtilis*. It has many health benefits including helping to prevent cardiovascular disease (Mine et al. 2005), cancer (Wang et al. 2007), intestinal diseases, and osteoporosis (Yamaguchi et al. 2000), which are due to various compounds that are produced by B. subtilis. The genus *Bacillus* has been used as putative probiotics in aquaculture, e.g. administering *Bacillus* as probiotics in the shrimp, *Penaeus monodon*, results in improvements in growth, survival, and immune states (Rengpipat et al. 1998, 2000). In the present study, the results observed for growth performance were further supported by feed utilization parameters such as feed efficiency and protein efficiency ratio. Although differences were not huge in feed utilization parameters, shrimp fed the fermented soy protein concentrate with *Bacillus* spp. (FSP_B) had lower values than shrimp fed the FSC_B diet. To assure optimal growth and feed efficiency, aquafeeds must be agreeable in flavour and acceptable in taste to the farmed shrimp. This guarantees the maximization of feed intake and the minimization of feed waste. Although it might be difficult to determine flavour perception and preference in shrimp (Lamb 2001), it is possible to quantify differences in the amounts of feed eaten (Jobling et al. 2001) and hence estimate feed palatability. From a shrimp sensorial viewpoint, it is known that shrimp are primarily attracted by a variety of nitrogen-containing compounds composed of free amino acids, nucleotides and nucleosides and quaternary ammonium bases and to a minor extent by other non-nitrogenous compounds, such as glucose, lactic acid and some alcohols (de la Higuerra 2001; Kasumayan & Døving 2003). Soy protein concentrate is made by removing a portion of the carbohydrates (sugars) from dehulled and defatted soybeans. Normally, about 750 kilograms of soybean protein concentrate can be obtained from one metric ton of defatted soybean flakes. There are different soy protein production methods. The most frequent method used is alcohol extraction although this method results in most loss of the soy isoflavones. Probably this is the reason for lower feed utilization of shrimp fed the soy protein concentrate diets.

The non-specific immune responses were determined in the present study. To resist the environmental stress, maintain health and ensure optimal survival, cultured fish and shrimp should benefit from optimized antioxidant and immune mechanisms (Castex et al. 2010; Li and Chen, 2008; Liu and Chen, 2004). An adaptive immune system is absent in crustaceans, thus non-specific immune responses are indispensable for defensive mechanisms against pathogens in shrimp (Vazquez et al. 2009). The SOD enzyme can control the reactive oxygen species in cells and catalyze the dismutation of hydrogen peroxide and normal oxygen from superoxide radicals (Fattman et al. 2003; Holmblad and Soderhall, 1999; Shao et al., 2010). On the other hand, the humoral innate immune response releases lysozyme which combats infections (Campa-Cordova et al. 2002). The results for lysozyme activity of shrimp fed the experimental diet corresponded to the presence of live Bacillus spp. in the diet. Shrimp fed the FSM_B and FSC_B diets had higher lysozyme activity than those of shrimp fed the CON and FSM_L diets. This could be related to the activity of *Bacillus* spp. by altering the immune responses. In a recent study by Lee et al. (2018), an increasing trend in lysozyme activity corresponding with the dietary *Bacillus* spp. inclusion in the diet of Japanese eel was observed. In line with this, Ye et al. (2011) reported the significant beneficial impact of *Bacillus* spp. on the lysozyme activity of Japanese flounder.

The farmed shrimp industry has experienced serious risks among which shrimp diseases caused by opportunistic bacteria such as *Vibrio* sp. and viruses are major problems that can lead to huge economic losses (Song et al. 1993; Liu et al. 2004; Teng et al. 2006). The results for eight days of challenge test against *Vibrio parahaemolyticus* corresponded with the presence or absence of live *Bacillus* spp. in feed. Differences is survival rate were observed from day three and shrimp fed the FSPB, FSCB and FSMB diets had significantly higher survival than those of shrimp fed the CON, FSC_{BS} and FSM_L. These differences were maintained until day six as more shrimp were dying due to pathogenic bacteria. At the final day, all the shrimp fed diets CON, FSM_L and SPC were dead and those shrimp fed the FSC_B had higher survival rate. There are some reports regarding the advantages of using probiotics in shrimp aquaculture which were reviewed by Gatesoupe (1999) and Farzanfar (2006). The beneficial effects of probiotic use in shrimp aquaculture are in the biological control of disease through improved shrimp immunity (Chiu et al. 2007; Tseng et al. 2009], pathogen inhibition (Vaseeharan and Ramasamy 2003; Decamp et al. 2008), and shrimp growth performance (Liu et al. 2009; Rengpipat et al. 200; Wang 2007). Among probiotics, *Bacillus* sp. has become more popular and widely used in a growing number of studies in shrimp

aquaculture. A high protease producing probiotic, *B. subtilis* E20 from the human health food, natto, was demonstrated to be beneficial to white shrimp, *L. vannamei*, immune response and disease resistance to the pathogenic bacterium (Tseng et al. 2009), and growth performance through improving its food digestion and absorption in the growthout phase. In addition, *B. subtilis* E20 was demonstrated to be a generally recognized as safe (GRAS) organism for *L. vannamei* (Liu et al. 2009). Our observations in this study could be supported by the intestinal *Bacillus* spp. count of shrimp fed the experimental diets. Shrimp fed diets containing unsterilized *Bacillus* spp.-fermented plant proteins (FSM_B, FSC_B and FSP_B) had significantly higher intestinal Bacillus spp. count. Administration of *B. subtilis* in *L. vannamei* larval breeding resulted in accelerated larval development and an increased larval survival rate compared to larvae in the control (Liu et al. 2010). Giant fresh water prawn, *Macrobrachium rosenbergii*, zoea larvae had a higher survival and a faster rate of metamorphosis when fed *B. subtilis* treated *Artemia* naupli compared to zoea larvae fed untreated *Artemia* (Keysami et al. 2007). Similarly, a higher survival rate and better growth of Indian white shrimp, *Fenneropenaeus indicus*, larvae were caused by increases in the activities of some digestive enzymes as investigated by immersion in a commercial probiotic (containing spores of 5 species of *Bacillus* spp.).

Production of fish, marine shrimp, and other species by aquaculture depends upon a supply of high-quality water. In cases of aquaculture projects sited where water quality is naturally impaired or polluted, the water quality limitations must be overcome or the aquaculture projects will fail. Water quality also declines in aquaculture systems because of waste accumulation and, thus, water quality management must be applied to avoid stress and mortality of aquaculture species, and to assure efficient production. Furthermore, the discharges from aquaculture facilities contain nutrients, organic matter, and suspended solids that pollute receiving water bodies. Many governments require aquaculture facilities to implement practices to minimize pollution (Boyd 2012). The purpose of this study was to compare different types of *Bacillus* spp.-fermented plant protein sources as FM replacers in whiteleg shrimp and evaluate the effects on water quality indices. Water temperature was measured every day and it was mostly consistent during the experiment period fluctuating between 26-28 °C in all tanks. Results for pH measurements in all experimental tanks are shown consistency in all tanks. For the first week, pH showed a value between 8-8.5 but as the days went on it dropped until pH 7.8 and it remained consistent until day 14-15. After two weeks of experiment, pH in all tanks dropped significantly until about 6.5. The

water was not changed in this experiment and only evaporated water was added to the tanks. The chemical reactions happening in the tanks due to respiration, ammonia excretion and microorganism activity could have resulted in depressed pH. The removal of nitrogen by biological nitrification and denitrification is a two-step process. In the first step (nitrification), ammonia is converted aerobically to nitrate (NO_3^-). In the second step (denitrification), nitrates are converted to N_2O or nitrogen gas (N_2) under anoxic conditions. Nitrification is an autotrophic process that means that the energy for bacterial growth is derived from the oxidation of nitrogen compounds, primarily ammonia. Both of these processes can produce H^+ in the water body and this will increase the pH level (Halling-Sorensen and Jorgensen. 1993). Total ammonia nitrogen (TAN) of water was influenced by both the dietary treatments and presence/absence of bacterial treatment. After 27 days of experiment, tanks fed with fermented soybean meal with Bacillus spp. and received bacterial treatment had lower TAN level than those tanks fed with CON diet and not treated with bacterial treatment. It is important to provide shrimp with a healthy environment and probiotics has a great deal of potential (Gomez-Gil et al., 2000).Wang et al. (2005) investigated the effect of commercial probiotics on water quality in shrimp, *P. vannamei*, ponds and the results showed that probiotics could significantly reduce the concentrations of nitrogen and phosphorus in pond water compared with the control. In the present study, the amount of NO_2-N in the experimental tanks after 27 days was more influenced by diets compared to the bacterial treatment. The control tanks which were not treated had higher NO_2-N than all other tanks but with no differences with treated control tanks. This could be related to higher digestibility of the fermented plant protein sources. Water quality parameters such as acidity, alkalinity, dissolved oxygen (DO), nitrate and ammonia play vital role in fish production in aquaculture industries. Low water quality (lowDO, high ammonia, high nitrate etc.) enhances the percentage of diseases susceptibility in fish farming industries. Probiotic bacteria not only trigger the defense system in fish, but also improve the water quality (Table 5). Several researchers have stated that probiotics can be used as an eco-friendly bio-control or bioremediation agents for sustainable development in aquaculture (Shariff et al., 2001; Dimitroglou et al., 2011; Iribarren et al., 2012). Until now, the use of probiotic candidates in fish culture ponds are not so popular, but in near future it would be randomly used in aquaculture industries and local fish farming sectors.

2. Future research directions

Further studies are required in evaluating different types of fermentation methods for soybean meal and corn gluten meal. The fermentation methods could be based on experiments with different types of bacteria, different methods of fermentation and different types of products. Also, other sources of plant proteins that have lower price than soybean meal or corn gluten meal are encouraged to be tested as potential fish meal replaces. The potential plant protein sources should have reasonable price and available throughout the year. *Bacillus* spp.-fermented plant protein sources can be used in fish and shrimp culture in recirculating aquaculture systems. Comparing *Bacillus* spp.-fermented plant protein sources in closed and flow-through systems could be interesting for water quality experiments.

3. References

Amaya E., Davis D.A., Rouse D.B. 2007. Alternative diets for the Pacific white shrimp *Litopenaeus vannamei*. Aquaculture 262:419-425.

Balcazar J.L., Rojas-Luna T. 2007. Inhibitory activity of probiotic Bacillus subtilis UTM 126 against vibrio species confers protection against vibriosis in juvenile shrimp (*Litopenaeus vannamei*). Curr Microbiol 55:409-12.

Boyd C. 2012. Water quality. In: Aquaculture, farming aquatic animals and plants (eds: Lucas J. S. and P. C. Southgate). Willey-blackwell, UK.

Campa-Cordova A.I., Hernandez-Saavedra N.Y., Ascencio F. 2002. Superoxide dismutase as modulator of immune function in American white shrimp (*Litopenaeus vannamei*). Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 133:557-565.

Castex M., Lemaire P., Wabete N., Chim L. 2010. Effect of probiotic Pediococcus acidilactici on antioxidant denfence and oxidant stress of *Litopenaeus stylirostris* under *Vibrio nigripulchritudo* challenge. Fish and Shellfish Immunology 28:622-631.

Chiu C.H., Guu Y.K., Liu C.H., Pan T.M., Cheng W. 2007. Immune responses and gene expression in white shrimp, Litopenaeus vannamei, induced by *Lactobacillus plantarum*. Fish Shellfish Immunol 23:364-77.

de la Higuerra M. 2001. Effects of nutritional factor and feed characteristics on feed intake. In: Houlihan D, Boujard T, Jobling M (eds) Food Intake in Fish, pp. 250–268. Blackwell Publishing, Oxford.

Decamp O., Moriarty D.J.W., Lavens P. 2008. Probiotics for shrimp larviculture: review of field data from Asia and Latin America. Aquac Res. 39:334-8.

Fattman C.L., Schaefer L.M., Oury T.D. 2003. Extracellular superoxide dismutase in biology and medicine. Free Radical Biology and Medicine 35:236-256.

Francis G., Makkar H.P., Becker K. 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture 199:197-227.

Farzanfar A. 2006. The use of probiotics in shrimp aquaculture. FEMS Immunol Med Microbiol 48:149-58.

Gatesoupe F.J. 1999. The use of probiotics in aquaculture. Aquaculture 180:147-65.

Gatlin D.M., Barrows F.T., Brown P., Dabrowski K., Gaylord T.G., Hardy R.W., Herman E., Hu G., Krogdahl Å., Nelson R. 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquac. Res. 38:551-579.

Gomez-Gil B., Roque A., Turnbull J.F. 2000. The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. Aquaculture 191:259-270.

Halling-Sorensen B., Jorgensen S.E. 1993. Studies in Environmental Science. Elsevier B.V.

Holmblad, T., & Soderhall, K. 1999. Cell adhesion molecules and antioxidative enzymes in a crustacean, possible role in immunity. Aquaculture, 172:111-123.

Jobling M., Cove´s D., Damsgard B., Kristiansen H.R., Koskela J., Petursdottir T.E. 2001. Techniques for measuring feed intake. In: Houlihan D, Boujard T, Jobling M (eds) Food Intake in Fish, pp. 49–87. Blackwell Science, Oxford.

Kasumayan A.O., Døving K.B. 2003. Taste preferences in fishes. Fish Fisheries 4:289-347.

Keysami M.A., Saad C.R., Sijam K., Daud H.M., Alimon A.R. 2007. Effect of Bacillus subtilis on growth development and survival of postlarvae *Macrobrachium rosenbergii* (de Man). Aquac Nutr 13:131-6.

Keysami M., Mohammadpour M., Saad C. 2011. Probiotic activity of Bacillus subtilis in juvenile freshwater prawn, *Macrobrachium rosenbergii* (de Man) at different methods of administration to the feed. Aquacult Int. 1-13.

Korenblum E, von Der Weid I, Santos ALS, Rosado AS, Sebastián GV, Coutinho CMLM, et al. 2005. Production of antimicrobial substances by Bacillus subtilis LFE-1, B. firmus H2O-1 and B. licheniformis T6-5 isolated from an oil reservoir in Brazil. J Appl Microbiol 98:667-75.

Krogdahl Å., Penn M., Thorsen J., Refstie S., Bakke A.M. 2010. Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. Aquac. Res. 41:333–344.

Lamb C.F. 2001. Gustation and feeding behaviour. In: Houlihan D, Boujard T, Jobling M (eds) Food Intake in Fish, pp. 108-130. Blackwell Science, Oxford.

Lee S., Katyac K., Hamidoghli A., Hong J., Kim D., Bai S.C. 2018. Synergistic effects of dietary supplementation of Bacillus subtilis WB60 and mannanoligosaccharide (MOS) on growth performance, immunity and disease resistance in Japanese eel, *Anguilla japonica*. Fish & shellfish immunology 83:283-291.

Li C., Chen J. 2008. The immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus* under low and high pH stress. Fish and Shellfish Immunology 25:701-709.

Lin Y., Mui J. 2017. Comparison of dietary inclusion of commercial and fermented soybean meal on oxidative status and non-specific immune responses in white shrimp, *Litopenaeus vannamei.* Fish & Shellfish Immunology 63:208-212.

Liu C.H., Chiu C.S., Lin P.L., Wang S.W. 2009. Improvement in the growth performance of white shrimp, *Litopenaeus vannamei*, by a protease producing probiotic, *Bacillus subtilis* E20 from natto. J Appl Microbiol 107:1031-41.

Liu C.H., Chen J.C. 2004. Effect of ammonia on the immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus*. Fish and Shellfish Immunology 16:321-334.

Liu X., Ye J., Kong J., Wang K., Wang A. 2013. Apparent digestibility of 12 protein-origin ingredients for Pacific white shrimp *Litopenaeus vannamei*. N. Am. J. Aquac. 75:90-98.

Liu K., Chiu C., Shiu Y., Cheng W., Liu C. 2010. Effects of the probiotic, Bacillus subtilis E20, on the survival, development, stress tolerance, and immune status of white shrimp, *Litopenaeus vannamei* larvae. Fish & Shellfish Immunology 28:837-844.

Mine Y., Wong A.H.K. Jiang B. 2005. Fibrinolytic enzymes in sian traditional fermented foods. Food Res Inst 38:243-250.

Moniruzzaman M., Bae J.H., Won S.H., Cho S.J., Chang K.H., Bai S.C. 2017. Evaluation of solid-state fermented protein concentrates as a fish meal replacer in the diets of juvenile rainbow trout, *Oncorhynchus mykiss*. Aquaculture nutrition 24:1198-1212.

Perez C., Suarez C., Castro G. 1993. Antimicrobial activity determined in strains of *Bacillus circulans* cluster. Folia Microbiol 38:25-8.

Rengpipat S., Phianphak W., Piyatiratitivorakul S. Menasvetam P. 1998. Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture 167:301-313.

Rengpipat S., Rukpratanporn S., Piyatiratitivorakul S., Menasaveta P. 2000. Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11). Aquaculture 191:271-288.

Shao X.P., Liu W.B., Xu W.N., Lu K.L., Xia W., Jiang Y.Y. 2010. Effects of dietary copper sources and levels on performance, copper status, plasma antioxidant activities and relative copper bioavailability in *Carassius auratus gibelio*. Aquaculture 308:60-65.

Shiu Y., Wong S., Guei W., Shin Y., Liu C. 2015. Increase in the plant protein ratio in the diet of white shrimp, *Litopenaeus vannamei* (Boone), using *Bacillus subtilis* E20-fermented soybean meal as a replacement. Aquaculture research 46:382-394.

Song YL, Cheng W, Wang CH. 1993. Isolation and characterization of Vibrio damsel infectious for cultured shrimp in Taiwan. J Invert Pathol 61:24-31.

Sookying D., Davis D.A., Soller Dias Da Silva F. 2013. A review of the development and application of soybean-based diets for Pacific white shrimp *Litopenaeus vannamei*. Aquac. Nutr. 19:441-448.

Teng P.H., Lee P.Y., Lee F.C., Chien H.W., Chen M.S., Sung P.F. 2006. Detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in *Litopenaeus vannamei* by ramification amplification assay. Dis Aquat Organ 73:103-11.

Tseng D.Y., Ho P.L., Huang S.Y., Cheng S.C., Shiu Y.L., Chiu C.S. 2009. Enhancement of immunity and disease resistance in the white shrimp, Litopenaeus vannamei, by the probiotic, *Bacillus subtilis* E20. Fish Shellfish Immunol 26:339-44.

Vaseeharan B., Ramasamy P. 2003. Control of pathogenic Vibrio spp. By *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Lett Appl Microbiol 36:83-7.

Vazquez L., Alpuche J., Maldonado G. 2009. Immunity mechanisms in crustaceans. Innate Immunity 15:179-188.

Verschuere L., Rombaut G., Sorgeloos P. Verstraete W. 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol Rev 64:655-671.

Wang Y.B. 2007. Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. Aquaculture 269:259-64.

Wang Y.B., Xu Z.R., Xia M.S. 2005. The effectiveness of commercial probiotics in Northern White Shrimp (*Penaeus vanname* iL.) ponds. Fish. Sci. 71:1034-1039.

Wang C.L., Ng T.B., Yuan F., Liu Z.K. Liu F. 2007. Induction of apoptosis in human leukemia K562 cells by cyclic lipopeptide from Bacillus natto T-2. Peptides 28:1344-1350.

Xie S., Liu Y., Zeng S., Niu J., Tian L. 2016. Partial replacement of fish-meal by soy protein concentrate and soybean meal based protein blend for juvenile Pacific white shrimp, *Litopenaeus vannamei*. Aquaculture 464:296-302.

Yamaguchi M., Gao T.Y., Igarashi A. Tsukamoto Y. 2000. Prolonged intake of fermented soybean (natto) diets containing vitamin K2 (menaquinone-7) prevents bone loss in ovariectomized rats. J Bone Miner Metab 18:71-76.

Yang Q., Zhou X., Zhou Q., Tan B., Chi S., Dong X. 2009. Apparent digestibility of selected feed ingredients for white shrimp Litopenaeus vannamei, Boone. Aquac. Res. 41:78-86.

Ye J.D., Wang K., Li F.-D. 2011. Single or combined effects of fructo- and mannan oligosaccharide supplements and *Bacillus clausii* on the growth, feed utilization, body composition, digestive enzyme activity, innate immune response and lipid metabolism of the Japanese flounder *Paralichthys olivaceus*, Aquacult. Nutr. 17:e902-e911.

CHAPTER V. Appendix

Raw data for growth performance of shrimp fed the experimental diets for 8 week

	Tank No.	Initial weight	Final Weight	WG	WG avg.	Total feed	SGR
CON	1	0.51	7.80	1428	1439	251.27	4.70
	15	0.50	7.10	1320		271.12	4.57
	26	0.48	8.01	1568		269.14	4.85
FSM_B	4	0.49	7.57	1445	1790	261.83	4.72
	18	0.51	11.11	2079		281.12	5.31
	28	0.49	9.54	1846		228.46	5.12
FSP_B	7	0.52	8.44	1523	1617	282.36	4.80
	25	0.49	8.88	1711		273.51	4.99
	29	0.50	8.66	1617		295.06	4.92
FSC_B	10	0.49	8.92	1720	1756	238.62	5.00
	17	0.50	9.95	1890		275.86	5.16
	22	0.50	8.79	1658		206.00	4.94
SPC	13	0.50	9.21	1741	1619	237.98	5.02
	16	0.51	7.30	1331		249.38	4.59
	19	0.50	9.42	1784		256.56	5.06
FSM_{BS}	3	0.49	7.76	1484	1480	268.61	4.76
	14	0.50	7.91	1481		285.03	4.76
	23	0.51	8.04	1476		301.57	4.75
FSC_{BS}	6	0.50	7.77	1455	1560	259.44	4.73
	11	0.51	8.58	1582		227.32	4.87
	24	0.51	8.89	1644		222.29	4.93
FSM_L	2	0.49	7.35	1399	1425	217.67	4.67
	9	0.52	7.85	1410		270.70	4.68
	21	0.51	7.99	1466		249.15	4.74

Water TAN value of non-recirculating system in juvenile whiteleg shrimp fed experimental diets for 4 weeks

	3	6	9	12	15	18	21	24	27
CON(X)	0.46	1.59	2.19	2.13	2.09	2.14	2.20	2.09	2.13
CON(O)	0.55	1.67	2.14	2.06	2.02	2.02	2.02	2.08	2.08
FSC_B(X)	0.43	1.48	2.10	2.18	2.18	1.99	1.99	2.04	2.04
FSC_B(O)	0.49	1.44	2.06	1.87	1.87	1.87	1.87	1.94	1.94
FSM_B(X)	0.48	1.37	2.10	1.91	1.87	1.87	1.87	1.99	1.99
FSM_B(O)	0.46	1.51	2.06	1.92	1.99	1.99	1.91	2.00	1.96
FSP_B(X)	0.45	1.48	2.13	1.88	1.88	1.88	1.87	1.97	2.01
FSP_B(O)	0.44	1.44	2.11	1.84	1.91	1.91	1.85	1.93	1.93

Water NO_2-N value of non-recirculating system in juvenile whiteleg shrimp fed experimental diets for 4 weeks.

	3	6	9	12	15	18	21	24	27
CON(X)	0.05	3.03	6.60	10.07	12.67	13.78	13.29	12.23	11.93
CON(O)	0.05	2.60	6.23	8.89	9.71	10.00	9.19	9.13	10.80
FSC_B(X)	0.04	2.50	5.28	7.37	10.93	8.74	8.46	8.65	9.10
FSC_B(O)	0.07	2.49	5.04	7.41	6.86	6.54	6.86	7.28	7.25
FSM_B(X)	0.04	2.58	6.01	7.53	7.20	7.17	6.87	7.49	8.04
FSM_B(O)	0.08	2.44	5.46	7.43	7.15	7.15	7.30	7.27	7.88
FSP_B(X)	0.06	2.77	5.55	8.74	9.52	8.96	7.96	8.34	6.84
FSP_B(O)	0.04	2.68	5.70	8.27	9.63	9.40	7.42	7.32	6.69

Water NO_3-N value of non-recirculating system in juvenile whiteleg shrimp fed experimental diets for 4 weeks.

	3	6	9	12	15	18	21	24	27
CON(X)	1.63	1.97	16.00	32.67	59.33	61.00	60.67	63.67	66.00
CON(O)	2.10	3.30	14.67	28.33	56.33	54.33	66.33	75.33	82.33
FSC_B(X)	1.13	1.40	10.00	37.00	55.00	57.00	61.67	67.33	67.00
FSC_B(O)	1.77	1.93	11.00	38.33	61.33	63.33	67.67	73.67	83.00
FSM_B(X)	4.63	3.77	14.00	33.67	51.00	54.00	59.33	64.33	63.00
FSM_B(O)	2.00	2.30	12.33	36.33	41.67	45.67	56.67	65.33	66.00
FSP_B(X)	1.37	1.23	13.00	38.00	74.00	69.67	67.67	66.00	63.67
FSP_B(O)	0.43	0.97	14.67	33.67	68.00	67.33	70.00	71.33	71.67