Thesis for the Degree of Doctor of Philosophy

Isolation and evaluation of dietary probiotics in whiteleg shrimp, *Litopaeneus vannamei* and Nile tilapia, *Oreochromis niloticus*

by

Seonghun Won

Department of Fisheries Biology

The Graduate School

Pukyong National University

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흰다리새우 및 나일 틸라피아에 있어 프로바이오틱스 분리 및 평가

Advisor: Prof. Sungchul C. Bai

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Seonghun Won

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Isolation and evaluation of dietary probiotics in whiteleg shrimp, *Litopaeneus vannamei* and Nile tilapia, *Oreochromis niloticus*

A dissertation

by

Seonghun Won

Approved as to style and content by:

(Chairman) In-Soo Kong

(Member) Chang-Hoon Kim

(Member) Jun-Ho Lee

(Member) Su-Hong Ko

(Member) Sungchul C. Bai

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TABLE OF CONTENTS

Isolation and evaluation of dietary probiotics in whiteleg shrimp, *Litopaeneus vannamei* and Nile tilapia, *Oreochromis niloticus*

Seonghun Won

Department of Fisheries Biology, Graduate School, Pukyong National University

Abstract

Three feeding trials were conducted to evaluate the effects of dietary probiotics on the growth performance, immune responses, hematology, enzyme activity, disease resistance and gene expression in Nile tilapia, *Oreochromis niloticus* and whiteleg shrimp, *Litopenaeus vannamei*. In experiment 1st and 2nd, the probiotic which was isolated from Japanese eel, *B. subtilis*, was compared with different dietary probiotics and investigated as an antibiotic replacer in whiteleg shrimp and nile tilapia, respectively. In the 1st experiment results indicated that *B. subtilis*, *Pediococcus pentosaceus* and *Lactococcus lactis* (isolated from whiteleg shrimp) at 1×10^8 CFU/g diet could improve the growth performance, immune responses, hematology, enzyme activity, disease resistance and gene expression, and could be a potential antibiotic replacer in whitleg shrimp. In the 2nd study, among *B. subtilis*, *B. licheniformis* (isolated from whiteleg shrimp), *L. lactis* (isolated from mice), and *Micrococcus luteus* (isolated from Nile tilapia), *B. subtilis* and *L. lactis* at the level of 1×10^8 CFU/g diet could improve the growth performance, immune responses, hematology, enzyme activity, disease resistance and gene expression, and be recognized as a potential antibiotic replacer in juvenile Nile tilapia. In the 3rd experiment, comparative evaluation of dietary probiotics with *B. subtilis* and *E. faecium* which was isolated from the intestine of Japanese eel and Nile tilapia respectively, were evaluated on the growth performance, immune responses, hematology, enzyme activity, disease resistance and gene expression in Nile tilapia, *Oreochromis niloticus*. In the 3rd experiment demonstrated that *B. subtilis* at 10^8 CFU/g and *E. faecium* at $10^{7\sim8}$ CFU/g diets could have an ideal probiotics in terms of

the growth performance, immune responses, hematology, enzyme activity, disease resistance and gene expression, while replacing the use of antibiotics in Nile tilapia.

First Experiment:

Effects of three different dietary probiotics on growth, immune responses, histology and gene expression in whiteleg shrimp, *Litopenaeus vannamei*

This study evaluated the effects of different dietary probiotic supplementations in juvenile whiteleg shrimp, *Litopenaeus vannamei*. A diet without supplementation of probiotics was used as control (CON); five other diets were prepared by supplementing *B. subtilis* at 1×10^7 CFU/g, *B. subtilis*, *P. pentosaceus* and *L. lactis* at 1×10^8 CFU/g (BS_8, PP_8 and LL_8, respectively), and oxytetracycline at 4g/kg of diet (OTC). Shrimp with initial body weight of 1.41 ± 0.05 g (mean ± SD) were distributed into 18 (40-L) capacity rectangular tanks (20 shrimp/tank), and were fed four times a day (09:00, 13:00, 17:00 and 21:00 h) at 5-6% of wet body weight/day for 8 weeks. Weight gain, specific growth, feed efficiency and protein efficiency ratio of shrimp fed BS_8 and LL_8 diets were significantly higher than those of shrimp fed CON diet ($P<0.05$). However, there were no significant differences between probiotics and OTC diets ($P>0.05$). Lysozyme activity of shrimp fed probiotics and OTC diets significantly improved compare to CON diet ($P<0.05$). Superoxide dismutase activity of shrimp fed BS_8 and PP_8 diets were significantly higher than those of fish fed CON diet ($P<0.05$). However, there were no significant differences between probiotics and OTC diets ($P>0.05$). The villi length of shrimp fed the probiotic-supplemented diets were longer than those of fish fed CON and OTC diets ($P<0.05$). Correspondingly, muscular layer thickness of shrimp fed PP_8 and LL_8 diets better than CON diet ($P<0.05$). However, there were no significant differences between probiotics and OTC diets ($P>0.05$). Expression of serine protein (SP), peroxinectin (PE) and prophenoloxidase (proPO) genes in shrimp fed BS_8, PP_8 and LL_8 diets were significantly higher than those of fish fed CON and OTC diets ($P<0.05$). At the end of 7 days of challenge test, shrimp fed BS_8, PP_8 and LL_8 significantly improved compared to CON diet. Trypsin, lipase and amylase activity of shrimp fed BS_8, PP_8 and LL_8 diets were significantly higher than those shrimp fed CON diet ($P<0.05$). Therefore, these results indicated that *B. subtilis* WB*60*, *P.*

pentosaceus and *L. lactis* at 1×10^8 CFU/g diets could potentially act as probiotics and antibiotics replacer in whiteleg shrimp.

Second Experiment:

Effects of four different dietary probiotics on growth, immune responses, histology and gene expression in Nile tilapia, *Oreochromis niloticus*

This study evaluated the effects of different dietary probiotic supplementations in juvenile Nile tilapia, *Oreochromis niloticus*. A diet without supplementation of probiotics was used as control (CON); seven other diets were prepared by supplementing *B. subtilis*, *B. licheniformis*, *L. lactis*, and *M. luteus* at 1×10^7 CFU/g (BS_7, BL_7, LL_7 and ML_7, respectively), *B. subtilis* and *L. lactis* at 1×10^8 CFU/g (BS_8 and LL_8, respectively), and oxytetracycline at 4g/kg of diet (OTC). Fish with initial body weight of 2.83 ± 0.05 g (mean ± SD) were distributed into 24 (40-L) rectangular tanks (20 fish/tank), and were fed twice daily (09:00 and 18:00 h) at 3-4% of wet body weight/day for 8 weeks. Weight gain and specific growth rate of fish fed BS_8, BL_7, ML_7, LL_7, LL_8 and OTC were significantly higher than those of fish fed CON (P<0.05). Feed efficiency and protein efficiency ratio were significantly higher in probiotic fed fish than those of fish fed CON diet. Superoxide dismutase and myeloperoxidase activity of fish fed BS_7, BS_8, ML_7, LL_7, LL_8 and OTC were significantly higher than those of fish fed CON. Meanwhile, lysozyme activity of fish fed BS_8, BL_7 and LL_8 diets was significantly higher than those of fish fed CON (P<0.05). There were no significant differences in immune responses among probiotics and OTC diets (P>0.05). Aspartate aminotransferase of fish fed OTC diet was significantly higher than fish fed all the other diets. Intestinal villi length and muscular layer thickness of fish fed BS_8, BL_7, ML_7 and LL_8 were significantly higher than those of fish fed CON and OTC. Expression of interferon-gamma (IFN-γ), interleukin (IL-1 ß), tumour necrosis factor (TNF-α) and heat shock protein 70 (HSP70) genes in fish fed BS_8, ML_7 and LL_8 diets were significantly higher than those of fish fed CON diet. At the end of 14 days of challenge test, fish fed LL_7 had significantly higher cumulative survival rate than those fed CON, BS_7, BL_7 and OTC diets. Trypsin activity of fish fed BL_7, LL_7 and LL_8 was significantly higher than those fed CON, OTC, BS_7 and ML_7 (P<0.05). Based on these results, *B. subtilis*

WB*60* and *L. lactis* at 1×10^8 CFU/g diet could have beneficial effects on growth, immunity, histology, gene expression, and disease resistance, while replacing the use of antibiotics in Nile tilapia.

Third Experiment:

Comparative evaluation of dietary probiotics *Bacillus subtilis* and *Enterococcus faecium* on growth, immune responses, histology and gene expression in Nile tilapia,

This study evaluated the effects of different dietary probiotic supplementations in juvenile Nile tilapia, *Oreochromis niloticus*. A diet without supplementation of probiotics was used as control (CON); three graded levels of *B. subtilis* at 10^6 (BS_6), 10^7 (BS_7), 10^8 (BS_8) and *E. faecium* at 10^6 (BS_6), 10^7 (BS_7), 10^8 (BS_8) CFU/g diet, and oxytetracycline at 4g/kg diet (OTC). Fish with initial body weight of 0.85 ± 0.01 g (mean ± SD) were distributed into 24 (40-L) rectangular tanks (20 fish/tank), and were fed twice daily (09:00 and 18:00 h) at 3-4% of wet body weight/day for 8 weeks. Weight gain, specific growth, feed efficiency and protein efficiency ratio of fish fed BS_8, EF_7 and EF_8 diets were significantly higher than those of fish fed CON diet (P<0.05). However, there were no significant differences between probiotics and OTC diets (P>0.05). Lysozyme activity and superoxide dismutase of fish fed BS_8, EF_8, EF_7 and EF_6 diets significantly improved compared to CON diet (P<0.05). However, there were no significant differences among fish fed probiotics and OTC diets (P>0.05). Myeloperoxidase of fish fed BS_8, EF_8, EF_7, EF_6 and OTC diets were significantly higher than those of fish fed CON diet. Likewise, there were no significant differences among fish fed probiotics and OTC diets (P>0.05). Expression of interferon-gamma (IFN-γ), interleukin (IL-1 ß), tumour necrosis factor (TNF-α) and heat shock protein 70 (HSP70) genes in fish fed EF_8, BS_8 and EF_7 diets were significantly higher than those of fish fed CON diet (P<0.05). At the end of 11 days of challenge test, cumulative survival rate of fish fed EF_8 and EF_7 diets were significantly higher than those of fish fed CON, BS_6 and OTC diets (P<0.05). However, there were no significance differences among fish fed BS_8, EF_7, EF_6 and OTC diets (P>0.05). Enzyme activity of fish fed EF_8, EF_7 and EF_6 diets significantly improved compared to CON and OTC diets (P<0.05). However, there were no significant differences among fish fed BS_8, EF_8, EF_7 and EF_6 diets (P>0.05).

Therefore, these results demonstrated that *B. subtilis* at 10^8 CFU/g and *E. faecium* at $10^{7\sim8}$ CFU/g diets could have an ideal probiotics in terms of the growth performance, immune responses, hematology, enzyme activity, disease resistance and gene expression, while replacing the use of antibiotics in Nile tilapia.

흰다리새우 및 나일 틸라피아에 있어 프로바이오틱스 분리 및 평가

원 성 훈

부경대학교 대학원 수산생물학과

요약

본 연구는 항생제 대체를 위한 연구로써 선행연구를 통해 개발된 뱀장어 장내 토착 probiotics가 국내외 주요 담수어종 (흰다리새우 및 틸라피아)에 있어서도 적용 가능한지를 확인하고, 틸라피아 장내 토착 probiotics 와의 비교평가를 통해 최적의 probiotics 평가하고자 수행되었다.

실험 1과 2에서는 뱀장어에서 개발된 probiotics (*B. subtilis*) 를 흰다리새우 및 틸라피아에서 각각 연구된 probiotics와의 비교실험을 통해, 각 어종별 적용가능성을 확인하기 위하여 실시되었다. 8주간의 사육실험 결과, 실험 1에서는 흰다리새우 사료 내 *B. subtilis, P. pentosaceus* 와 *L. lactis* (새우 장내 추출) at 1×10^8 CFU/g 첨가 시 성장, 면역, 장조직, 유전자발현 및 소화율 향상에 효과를 나타내었다. 실험 2에서는 틸라피아 사료 내 *B. subtilis, B. licheniformis* (새우 장내 추출), *L. lactis* (쥐 장내 추출), and *Micrococcus luteus* (틸라피아 장내 추출) 중 *B. subtilis* 와 *L. lactis* 를 각 1×10^8 CFU/g 첨가 시 성장, 면역, 장조직 및 유전자발현 향상에 효과를 나타내었다.

실험 3에서는 틸라피아 장내 토착 probiotics를 선별하였으며, *B. subtilis* 와의 사육실험을 통해 첨가효과를 평가하였다. 8주간의 사육 실험결과, 사료 내 *B. subtilis* 10^8 CFU/g 와 *E. faecium* 를 10^7 또는 10^8 CFU/g 첨가 시 성장, 면역, 장조직, 유전자발현 및 소화율 향상에 효과를 나타내었다. 따라서, 흰다리 새우 및 틸라피아 사료 내 뱀장어 및 틸라피아 장내 토착 *B. subtils*와 *E. faecium* 를 각

10^8 CFU/g 또는 $10^{7\sim8}$ CFU/g 첨가 시 성장, 면역, 장조직, 유전자발현, 소화율 향상 및 항생제 대체효과를 나타내는 것을 확인할 수 있었다.

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Seonghun Won

List of Tables

List of Figures

CHAPTER I. General introduction

Over the years numerous studies to modulate the composition of the gut microbiota for better growth performance, immune responses, enzyme activity and disease resistance of the host have been developed in human, livestock as well as in fish [1]. The manipulation of microbiota using probiotic is a novel approach not only from nutritional point of view but also as an alternate viable therapeutic modality to overcome the adverse effects of antibiotics [2]. Those beneficial microorganisms are usually referred as “probiotics” which after administration can able to colonize and multiply in the gut of host and execute various beneficial effects by modulating various biological systems in host [3]. Probiotics have been defined as ‘a viable microbial food supplement which beneficially influences the health of the host’. The term probiotic was originated from the Greek words “pro” and “bios” which mean “for life” [4] and are often called as promoter of life that help in a natural way to improve the overall health status of the host organism. According to the previous studies, probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host [5, 6, 7].

Many definitions have been established to describe probiotics [8, 9]. However, Gatesoupe et al. [10] identified survival and colonization of the gastrointestinal (GI) tract as important characteristics of probiotic selection. Therefore, it would seem sensible that probiotic species that would be best suited to perform this task would originate from the fish. In addition to a probiotic's ability to survive the harsh effects of the GI tract, Balcazar et al. [11] suggested that probiotic strains that have been isolated from the host are preferable. Furthermore, additional studies have indicated that bacteria isolated from the appropriate fish species can be an efficient method of selecting effective probiotics [12, 13, 14, 15]. Indeed, many studies on isolated from the intestine of probiotics have been published in fish and shellfish aquaculture worldwide.

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CHAPTER II. Effects of three different dietary probiotics on growth, immune responses, histology and gene expression in whiteleg shrimp, *Litopenaeus vannamei*

1. Introduction

The whiteleg shrimp, *Litopenaeus vannamei* is commercially important shrimp species which accounts 80% of global shrimp production. The global production of whiteleg shrimp has increased rapidly and reached 445 million metric tons in 2017 with an estimated total value of 26.7 billion US dollars [1]. With the high demand of whiteleg shrimp, it has become intensive aquaculture that leads to serious a problem such as infectious disease outbreaks by the parasite. In particular, Vibrio species are the most frequent pathogens in shrimp farming, which have a serious impact on survival, immune responses and production losses in shrimp aquaculture [2]. The emergence of infectious diseases in shrimp aquaculture causes the abuse or misuse of antibiotics and antibiotic resistance [3, 4]. Antibiotics are used as a chemotherapeutic agent for shrimp because they are active from an extensive range of gram-negative/positive organisms. However, the excessive use of antibiotic can result in the occurrence of bacterial resistance [5]. Moreover, Use of antibiotics and their consequences in shrimp farming has received attention from public health point of view due to the potential exposure of human consumers to antibiotic residues. Hence, studies to replace antibiotics have been continuously required.

The potential benefits of probiotic in shrimp aquaculture have been reported; improvement of growth, immunity, disease resistant and digestive enzyme activity [5, 6, 7]. In addition, according to previous studies [8, 9], probiotic, which is a safe approach used as an antibiotic replacer in shrimp aquaculture practices. In fact, probiotics play an important role in preventing and maintaining the microbial balance between necessary and excessive defense mechanisms in terms of innate immune responses [10]. Many bacteria species have been demonstrated that growth,

immune responses and disease resistance of shrimp could be elevated with the administration of probiotics such as *Bacillus subtilis* [11, 12], *Pediococcus pentosaceus* [13, 14] and *Lactococcus lactis* [15, 16]. However, previous studies only evaluated each probiotic effects and have not been performed comparative evaluation of probiotics and antibiotics.

Therefore, in line with the dearth of knowledge on dietary probiotics in shrimp aquaculture, the present study was conducted to investigate the different dietary effects of probiotic to replace dietary antibiotics in whiteleg shrimp, *Litopenaeus vannamei.*

2. Materials and methods

The study was conducted under the guidelines of the Animal Ethics Committee Regulations, No.18-0145 issued by the Pukyong National University, Busan, Rep. Korea.

2.1 Probiotic conditions

The candidate probiotic strains evaluated in this study were isolated from the intestine of fish in Japanese eel [17] and shrimp [14, 16], respectively. Among the these probiotic, *B. subtilis* was isolated from the intestine of juvenile Japanese eel and identified as *B. subtilis* WB60 according to [17]. This probiotic was incubated at 30 ºC for 72h in LB broth (Sigma-Aldrich, St. Louis, USA) and measured at 600 nm optical density (OD_{600}) using spectrophotometer. *P. pentosaceus* and *L. lactis* was isolated from the intestine of juvenile whiteleg shrimp [14, 16]. The strain *P. pentosaceus* was cultured and incubated at 25 ºC for 48h in TSA. While *L. lactis* was grown in MRS (De Man Rogosa & Sharp broth) medium and incubated at 30 ºC for 48h. Three probiotics were washed in sterile saline and the concentration of the final suspension was calculated to 1 x 10^7 and 10^8 in the diets.

2.2 Experimental diets

Feed formulation and proximate composition of the basal diet is shown in Table 1. A diet without supplementation of probiotics was used as the control diet (CON), and four diets with each of four different probiotics were prepared by supplementing two graded levels of *B. subtilis* at 1×10^7 (BS_7) and *B. subtilis, P. pentosaceus* and *L. lactis* at 1×10^8 (BS_8, PP_8 and LL_8) CFU/g diet respectively, and oxytetracycline at 4g/kg diet (OTC). Fishmeal, soybean meal, wheat gluten meal and squid liver powder were used as the protein sources. Fish oil served as the lipid source, wheat flour and corn starch as the carbohydrate source. The preparation and storage of experimental diets were followed by Bai & Kim [17]. Briefly, all the dry ingredients were weighted and mixed in a mixing machine, followed by the addition of fish oil and water until dough was formed. Experimental diets were pelleted using a laboratory pelleting machine with a 1 to 2-mm diameter module (Baokyong Cmmercial Co., Busan, Republic of Korea). The pellets were air-dried for 72 hours stored at -20ºC in the refrigerator until use.

2.3 Experimental fish and feeding trial

The feeding trial was carried at the Feeds and Foods Nutrition Research Center (FFNRC), Pukyoung National University, Busan, Republic of Korea. Juvenile whiteleg shrimp were purchased from Palddak shrimp farm (Goseong, Republic of Korea) and were transported to the laboratory. Shrimp were acclimatized to the FFNRC for two weeks and fed a basal diet. Prior to the feeding trial, shrimp were examined for external abnormalities and starved for 24 h. At the begging of the experiment, shrimp with the initial body weight of 1.41 ± 0.05 g (mean ± SD) were distributed into 18(40-L) capacity rectangular tanks (20 shrimp/tank) receiving seawater at the a constant flow (1.8L/min) from the main tank. Each tank was randomly distributed into one of the three replicates of the eight dietary treatments.

Shrimp were fed four times a day (09:00, 13:00, 17:00 and 21:00 h) at 5-6% of wet body weight/day for 8 weeks. Supplemental aeration was provided to maintain the dissolved oxygen. The temperature of aquarium was maintained at 27.0 ± 1.0ºC and pH remained at 7.68 ± 0.05. The condition of tank were maintained by siphoning off uneaten feeds 2 h after feeding and the walls and bottom of the tanks were scrubbed once a week.

Preferred Table 2-1.

2.4 Sample collection and analysis

At the end of the feeding trial, all the shrimp were starved for 24 h. The count and weight of total shrimp in each tanks were measured to calculate final body weight (FBW), weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER) and survival. Four fish from each treatment group was sacrificed to collect the blood samples. Serum samples were separated by centrifugation at 8,000 *g* for 15 min and stored at -70°C for the analysis of non-specific immune responses including superoxide dismutase (SOD), myeloperoxidase (MPO) and lysozyme activity (LYZ), as well as biochemical parameters such as aspartate aminotransferase activity (AST), aminotransferase activity (ALT), total protein (TP) and glucose. In addition, intestine of shrimp samples were collected for histological sections and digestive enzyme activity measurements.

The proximate composition of experimental diets and whole-body samples were analysed following the standard methods of AOAC [18]. Moisture content was measured after oven-drying the samples at 105°C to constant weight, while crude ash was estimated after incineration at 550°C for 3 h. Crude protein was measured using the Kjeldahl method (N × 6.25) after acid digestion, and crude lipid was determined by soxhlet extraction using the soxhlet system 1046 (Tacator AB, Hoganas, Sweden) after freeze-drying the samples for 20 h.

2.5 Non-specific immune responses analysis

Lysozyme activity was measured by supplementing 0.1 ml serum sample to 2 ml of *Micrococcus lysodeikticus* (0.2 mg/ml, Sigma) in a 0.05 M sodium phosphate buffer (pH 5.5). The reactions were performed at room temperature (20℃) and the absorbance of sample at 450 nm wavelength was measured between 0.5 min and 4.5 min with a spectrophotometer. The sample unit was defined as the amount of enzyme yielding a decrease in absorbance of 0.001/min. SOD activity was obtained by the superoxide radical dependent reaction inhibition rate of enzyme with Water Soluble Tetrazolium dye substrate and xanthine oxidase with the SOD Assay Kit (Sigma-Aldrich, 19160) according to the manufacturer's instructions. Each endpoint assay was estimated at 450 nm absorbance after incubating for 37°C at 20 min. The percent inhibition was normalized by mg protein and expressed as SOD unit/mg. Additionally, MPO activity was determined as described by Quade & Roth [19]. Briefly, 20 µL of serum was diluted with HBSS (Hanks Balanced Salt Solution) without Ca^{2+} or Mg^{2+} (Sigma-Aldrich, USA) in separated 96-well plates to which 35 µL of 3, 3', 5, 5' tetramethylbenzidine hydrochloride (TMB, 20 mM; Sigma-Aldrich) and H_2O_2 (5 mM) were added afterwards. The colour change reaction after 2 min was completed by adding 35 µL of 4 M sulphuric acid. The optical density was measured by using spectrophotometer at 450 nm.

2.6 Real-time PCR

Five fish per experimental diets were used for sample analysis after anesthetized. Total RNA was extracted from mid intestine (50mg) of fish using RiboEx™ (GeneALL, Seoul, Rep. Korea) following standard procedure (Riboclear plus, GeneAll, South Korea). RNA concentration (ng/µl) and purity (OD 260:280) were quantified with nanodrop measurement (Thremo Fisher Scientific, USA). The cDNA was synthesized from 1 µg of RNA using to the manufacturers` instructions cDNA

synthesis Kit (Takara, Japan). RNA isolation and preparation of cDNA by 1 μg of RNA was performed following Hasan et al. [20]. Then, primer and target gene were prepared by BIONICS company (Seoul, Rep. Korea) (Table 2-2). Relative RNA level of the target genes (IL-1ß, TNF-α, IFN-γ, HSP90) were evaluated and calculated using endogenous β-actin RNA level.

Preferred Table 2-2.

2.7 Histomorphology of the intestine

The mid-intestines of the shrimp were sampled from experimental diets (n=3) and were preserved in 10% buffered formaldehyde for 24h, then dehydrated in a graded ethanol series and embedded in paraffin. Tissue blocks were sectioned (5 μm) and stained with hematoxylin and eosin (H&E). The evaluation of villi height (VL) and muscular thickness (MT) was observed by using light microscope (AX70 Olympus, Tokyo, Japan) equipped with scientific digital camera for microscopy (DIXI Optics, Daejeon, Republic of Korea) and processed following image analysis software (Image J 1.32j, National Institute of Health, USA).

2.8 Challenge test

Vibrio parahaemolyticus is a pathogen that is commonly occurred in shrimp environment. The pathogenic bacterium, *V. parahaemolyticus* KCCM 11965, was obtained from the Department of Biotechnology, Pukyong National University, Busan, Republic of Korea. At first, bacteria was grown in 10 ml brain heart infusion (BHI; Becton, Dickinson and Company, USA) broth and incubated at 37 °C for 24 h with a shaking incubator. Growth of *V. parahaemolyticus* was observed by optical density of 600 (OD_{600} nm) using a spectrophotometer (Mecasys, Optizen, Republic of Korea), harvested by centrifugation and washed two times with 0.1 M PBS for further use. At the end of the experiment, eight shrimp from each tank were randomly collected and distributed based on their previous dietary treatment groups

in 12-L tanks. Fish were injected intraperitoneally with 0.1 mL per shrimp of *V. parahaemolyticus* KCCM 11965 at 2×10^7 CFU/mL ($2 \times LD_{50}$). Fish mortality was recorded daily up to 7 days and water temperature was maintained at 27 ± 1.0℃ (mean ± SD).

2.9 Enzyme activities

The enzyme activities of trypsin, lipase and amylase were followed by manufacturer`s instructions with enzyme assays kit (Biovision, USA) and spectrophotometer with the linear range. The pre-treatment of each specific enzyme assays kit was carried out with substrate and assay buffer. Trypsin activity was prepared with a mixed solution and measured by spectrophotometer at 405 nm wavelength for 40min. Lipase activity was measured with spectrophotometer at a wavelength of 412 nm for 20 min after mixing lipase substrate and assay buffer. Amylase activity was reacted with assay buffer and substrate mix, and was measured by absorbance of shrimp samples at a wavelength of 402 nm for 40 min. Specific enzyme activities were defined as the amount of enzyme that catalysed the conversion of 1 μmol of substrate per minute per mg of protein (i.e. U mg soluble protein− 1) at the respective temperature.

2.10 Statistical analysis

The values from this study were statistically analysed by using the one-way ANOVA (Statistix3.1; Analytical Software, St. Paul, MN, USA) in order to test the effects of dietary probiotic treatments. When a significant treatment effect was observed, an LSD post hoc test was used to compare means. Treatment effects were considered to be significant at $P < 0.05$.

3. Results

3.1 Growth performance and whole body proximate composition

The growth performances and survival of juvenile whiteleg shrimp fed different probiotics diets are displayed in Table 3. Weight gain (WG), specific growth rate (SGR), Feed efficiency (FE) and protein efficiency ratio (PER) of fish fed BS_8 and LL_8 diets were significantly higher than those of fish fed CON diet (P<0.05). However, there were no significant differences among fish fed BS_8, PP_8, LL_8, BS_7 and OTC diets (P>0.05). Shrimp survival rate of LL_8 diet showed significantly higher than CON and OTC diets (P<0.05). However, there were no significant differences among fish fed BS_8, PP_8, LL_8 and BS_7 diets (P>0.05). On the other hands, no significant differences were observed in terms of whole body protein, lipid, moisture and ash content among all diets (P>0.05; Table 3).

Preferred Table 2-3.

Preferred Table 2-7.

3.2 Non-specific immune responses

The non-specific immune responses (Lysozyme, superoxide dismutase; SOD and myeloperoxidase; MPO activity) were showed in Table 2-4. Lysozyme activity of fish fed probiotics and OTC diets significantly improved compare to CON diet (P<0.05). SOD activity of fish fed BS_8 and PP_8 diets were significantly higher than those of fish fed CON diet (P<0.05). However, there were no significant differences among probiotics and OTC diets (P>0.05). Meanwhile, MPO did not show any significant differences among treatment diets (P>0.05).

Preferred Table 2-4.

3.3 Intestinal histology

The intestinal histology of whiteleg shrimp fed experimental diets for 8 weeks were described (Fig 2-1, Table 2-5). The villi length of shrimp fed the probiotic-supplemented diets were longer than those of fish fed CON and OTC diets ($P<0.05$). Correspondingly, muscular layer thickness of fish fed PP_8 and LL_8 diets better than CON diet ($P<0.05$). However, there were no significant differences among fish fed BS_7, BS_8, PP_8, LL_8 and OTC diets ($P>0.05$).

Preferred Table 2-5.

Preferred Figure 2-1.

3.4 Haematological analysis

As shown in table 2-6, there were no significant differences among treatment groups in terms of alanine aminotransferase activity (ALT), aspartate aminotransferase activity (AST), glucose and total protein ($P>0.05$).

Preferred Table 2-6.

3.5 Immune-related gene expressions

The gene expressions of the immunological expression in the intestine of whiteleg shrimp fed experimental diets are demonstrated in Fig 2-2. The expression level of serine protein (SP) from fish fed probiotic diets were significantly higher than those of fish fed CON and OTC diets ($P<0.05$). Also, peroxinectin (PE) expression of fish fed probiotic diets were higher compare to CON and OTC diets ($P<0.05$). Meanwhile, prophenoloxidase (proPO) expression in BS_8, PP_8 and LL_8 were significantly improved compare to CON, BS_7 and OTC diets. However, there were no significant differences between BS_7 and OTC diets ($P>0.05$).

Preferred Figure 2-2.

3.6 Challenge test

Cumulative survival rate of juvenile whiteleg shrimp challenged with *Vibrio parahaemolyticus* for 7 days is presented Fig 2-3. During the challenge test, the first shrimp mortalities occurred on the first day post-injection. Statistical analysis at the end of 7 days of the *V. parahaemolyticus* challenge showed that fish fed BS_8, LL_8 and PP_8 diets have significantly better cumulative survival rate than those of fish fed CON diet ($P<0.05$). However, there were no significant differences among probiotic and OTC diets ($P>0.05$).

Preferred Figure 2-3.

3.7 Enzyme activities

Digestive activity of juvenile whiteleg shrimp showed in Fig 2-4. Trypsin activity of shrimp fed BS_8, PP_8 diets were significantly higher than those of CON diet ($P<0.05$). However, there were no significant differences among BS_8, PP_8, LL_8, BS_7 and OTC diets ($P>0.05$). Lipase activity of shrimp fed BS_8, PP_8 and LL_8 diets were significantly improved compare to CON, BS_7 and OTC diets ($P<0.05$). Moreover, amylase activity in probiotic and OTC groups were significantly higher than the CON group ($P>0.05$).

Preferred Figure 2-4.

4. Discussion

The application of beneficial probiotics has been investigated as a valuable practical study in shrimp diets regarding elevating the growth [21], enhancing immune response [22] and improving disease resistance [23]. Among the probiotics, *Bacillus subtilis* WB60 which isolated from the intestine of juvenile Japanese eel, *Anguilla japonica* reported as a potential probiotic as well as antibiotic replacer in the diet [17]. In this study, the effects of three different dietary probiotics supplementation

on growth, immune responses, histology and gene expression in whiteleg shrimp were investigated.

The results of our study indicated that dietary *B. subtilis* and *Lactococcus lactis* at 10^8 cfu/g had a significant influence on growth and feed utilization of whiteleg shrimp. The similar results were shown in previous studies on the improved growth and feed utilization by probiotic supplementation such as *B. subtilis* [12], *L. lactis* [16] and P. *pentosaceus* [14], which was improved compared to CON diet. The enhanced growth performance and feed-utilization of whiteleg shrimp fed probiotic diets were affected by increased enzyme activities. According to previous studies, administration of probiotics could have the beneficial effects on shrimp digestive enzyme activity [24, 25]. Furthermore, our results are similar with the observation of Adel et al. [14], who demonstrated that the higher level of enzyme activity obtained with diets containing probiotics improved the digestion of trypsin, amylase, and lipase, which might, in turn, explain the better growth observed with the probiotic-supplemented diets when compared with CON diet.

The invertebrate depends on non-specific immune responses for combating pathogenic organisms, and it has been observed that it can be stimulated by probiotics which produce transduction signaling molecules that have the ability to alert the immune system against assaults by pathogenic agents [26]. Various studies revealed that probiotic induce elevated immune responses in shrimp diet [23, 27, 28]. In the current study, the dietary probiotic groups improved in Lysozyme activity compare to CON diet as well as did not showed significant differences with OTC diet. Moreover, SOD activity of fish fed BS_8 and PP_8 diets were significantly higher than those of fish fed CON diet and did not differ between probiotic and OTC diets. Previous studies have reported that the supplementation of probiotic that complements each other and occupies different niches within the gut microflora environment could result in an enhancement or prolongation of the desirable effects on the host immune response and health [29, 30].

The intestinal histology has been determined to estimate the gut condition [31]. The villi height of whiteleg shrimp fed probiotic diets revealed significantly higher in the mid-intestine than those of shrimp fed CON and OTC diets ($P<0.05$). On the other hands, muscular layer thickness showed significantly higher in the mid-intestine of PP_8 and LL_8 diets compared to CON diet ($P<0.05$). However, there were no significant differences between supplemented-probiotic and OTC diets ($P>0.05$). Our findings are in agreement with [32], who reported that enhanced villi height and muscular layer thickness have been associated with probiotic administration which could improve its nutrient absorptive ability. Indeed, although several studies have been carried out on the effect of probiotics in shrimp, a few study evaluated histology analysis in shrimp.

The immune gene expression of shrimp increased with probiotic supplementation as shown in previous studies [11, 23]. In the current experiment, BS_8, PP_8 and LL_8 diets significantly improved compared to CON and OTC diets. Chiu et al. [23] reported that the expression of proPO and PE can elevate the biological activity of cell adhesion [33], opsonin [34], degranulation [35] and peroxidase [36] of shrimp. These biological activities would be achieved in the present study with the probiotic treatments, gene up-regulation was enhanced in treated shrimp compared to CON diet.

Disease resistance against *V. parahaemolyticus* for 7 day was significantly enhanced by administration of *B. subtilis*, WB*60*, *P. pentosaceus* and *L. lactis* at 1×10^8 CFU/g diet. This result is in agreement with previous suggestion affected by pathogens [11, 22, 28]. Generally, administration of probiotic in shrimp diet could induce decreased mortality rate compared to CON diet [27, 37, 38]. Furthermore, the results of this study corroborated with survival rate. The enhanced mortality and survival rate may be due to probiotic supplementation.

The digestive enzyme activities of shrimp are an important indicator for estimating the organism to metabolize given nutrients as well as the types, properties, and

modulation [39, 40]. The results of this study indicated that BS_8, PP_8 and LL_8 diets significantly improved compared to CON diet (P<0.05). According to Zheng et al. [31], probiotic supplementation may be related to the improved height and density of enterocytes. Moreover, the microvilli of the intestine cell contribute to an extensive absorptive ability and surface area of nutrients [41]. A few studies conducted on shrimp with probiotic supplementation with *Bacillus* sp. [42, 43], *L. latics* [16], *Lactobacillus plantarum* [44] and *P. acidilactici* [45] demonstrated the response similar to our results. As it was mentioned before, the improved growth performance, feed utilization and survival rate of shrimp may be due to enhancing digestive enzyme activity induced by the probiotics.

In conclusion, the present study demonstrated that *B. subtilis*, WB*60 P. pentosaceus* and *L. lactis* at 1×10^8 CFU/g diet could be beneficial feed additives, replacing antibiotic replacer to improve growth, immune responses, intestinal morphology and gene expression in whtieleg shrimp, *Litopenaeus vannamei*.

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Figure legends

Figure 1. Histological sections of juvenile whiteleg shrimp intestine fed the experimental diets for 8 weeks. A-CON, basal diet; B-BS_7, *Bacillus subtilis* at 1 x 10^7 CFU/g; C- BS_8, *Bacillus subtilis* at 1 x 10^8 CFU/g; D-PP_8, *Pediococcus pentosaceus* at 1 x 10^8 CFU/g; E- LL_7, *Lactococcus lactis* at 1 x 10^8 CFU/g; H-OTC, oxytetracycline at 4g/kg. (Scale bar = 100 μm; Original magnification × 4).

Figure 2. Intestinal gene expression levels of serine protein (SP), peroxinectin (PE) and prophenoloxidase (proPO) were evaluated in juvenile whiteleg shrimp fed the experimental diets for 8 weeks. Bars with range represent mean ± SD of triplicate samples, and diets refer to Fig 1.

Figure 3. Cumulative survival rate of juvenile whiteleg shrimp fed the experimental diets with different probiotics for 8 weeks and experimentally challenged with *V. parahaemolyticus* for 7 days. Each value represents mean of triplicate groups. Significant differences among means are indicated by different superscripts ($P<0.05$), and diets refer to Fig 1.

Figure 4. Specific enzyme activities of 1. Trypsin, 2. Amylase and 3. Lipase measured in the intestines of juvenile whiteleg shrimp fed the experimental diets with different probiotics for 8 weeks, and diets refer to Fig 1.

6. Table and figures

Table 2-1. Formulation and composition (% dry matter) of the basal diet for Whiteleg shrimp

Ingredients	%
Fishmeal, Chile[1]	30.0
Soybean meal[2]	25.0
Wheat flour[2]	12.0
Wheat gluten meal[2]	8.00
Corn starch[2]	7.00
Squid liver powder[2]	4.00
Fish oil[3]	4.00
Calcium phosphate[6]	2.50
Lecithin[2]	2.00
Vitamin premix[4]	2.00
Mineral premix[5]	2.00
Cholesterol	0.50
Cellulose[6]	1.00
Proximate composition	
Moisture	9.60
Crude protein	42.8
Crude lipid	9.72
Crude ash	8.15

[1]Suhyup Feed Co. Uiryeong, Korea

[2]The Feed Co. Goyang, Korea

[3]Jeil Feed Co. Hamman, Korea

[4]Contains (as mg/kg in diets) : Ascorbic acid, 300; dl-Calcium pantothenate, 150; Choline bitate, 3000; Inositol, 150; Menadion, 6; Niacin, 150; Pyridoxine · HCl, 15; Rivoflavin, 30; Thiamine mononitrate, 15; dl-α-Tocopherol acetate, 201; Retinyl acetate, 6; Biotin, 1.5; Folic acid, 5.4; Cobalamin, 0.06

[5]Contains (as mg/kg in diets) : NaCl, 437.4; $MgSO_4 \cdot 7H_2O$, 1379.8; $ZnSO_4 \cdot 7H_2O$, 226.4; Fe-Citrate, 299; $MnSO_4$, 0.016; $FeSO_4$, 0.0378; $CuSO_4$, 0.00033; $Ca(IO)_3$, 0.0006; MgO, 0.00135; $NaSeO_3$, 0.00025

[6]Sigma-Aldrich Korea, Yongin, Korea

Table 2-2. Primers used to quantify relative gene expression

Primers	Sense	Sequences
SP[1]	F	5′-CCGTCTTGGAGAATACGACTTGAG-3′
	R	5′-GCTACAGGTAGGCTGGATAACTTG-3′
PE[2]	F	5′-GTGAACGGTAGTCCTTTACCTAAT-3′
	R	5′-CGAGGTCCATAGAAAGCATCTC-3′
proPO[3]	F	5′-CAAGCCCTTCGACTACCATATAC-3′
	R	5′-CTGACTGTTCACTTGAGTTCCC-3′
actin[4]	F	5′-TGGCAATGAGAGGTTCCG-3′
	R	5′-TGCTGTTGTAGGTGGTTTCG-3′

[1] SP, serine protein (AU_368151.1)

[2] PE, peroxinectin (AF188840.1)

[3] proPO, prophenoloxidase (EU284136)

[4] actin (AF100986)

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

	Diets[1]					
	CON	BS_7	BS_8	PP_8	LL_8	OTC
IBW (g)[2]	1.40±0.05	1.42±0.08	1.40±0.09	1.42±0.06	1.39±0.03	1.42±0.08
FBW (g)[3]	6.42±0.12^{b}	6.67±0.44^{ab}	7.08±0.51^{a}	6.85±0.14^{ab}	7.15±0.26^{a}	6.71±0.65^{ab}
WG (%)[4]	359±22.1^{b}	373±24.8^{ab}	406±28.7^{a}	378±16.2^{ab}	417±29.8^{a}	373±24.8^{ab}
FE (%)[5]	83.7±4.57^{b}	87.9±5.82^{ab}	94.6±6.88^{a}	88.7±3.20^{ab}	97.1±6.47^{a}	87.2±6.49^{ab}
SGR(%/day)[6]	2.72±0.09^{b}	2.79±0.10^{ab}	2.89±0.10^{a}	2.79±0.06^{ab}	2.93±0.10^{a}	2.77±0.10^{ab}
PER[7]	2.31±0.08^{b}	2.58±0.12^{ab}	2.51±0.09^{a}	2.57±0.08^{ab}	2.59±0.09^{a}	2.60±0.06^{ab}
Survival[8]	73.3±5.00^{b}	76.7±2.89^{ab}	83.3±5.00^{ab}	76.7±5.00^{ab}	88.3±2.89^{a}	73.3±5.77^{b}

[1]Data are means ± SD of triplicate groups of fish. Values in each row with different superscripts are significantly different (P<0.05)

Diets: CON = the basal diet, refer to Table 1; BS_7 = *Bacillus subtilis* at 1 x 10^7 CFU/g; BS_8 = *Bacillus subtilis* at 1 x 10^8 CFU/g; PP_8 = *Pediococcus pentosaceus* at 1 x 10^8 CFU/g; LL_8 = *Lactococcus lactis* at 1 x 10^8 CFU/g; OTC = oxytetracycline at 4g/kg.

[2] Initial body weight

[3] Final body weight

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

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

[6] Specific growth rate (SGR, %) = [($\log_e$ final wt. - $\log_e$ initial wt.) × 100] / days

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

[8] Survival rate = [(total fish – dead fish) × 100] / total fish

Table 2-4. Non-specific immune responses of juvenile whiteleg shrimp fed the experimental diets for 8 weeks[1]

	Diets					
	CON	BS_7	BS_8	PP_8	LL_8	OTC
Lysozyme (U/ml)	0.20±0.03[b]	0.32±0.02[a]	0.32±0.05[a]	0.34±0.04[a]	0.34±0.02[a]	0.30±0.03[a]
SOD[2]	93.9±2.15[b]	95.6±1.38[ab]	98.6±3.69[ab]	98.7±2.66[a]	95.8±2.71[a]	94.7±2.28[ab]
MPO[3]	3.41±0.40	3.76±0.38	3.81±0.09	3.77±0.21	3.74±0.29	3.69±0.33

[1]Values are means ± SD of triplicate groups of fish. Values in each row with different superscripts are significantly different (P<0.05). Diets refer to Table 3.

[2]SOD: Superoxide dismutase activity (% inhibition)

[3]MPO: Myeloperoxidase activity (OD at 450 nm)

Table 2-5. Intestinal morphology of juvenile whiteleg shrimp fed the different probiotics diets for 8 weeks[1]

	Diets					
	CON	BS_7	BS_8	PP_8	LL_8	OTC
Villi height (μm)	134±13.9^{d}	225±15.2^{c}	365±37.5^{a}	320±19.0^{b}	331±52.0^{ab}	129±25.7^{d}
Muscular layer thickness (μm)	87.6±11.9^{b}	107±5.54^{ab}	108±19.4^{ab}	112±17.8^{a}	121±17.8^{a}	99.2±13.7^{ab}

[1]Data are means ± SD of triplicate groups of fish. Values in each row with different superscripts are significantly different (P<0.05)

Table 2-6. Haematological analysis of juvenile whiteleg shrimp fed the different probiotics diets for 8 weeks[1]

	Diets					
	CON	BS_7	BS_8	PP_8	LL_8	OTC
AST (U L^{-1})	118±2.08	123±4.16	113±10.6	119±2.89	122±8.39	122±2.89
ALT (U L^{-1})	175±12.6	171±18.0	2.89±0.10	2.79±0.06	2.93±0.10	2.77±0.10
T-protein (g dL^{-1})	11.3±0.58	11.0±1.00	10.7±0.58	11.3±0.58	11.3±0.58[a]	11.7±0.58
Glucose	74.7±5.13	76.3±4.04	71.3±5.86	70.7±5.13	69.3±5.69	75.3±3.06

[1]Data are means ± SD of triplicate groups of fish. Values in each row with different superscripts are significantly different (P<0.05)

[2]AST (U L^{-1}): Aspartate aminotransferase

[3] ALT (U L^{-1}): Alanine aminotransferase

[4]T-protein (g dL^{-1}): Total protein

Table 2-7. Whole-body proximate composition (% dry matter) of juvenile whiteleg shrimp fed the different probiotics diets for 8 weeks[1]

	Diets					
	CON	BS_7	BS_8	PP_8	LL_8	OTC
Moisture	75.4±1.08	76.0±1.24	75.2±1.10	75.8±1.28	75.5±1.32	76.2±1.05
Protein	17.8±0.45	18.4±0.33	17.9±0.29	18.2±0.52	18.7±0.44	18.6±0.38
Lipid	2.25±0.08	2.16±0.10	2.19±0.05	2.20±0.07	2.21±0.03	2.22±0.05
Ash	3.54±0.12	3.62±0.10	3.60±0.09	3.58±0.12	3.62±0.08	3.55±0.10

[1]Data are means ± SD of triplicate groups of fish. Values in each row with different superscripts are significantly different (P<0.05)

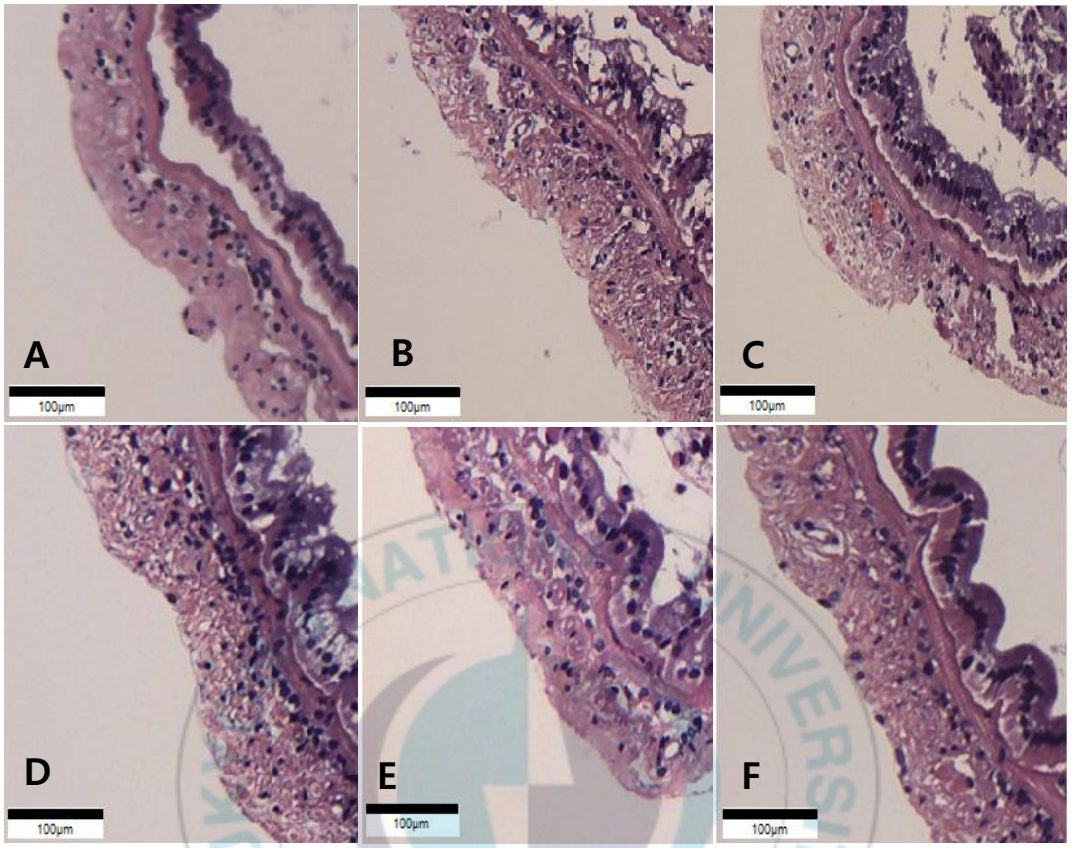


Fig 3-1.

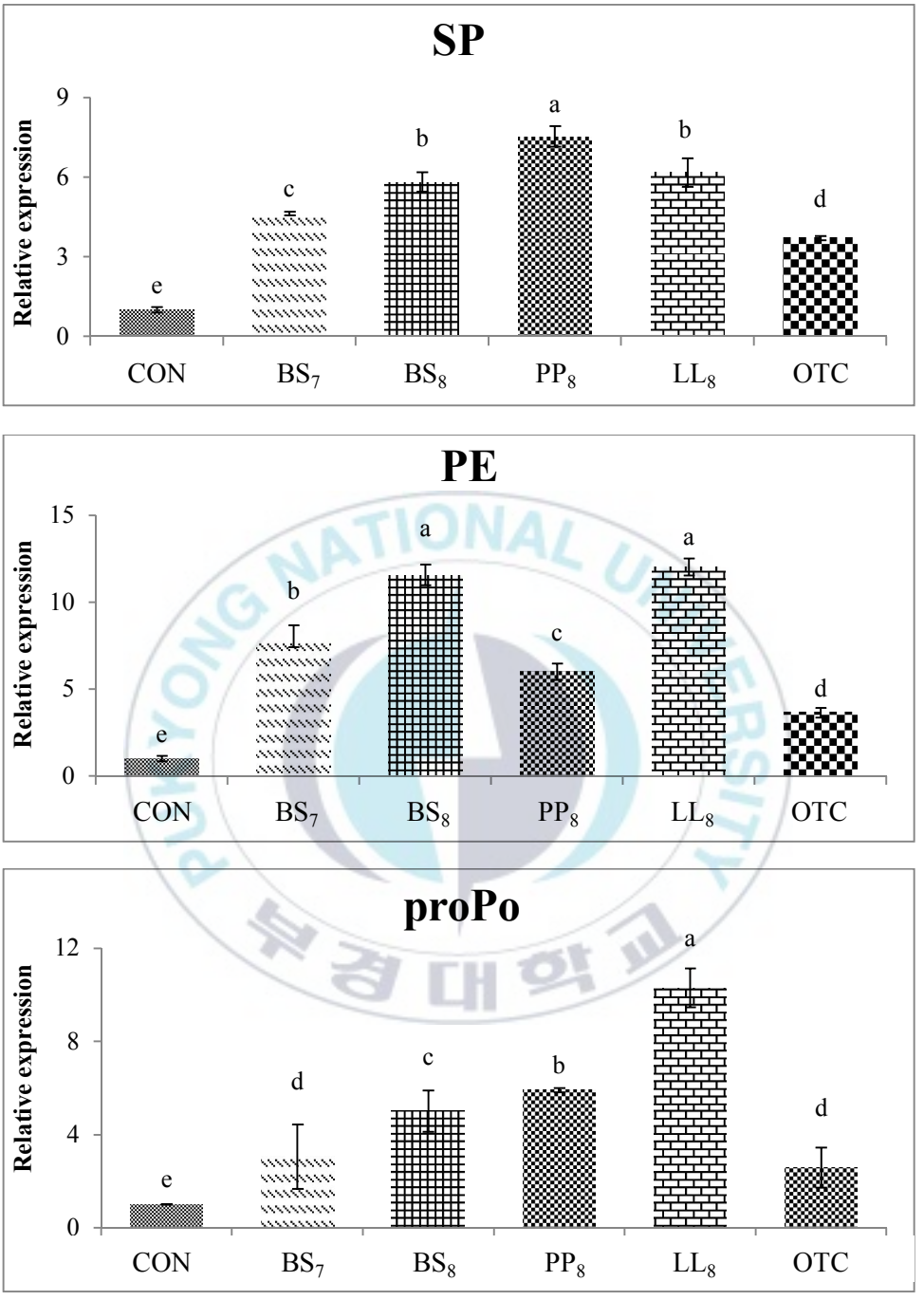

SP
Relative expression
9
6
3
0
e
c
b
a
b
d
CON
BS7
BS8
PP8
LL8
OTC
PE
Relative expression
15
10
5
0
e
b
a
c
a
d
CON
BS7
BS8
PP8
LL8
OTC
proPo
Relative expression
12
8
4
0
e
d
c
b
a
d
CON
BS7
BS8
PP8
LL8
OTC

Fig 3-2.

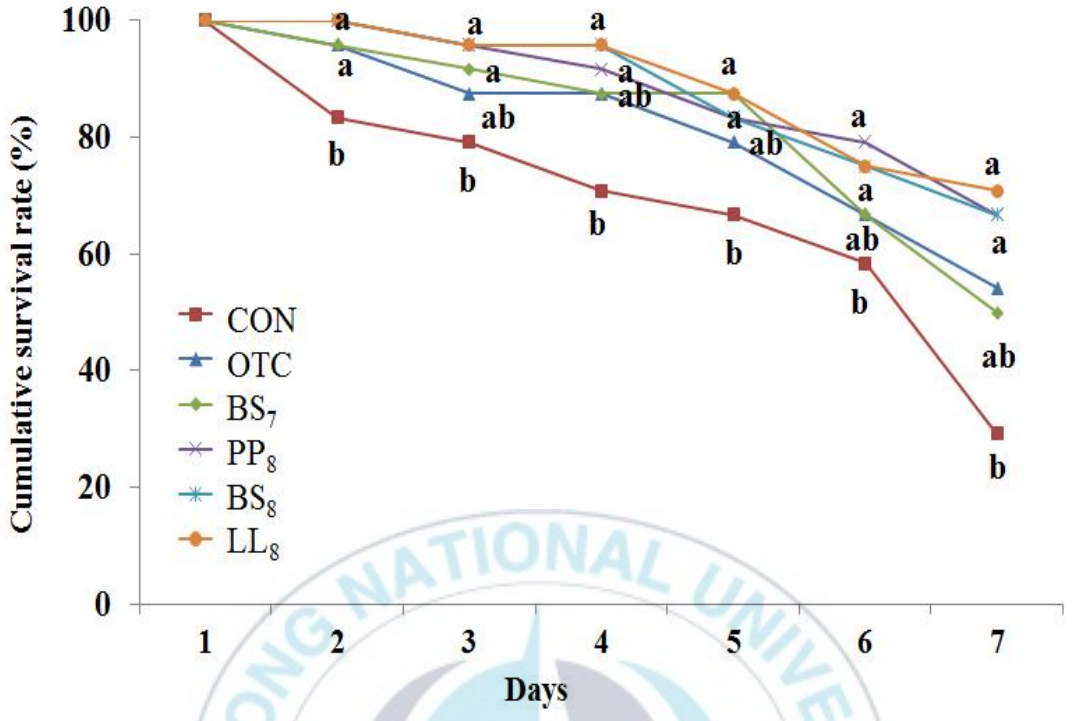
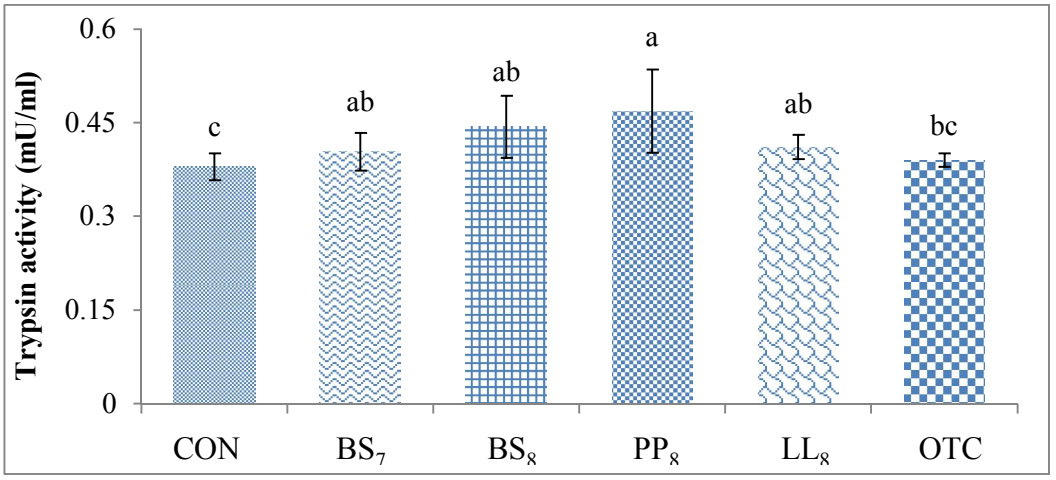
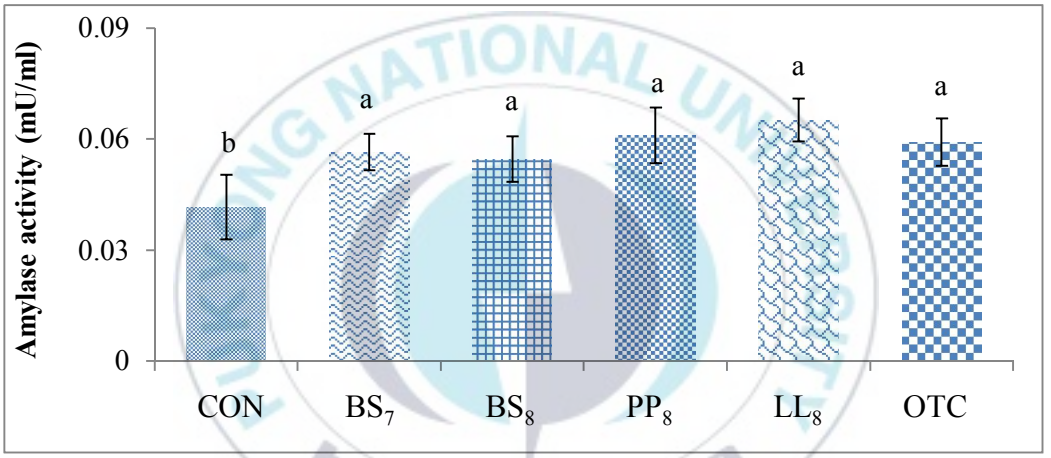


Fig 3-3.


Trypsin activity (mU/ml)
0.6
0.45
0.3
0.15
0
c
ab
ab
a
ab
bc
CON
BS7
BS8
PP8
LL8
OTC


Amylase activity (mU/ml)
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0.06
0.03
0
b
a
a
a
a
a
CON
BS7
BS8
PP8
LL8
OTC

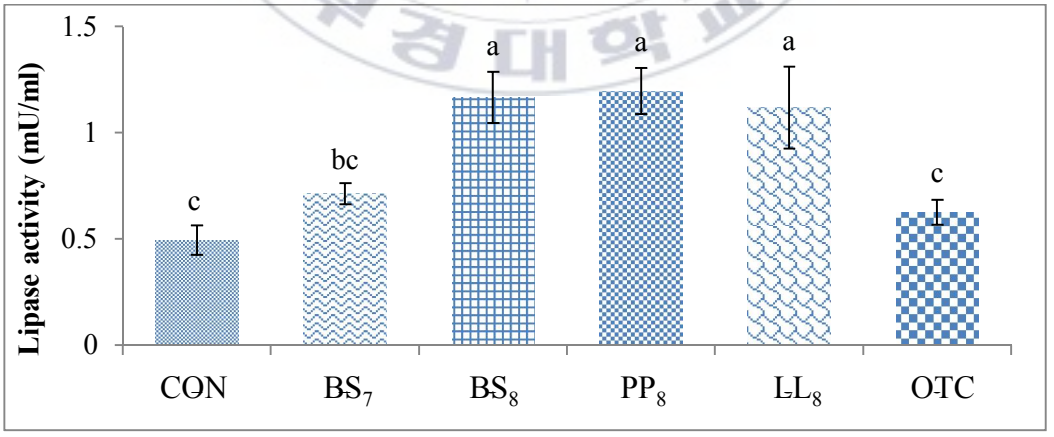
Lipase activity (mU/ml)
1.5
1
0.5
0
c
bc
a
a
a
c
CON
BS7
BS8
PP8
LL8
OTC

Fig 3-4.

CHAPTER III. Effects of four different dietary probiotics on growth, immune responses, histology and gene expression in Nile tilapia, *Oreochromis niloticus*

1. Introduction

Aquaculture, consisting of aquatic animals and plants, is one of the fastest growing food-producing sectors worldwide [1]. Along with the escalating demand for fish, the world aquaculture has become intensified with minimum land and water usage. However, the intensive aquaculture is confronted with problems that have led to concerns about fish welfare regarding growth problems, mortality and disease outbreaks [2, 3]. As a consequence, most of the farmers have been using antibiotics prophylactically to overcome these problems [4]. Antibiotics are generally used to control disease and promote growth in aquaculture industry. However, the use of antibiotics in aquaculture may alter the intestinal microbiota and give rise to resistant bacteria, which could be harmful to aquatic organisms [5]. On account of these reasons, several previous studies have been carried out on feed additives to replace antibiotics in aquaculture [6-8].

Recently, the potential of probiotics to reduce the use of antimicrobials and antibiotics in disease control has been highlighted. Probiotics have been used as an alternative environment-friendly strategy to develop sustainable aquaculture. Probiotics have been defined in several ways, as the live and viable microbial food supplements that provide beneficial effects on fish in terms of growth performances, immune responses, intestinal microbial balance and digestive enzyme activities [9, 10]. Probiotics such as *Bacillus subtilis* [11], *Baillus licheniformis* [12], *Lactococcus lactis* [13] and *Micrococcus luteus* [14] have been identified as beneficial for aquaculture. But studies on suitable probiotics for each fish and shrimp species are still ongoing [14, 15, 16].

Therefore, in line with the dearth of knowledge on dietary probiotics in freshwater aquaculture, the present study was conducted to investigate effects of different dietary probiotics to replace dietary antibiotics in Nile tilapia, *Oreochromis niloticus*.

2. Materials and methods

The study was conducted under the guidelines of the Animal Ethics Committee Regulations, No.18- 0125 issued by the Pukyong National University, Busan, Rep. Korea.

2.1 Probiotic conditions

The probiotic strains tested in this study as antibiotic replacers were *B. subtilis*, *B. licheniformis*, *L. lactis*, and *M. luteus*. Among these probiotic, *B. subtilis* was isolated from the intestine of juvenile Japanese eel and identified as *B. subtilis* WB60 according to Lee et al. [11]. This probiotic was incubated at 30 ºC for 72h in LB broth (Sigma-Aldrich, St. Louis, USA) and measured at 600 nm optical density (OD_{600}) using spectrophotometer. *B. licheniformis* was isolated from the shrimp culture environment and identified by Gobi et al. [12]. The strain *B. licheniformis* was grown at a shaking incubator at 36 ºC for 24h in LB broth. The cell density was estimated at OD_{600}. *M. luteus* was isolated from the internal organs of juvenile Nile tilapia and purified as *M. luteus* according to El-Rhman et al. [14]. On the other hand, *L. lactis* was grown in MRS broth at 36 ºC for 48h following Xia et al. [13]. All probiotics were washed in sterile saline and the concentration of the final suspension was calculated at 1×10^7 and 10^8 in the diets.

2.2 Experimental diets

Feed formulation and proximate composition of the basal diet is shown in Table 1. A basal control diet without supplementation of probiotics (CON), and seven other diets were prepared by supplementing *B. subtilis*, *B. licheniformis*, *L lactis* and *M. luteus* at 1×10^7 CFU/g diet (BS_7, BL_7, LL_7 and ML_7, respectively), *B. subtilis* and *L. lactis* at 1×10^8 CFU/g diet (BS_8 and LL_8, respectively), or oxytetracycline at 4g/kg diet (OTC). Tuna by-product and soybean meal served as the major protein sources, soybean oil as the lipid source, while wheat flour and corn starch as the carbohydrate sources. The preparation and storage of experimental diets were followed by Bai & Kim [17]. Briefly, all the dry ingredients were measured and mixed in an electric mixing machine, followed by the addition of corn oil and water until dough was formed. Experimental diets were pelleted using a laboratory pelleting machine with a 1 mm diameter module (Baokyong Cmmercial Co., Busan, Republic of Korea). The pellets were air-dried for 72 hours and stored at -20ºC until use.

Preferred Table 3-1.

2.3 Experimental fish and feeding trial

The feeding trial was carried at the Feeds and Foods Nutrition Research Center (FFNRC), Pukyoung National University, Busan, Republic of Korea. Juvenile Nile tilapia were obtained from the Docheon fishfarm (Gyeongsangnam-do, Republic of Korea) and were transported to FFNRC. Fish were acclimatized to the FFNRC condition for two weeks and fed a basal diet. Prior to the feeding trial, fish were examined for external abnormalities and starved for 24 h. At the begging of the experiment, fish with the initial body weight of 2.83 ± 0.05 g (mean ± SD) were distributed into 24 (40-L) rectangular tanks (20 fish/tank) receiving filtered freshwater at the a constant flow (1.5 L/min) from the main tank. Each tank was randomly allocated to one of the three replicates of the eight dietary treatments. Fish were fed twice daily (09:00 and 18:00 h) at 3-4% of wet body weight/day for 8

weeks. Supplemental aeration was provided to maintain the dissolved oxygen. The temperature of aquarium was maintained at 27.0 ± 0.8ºC and pH remained at 7.50 ± 0.05 throughout the feeding trial. The condition of tank were maintained by siphoning off uneaten feeds 2 h after feeding and the walls and bottom of the tanks were scrubbed once a week.

2.4 Sample collection and analysis

At the end of the feeding trial, all the fish were starved for 24 h. The number and weight of total fish in separated tanks were measured to calculate final body weight (FBW), weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER) and survival. Three fish from each tank were randomly selected, individually weighed and dissected to estimate liver and gastro-intenstinal organs for the measurement of hepatosomatic index (HSI), viscerosomatic index (VSI) and condition factor (CF). Additionally, three fish from each treatment group was anesthetized by tricaine methanesulfonate (MS 222, 100ppm) to collect the blood samples. Serum samples were separated by centrifugation at 5,000 *g* for 10 min and stored at -70°C for the analysis of non-specific immune responses including superoxide dismutase (SOD), myeloperoxidase (MPO) and lysozyme, as well as biochemical parameters such as glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), total protein (TP) and glucose. Intestine of fish samples were used for the preparations of histological sections and measurements of digestive enzyme activity.

The proximate composition of experimental diets and whole-body samples were analysed according to standard methods of AOAC [18]. Moisture content was measured after oven-drying the samples at 105°C to constant weight, while crude ash was estimated after incineration at 550°C for 3 h. Crude protein was measured using the Kjeldahl method (N × 6.25) after acid digestion, and crude lipid was determined by soxhlet extraction using the soxhlet system 1046 (Tacator AB, Hoganas, Sweden) after freeze-drying the samples for 20 h.

2.5 Non-specific immune responses analysis

Lysozyme activity was determined by adding 0.1 ml serum sample to 2 ml of *Micrococcus lysodeikticus* (0.2 mg/ml) suspension in a 0.05 M sodium phosphate buffer (pH 5.5). The reactions were carried out at 20℃ and the absorbance of sample at 530 nm wavelength was measured between 0.5 min and 4.5 min with a spectrophotometer. The sample unit was defined as the amount of enzyme yielding a decrease in absorbance of 0.001/min. On the contrary, activity of SOD was analysed by the superoxide radical dependent reaction inhibition rate of enzyme with water soluble tetrazolium dye substrate and xanthine oxidase using the SOD Assay Kit (Sigma-Aldrich, 19160) following the manufacturer's instructions. Each endpoint assay was monitored at 450 nm absorbance after incubating at 37°C for 20 min. The percent inhibition was normalized by mg protein and expressed as SOD unit/mg. In addition, MPO activity was determined according to the method described by Quade & Roth [19]. Briefly, 20 µL of serum was diluted with HBSS (Hanks Balanced Salt Solution) without Ca^{2+} or Mg^{2+} (Sigma-Aldrich) in each well of a 96-well plate to which 35 µL of 3, 3', 5, 5' tetramethylbenzidine hydrochloride (TMB, 20 mM; Sigma-Aldrich) and H_2O_2 (5 mM) were subsequently added. The colour change reaction after 2 min was completed by supplementing 35 µL of 4 M sulphuric acid. Finally, the optical density was read at 450 nm in a spectrophotometer.

2.6 Real-time PCR

Five fish per experimental diets were used for sample analysis after being anesthetized. Total RNA was extracted from mid intestine (50mg) of fish using RiboEx™ (GeneALL, Seoul, Rep. Korea) following standard procedure (Riboclear plus, GeneAll, South Korea). RNA concentration (ng/µl) and purity (OD 260:280) were quantified with nanodrop measurement (Thremo Fisher Scientific, USA). The cDNA was synthesized from 1 µg of RNA using manufacturers` instructions of cDNA

synthesis Kit (Takara, Japan). RNA isolation and preparation of cDNA by 1 μg of RNA was performed following Hasan et al. [20]. Then, primer and target gene were prepared by BIONICS company (Seoul, Rep. Korea; Table 2). Relative RNA level of the target genes (IL-1ß, TNF-α, IFN-γ and HSP90) were evaluated and calculated using endogenous β-actin RNA level.

Preferred Table 3-2.

2.7 Histomorphology of the intestine

The mid-intestinal samples were collected from experimental groups (n=5) and were preserved in 10% neutral buffered formalin, dehydrated in a graded ethanol series and embedded in paraffin according to standard histological process. Sections series of 6 μm were made with a microtome and stained with hematoxylin and eosin (H&E). The evaluation of villi height (VL) and muscular thickness (MT) was observed using light microscope (AX70 Olympus, Tokyo, Japan) equipped with scientific digital camera for microscopy (DIXI Optics, Daejeon, Republic of Korea) and processed by image analysis software (Image J 1.32j, National Institute of Health, USA).

2.8 Challenge test

Aeromonas hydrophila is a well-known pathogen that generally causes disease in Nile tilapia [21]. The pathogenic bacterium, *A. hydrophila* KCTC2358, was received from the Department of Biotechnology, Pukyong National University, Busan, Republic of Korea. At first, bacteria was grown in 10 ml brain heart infusion (BHI; Becton, Dickinson and Company, USA) broth and incubated at 37 °C for 24 h in a shaking incubator. Growth of *A. hydrophila* was observed by optical density of 600 (OD_{600} nm) using a spectrophotometer (Mecasys, Optizen, Republic of Korea), harvested by centrifugation and washed two times with 0.1 M PBS for further use. At the end of the experiment, eight fish from each tank were randomly collected and

reorganized based on their previous dietary treatment groups in 12-L tanks and allowed to maintain for 24 h. Fish were injected intraperitoneally with 0.1 mL per fish of *A. hydrophila* KCTC2358 at 2×10^7 CFU/mL ($2 \times LD_{50}$). Fish mortality was documented daily up to 13 days. Water temperature was controlled at 27 ± 0.5°C (mean ± SD) during the bacterial challenge test.

2.9 Enzyme activities

The enzyme activities of trypsin, lipase and amylase were determined in the linear range by using enzyme assays kit (Biovision, USA) and spectrophotometer. The preparation of each specific enzyme assays kit was carried out with standard solutions, substrate and assay buffer. Trypsin activity was measured after reading the absorbance of samples at 405 nm wavelength at 0 and 20 min. Lipase activity was prepared by reaction mix including assay buffer, DTNB probe and lipase substrate and was read by spectrophotometer at a wavelength of 412 nm for 20 min. Amylase activity was reacted with assay buffer and substrate mix, and was determined by absorbance of samples at a wavelength of 402 nm at 0 and 20 min. Specific enzyme activities were defined as the amount of enzyme that catalysed the conversion of 1 μmol of substrate per minute per mU (i.e. mU/ml).

2.10 Statistical analysis

The obtained data were statistically analysed by using the one-way ANOVA (Statistix3.1; Analytical Software, St. Paul, MN, USA) in order to test the effects of dietary probiotic treatments. When a significant treatment effect was observed, an LSD post hoc test was used to compare means. Treatment effects were considered to be significant at $P < 0.05$.

3. Results

3.1 Growth performance and whole body proximate composition

The growth performances and survival of juvenile Nile tilapia fed different probiotic diets are shown in Table 3-3. Weight gain, specific growth rate, feed efficiency and protein efficiency ratio of fish fed BS_8, LL_8, BL_7, LL_7, ML_7 diets were significantly higher than those of fish fed CON diet (P<0.05), however, there were no significant difference among fish fed BS_8, LL_8, BL_7, LL_7, ML_7 and OTC diets (P>0.05). Fish survival varied from 90 to 96.7%, and no significant differences were shown among the all diets. On the other hand, there were no significant differences in hepatosomatic indices, viscerosomatic indices, and condition factor in all diets (P>0.05). No significant differences were observed on body protein, lipid, moisture and ash contents among treatment groups (P>0.05; Table 3-7).

Preferred Table 3-3.

Preferred Table 3-7.

3.2 Non-specific immune responses

The non-specific immune responses including lysozyme activity (LYZ), superoxide dismutase (SOD) and myeloperoxidase (MPO) were presented in Table 3-4. LYZ of fish fed BS_8, LL_8 and BL_7 diets were significantly higher than those of fish fed CON diet (P<0.05). SOD of fish fed BS_8, LL_8, BS_7, LL_7, ML_7 and OTC diets were significantly higher than those of fish fed CON diet (P<0.05). MPO activity of fish fed BS_8, LL_8, BS_7, LL_7, BL_7, ML_7 and OTC diets were significantly higher than those of fish fed CON diet (P<0.05). However, there were no significant differences for LZY, SOD and MPO among fish fed probiotics and OTC diets (P>0.05).

Preferred Table 3-4.

3.3 Intestinal histology

The intestinal histology of Nile tilapia fed the experimental diets for 8 weeks are illustrated in Fig 3-1 and Table 3-5. Intestinal villi length and muscular layer thickness of fish fed BS_8, LL_8, BL_7 and ML_7 diets were significantly higher than those of fish fed CON and OTC diets (P<0.05). However, there were no significant differences among fish fed probiotics diets.

Preferred Table 3-5.

Preferred Figure 3-1.

3.4 Haematological analysis

As shown in table 5, there were no significant differences in haematological analysis regarding alanine aminotransferase activity (ALT), glucose and total protein among fish fed the experimental diets (P<0.05). However, aspartate aminotransferase activity (AST) of fish fed OTC diet were significantly higher than those of fish fed the other diets (P<0.05).

Preferred Table 3-6.

3.5 Immune-related gene expressions

The immune-related gene expressions of the Nile tilapia intestine fed experimental diets are displayed in Fig 3-2. Heat shock protein 70 (HSP70) levels of BS_8, LL_8, ML_7 and LL_7 fed fish were significantly higher than those of fish fed CON and BS_7 diets (P<0.05). Moreover, there was no significant difference among OTC, BS_8, BL_7, ML_7 and LL_8 groups (P>0.05). The expression of interleukin (IL-1β) was higher in LL_8, BL_7 and ML_7 fed groups compared to those of fish fed CON, BS_7, LL_7 and OTC diets (P<0.05). However, IL-1β did not differ among the fish fed BS_8, LL_8, BL_7 and ML_7 and diets (P>0.05). On the other hand, tumour necrosis factor (TNF-α) and interferon-gamma (IFN-γ) expression in LL_8 and ML_7 fed fish were

significantly higher compared to other experimental diets (P<0.05), while they showed no significant differences among fish fed BS_8, BL_7 and OTC diets (P>0.05). ***Preferred Figure 3-2.***

3.6 Challenge test

Cumulative survival rate of juvenile Nile tilapia against *Aeromonas hydrophila* for 13 days is provided in Fig 3-3. Fish mortalities initially occurred on the third day post injection. At the end of 13 days of challenge test, cumulative survival rate of fish fed BS_8 and LL_8 diets were significantly higher than those of fish fed CON, BS_7, BL_7 and OTC diets (P<0.05). However, there were no significance differences among fish fed BS_8, LL_8, LL_7 and ML_7 diets (P>0.05).
Preferred Figure 3-3.

3.7 Enzyme activities

The specific digestive activity of juvenile Nile tilapia is shown in Fig 3-4. Trypsin activity of fish fed LL_8, BL_7 and LL_7 diets were significantly higher than those of fish fed CON, BS_7, ML_7 and OTC diets (P<0.05). However, there were no significant difference among BS_8, LL_8, BL_7 and LL_7 groups (P>0.05). Lipase and amylase activity did not differ among the all groups (P>0.05).
Preferred Figure 3-4.

4. Discussion

The role of probiotics in improving the growth and immune system has been considered and reviewed previously in aquaculture research [22, 23]. Probiotics including *Bacillus* sp., *Lactococcus* sp., and *Micrococcus* sp. are commonly used as dietary additives where they can provide beneficial effects on the health condition,

modulate intestinal homeostasis and promote gut health. To the best of our knowledge, the present study is first to compare potential probiotics such as *B. licheniformis*, *L. lactis*, *M. luteus* and *B. subtilis*, that could have beneficial effects on growth performance, health condition and disease resistance in Nile tilapia.

Based on our findings, probiotics improved growth performance and feed efficiency of Nile tilapia, which is in agreement with previous observations suggesting growth enhancement by *B. subtilis* [11], *B. licheniformis* [12], *L. lactis* [13] and *M. luteus* [14]. The improved growth performance and feed-utilization of Nile tilapia fed probiotic diets could be due to enhanced intestinal histology and enzyme activities. Probiotics may have facilitated effective nutrient absorption by improved intestinal villi length, muscle layer thickness and trypsin activity which have been proven by our results shown in Fig 3-1 and Table 3-6. Xia et al. [13] reported that *L. lactis* can improve intestinal health and increase growth performance of Nile tilapia compared to CON diet. Furthermore, our results are similar with the finding of Liu et al. [24], who reported that *B. subtilis* supplementation could enhance the digestive enzyme activities of Nile tilapia and thus improve fish growth performance. Also, probiotic supplementation have led to increased digestive enzyme activities in shrimp *Penaeus vannamei* and improved growth performance [25]. In rainbow trout *Onchorhynchus mykiss*, probiotic supplementation increased digestive enzyme activity and improved intestinal morphology which resulted in enhanced growth performance [26].

The non-specific immune parameters have been commonly used as indicators of health conditions in fish. Numerous studies [10, 27-29] have investigated probiotics as important compounds in immune system modulation and disease prevention. Superoxide dismutase (SOD) and myeloperoxidase (MPO) are known to regulate the reactive oxygen species in numerous target cells and catalyze the conversion of hydrogen peroxide and superoxide radicals to normal oxygen [30, 31]. In the current study, dietary probiotic groups improved SOD activity compared to CON group, but did not show significant differences with OTC. Moreover, MPO activity clearly showed a

similar trend with SOD activity. Among the innate immune system parameters, lysozyme plays a key role regarding protection against host infections and constitutes a chemical and biological barrier of first defence against pathogens in fish [32]. In the present study, lysozyme activity of fish fed BS_8, BL_7 and LL_8 were significantly higher than those of fish fed CON diet. However, there were no differences among probiotics and OTC diets. Previous studies demonstrated probiotics can enhance immune responses such as SOD, MPO and lysozyme in Nile tilapia [33, 34], lysozyme in rainbow trout [35], and SOD and lysozyme in grouper *Epinephelus malabaricus* [36]. One of the reasons for the improved immunity is the enhancement of phagocytic activity and reactive oxygen metabolites by macrophages. Observations from this study are in complete agreement with the findings of Newaj-Fyzul et al. [37] who found the number of leucocytes, respiratory burst and phagocytic activity increased significantly when *B. subtilis* was supplemented in rainbow trout diets. Ultimately, respiratory burst activity may have enhanced immunity by extracellular protection against pathogens [38, 39]. Because of these reasons, numerous studies on probiotics have been demonstrated that they are not only a potential antibiotic replacer in aquaculture but also elevate the growth, immune response and disease resistance of fish [40, 41].

Interestingly, in the present study, antibiotic fed fish showed significantly higher aspartate aminotransferase (AST) than other treatment groups. Haematological parameters are known to determine health conditions and are important indicators of fish health. Previous studies suggested that antibiotics can influence the liver by hepatotoxic and lead to damage through physiological stress [42, 43]. The AST value was not affected by probiotic supplementations in our study.

Cytokines (e.g., IL-1β, TNF- α and IFN- γ) are signaling proteins that regulate a wide range of biological functions such as stress, immune responses, hematopoiesis and inflammation mainly through extracellular signaling [44]. Specially, they are secreted by cells of both innate and adaptive immune systems [45]. Interferon-γ,

known as immune interferon, is primarily provided by activated T lymphocytes and possibly by natural killer cells. It has a pivotal mediator role for macrophage activation and has immunomodulatory properties [46]. The results of the present study showed that probiotic groups including BS_8, BL_7, ML_7 and LL_8 had significantly higher IFN- γ expression compared to the CON group. However, there were no differences with OTC diet. These results are similar with Xia et al. [13], which reported probiotic supplementation may influence the IFN-related gene, and improve disease resistance against *A. hydrophila.* IL-1β is an indicator of immune responses against pathogens and virus and participates in disease resistance, microbial invasion and tissue injury in fish, while TNF- α is mainly secreted by activated macrophages, and is involved in the immune response [46]. Observations of our study showed higher expression for IL-1β and TNF- α in fish fed LL_8 and ML_7 diets compared to CON, BS_7, LL_7 and OTC, but they did not show any differences among BS_8, LL_8, BS_7 and ML_7 diets (fig 3-2). This means that optimum probiotic levels in the diet of Nile tilapia can up regulate the expression of IL-1β and TNF- α genes. It was reported that the lymphoid cells in intestine were elevated by stimulation of probiotics [47]. Furthermore, Gatesoupe [15] concluded that probiotic supplementation in the diet can improve antagonistic activity against pathogens and increase the resistance of the host. Heat shock protein 70 (HSP70) has been lately recognized as a potential immune response regulator [48]. In this study, probiotic supplemented diets increased the expression of the HSP70 genes in the intestine of Nile tilapia, while this gene was not influenced by the OTC diet. According to previous research, the probiotic bacterial communities can improve immune system in terms of HSP70 in Japanese eel [11] and Nile tilapia [13]. Overall gene expressions showed that optimum probiotics in Nile tilapia diet can improve immune responses and stimulated cytokine and protein enzymes.

The intestinal histology including villi length and muscular layer thickness have been determined to estimate the gut condition of fish [49]. The villi length of Nile

tilapia fed probiotic diets was significantly higher in the mid-intestine than those of fish fed CON and OTC diets. Specially, BS_8 and LL_8 diets showed highest length among the experimental groups, which was in agreement with the previous reports [11, 13]. Likewise, muscular layer thickness was significantly higher in the mid-intestine of the probiotic supplemented groups compared to CON. However, there were no differences between probiotic and OTC groups. These results are in agreement with Lazado & Caipang [50], who reported that enhanced muscular layer thickness has been associated with probiotic administration. In addition, probiotics can modulate the physiological activities of gut mucosal cells. As previously stated, improvement of intestinal morphology increases the absorption of nutrients in surface area, and ultimately improves the growth performance.

The cumulative survival rate against *A. hydrophila* for 13 days were significantly higher in Nile tilapia when fed dietary probiotics compared to CON. Previous studies have reported that probiotic diets such as *B. subtilis*, *M. luteus* and *L. lactics* elevate disease resistance of Nile tilapia against bacteria [13, 14, 51]. According to what was mentioned, probiotics may have improved non-specific immunity of Nile tilapia and thus enhanced disease resistance against *A. hydrophila* infection. Further, the results of the current study corroborated with the previous studies on the increased disease resistance by probiotic supplementations [52, 53]. Besides, BS_8 and LL_8 diets showed potential effects as antibiotic replacers in Nile tilapia. The differing impact of probiotics with each concentration on disease resistance in tilapia can be related to differences in the antibiotic activities of specific probiotic strains.

Digestive enzyme activities are useful in estimating the potential of an organism to metabolize a given substrate, and also for establishment of feeding rhythms [54]. In the current study, trypsin activity of Nile tilapia was significantly influenced by probiotic supplementation (fig 3-4). According to Bowyer et al. [55], it was demonstrated that the response of the digestive enzyme activity is closely correlated with that of growth performance. Furthermore, probiotics secrete protease enzymes

that can digest the peptide bonds in proteins and break down the proteins into their constituent monomers and free amino acids; this process can benefit fish nutritionally [56]. In general, enzyme activity increases in fish when fed diets with probiotics, which was proven in common carp *Cyprinus carpio* [57], sea bass *Dicentrarchus labrax* [58] and Javanese carp *Puntius gonionotus* [59]. Meanwhile, lipase and amylase activities in our study were not affected by treatment diets. Some previous studies demonstrated that enzyme activities were not influenced by dietary probiotic supplementations in paddlefish *P. spathula* after 80 days [60] and in catla *C. catla* after 60 days [61]. The previous studies suggested that continuous feeding with excessive probiotics could inhibit the endogenous enzyme activities [60]. Apart from trypsin activity, further studies are needed to determine how probiotics improve enzyme activities such as lipase and amylase.

In conclusion, the current study suggested that dietary supplementation of *B. subtilis* (BS_8) and *L. lactis* (LL_8) at 1 x 10^8 (CFU/g) could replace antibiotic and enhance growth performance, immune responses, intestinal morphology, disease resistance and gene expression in Nile tilapia. *Oreochromis niloticus.*

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Figure legends

Figure 1. Histological sections of juvenile Nile tilapia intestine fed the experimental diets for 8 weeks. A-CON, basal diet; B-BS_7, *Bacillus subtilis* at 1 x 10^7 CFU/g; C-BL_7, *Bacillus licheniformis* at 1 x 10^7 CFU/g; D-LL_7, *Lactococcus lactis* at 1 x 10^7 CFU/g; E-ML_7, *Micrococcus luteus* at 1 x 10^7 CFU/g; F-BS_8, *Bacillus subtilis* at 1 x 10^8 CFU/g; G-LL_7, *Lactococcus lactis* at 1 x 10^8 CFU/g; H-OTC, oxytetracycline; oxytetracycline at 4g/kg. (Scale bar = 100 μm; Original magnification × 4).

Figure 2. Intestinal gene expression levels of Heat shock protein 70 (HSP70), interleukin (IL-1 ß), interferon-gamma (IFN-γ) and tumour necrosis factor (TNF-α) were evaluated in juvenile Nile tilapia fed the experimental diets for 8 weeks. Bars with range represent mean ± SD of triplicate samples, and diets refer to Fig 1.

Figure 3. Cumulative survival rate of juvenile Nile tilapia fed the experimental diets with different probiotics for 8 weeks and experimentally challenged with *A. hydrophila* for 13 days. Each value represents mean of triplicate groups. Significant differences among means are indicated by different superscripts (P<0.05), and diets refer to Fig 1.

Figure 4. Specific enzyme activities of 1 Trypsin, 2 Amylase and 3 Lipase measured in the intestines of juvenile Nile tilapia fed the experimental diets with different probiotics for 8 weeks, and diets refer to Fig 1.

6. Table and figures

Table 3-1. Formulation and composition (% dry matter) of the basal diet for Nile tilapia

Ingredients	%
Tuna by-product[1]	17.0
Squid liver powder[2]	3.0
Blood meal[2]	5.0
Soybean meal[2]	31.5
Wheat gluten meal[2]	6.1
Wheat flour[2]	19.5
Corn starch[2]	10.5
Soybean oil[3]	2.4
Vitamin premix[4]	1.0
Mineral premix[5]	1.0
Calcium phosphate[6]	2.0
Cellulose[6]	1.0
Proximate composition	
Moisture	9.89
Crude protein	36.5
Crude lipid	6.19
Crude ash	6.24

[1]Suhyup Feed Co. Uiryeong, Korea

[2]The Feed Co. Goyang, Korea

[3]Jeil Feed Co. Hamman, Korea

[4]Contains (as mg/kg in diets) : Ascorbic acid, 300; dl-Calcium pantothenate, 150; Choline bitate, 3000; Inositol, 150; Menadion, 6; Niacin, 150; Pyridoxine · HCl, 15; Rivoflavin, 30; Thiamine mononitrate, 15; dl-α-Tocopherol acetate, 201; Retinyl acetate, 6; Biotin, 1.5; Folic acid, 5.4; Cobalamin, 0.06

[5]Contains (as mg/kg in diets) : NaCl, 437.4; $MgSO_4 \cdot 7H_2O$, 1379.8; $ZnSO_4 \cdot 7H_2O$, 226.4; Fe-Citrate, 299; $MnSO_4$, 0.016; $FeSO_4$, 0.0378; $CuSO_4$, 0.00033; $Ca(IO)_3$, 0.0006; MgO, 0.00135; $NaSeO_3$, 0.00025

[6]Sigma-Aldrich Korea, Yongin, Korea

Table 3-2. Primers used to quantify relative gene expression

Primers	Sense	Sequences
HSP90[1]	F	5′-CATCGCCTACGGTCTGGACAA-3′
	R	5′-TGCCGTCTTCAATGGTCAGGAT-3′
IL-1ß[2]	F	5′-CAAGGATGACGACAAGCCAACC-3′
	R	5′-AGCGGACAGACATGAGAGTGC-3′
IFN-γ[3]	F	5′-AAGAATCGCAGCTCTGCACCAT-3′
	R	5′-GTGTCGTATTGCTGTGGCTTCC-3′
TNF-α[4]	F	5′-GGAAGCAGCTCCACTCTGATGA-3′
	R	5′-CACAGCGTGTCTCCTTCGTTCA-3′
ß-actin[5]	F	5′-CCACACAGTGCCCATCTACGA-3′
	R	5′-CCACGCTCTGTCAGGATCTTCA-3′

[1]HSP90, heat shock protein 90 (FJ207463.1)

[2] IL-1ß, interleukin (XM_003460625.2)

[3] IFN-γ, interferon-gamma (XM_005448319.1)

[4]TNF-α, tumour necrosis factor (JF957373.1)

[5]ß-actin (HQ386788.1)

Table 3-3. Growth performance and feed utilization of juvenile Nile tilapia fed the experimental diets for 8 weeks.

	Diets[1]							
	CON	BS_7	BL_7	LL_7	ML_7	BS_8	LL_8	OTC
IBW (g)[2]	2.85±0.05	2.83±0.06	2.83±0.08	2.82±0.08	2.82±0.03	2.80±0.05	2.85±0.05	2.83±0.03
FBW (g)[3]	9.77±0.27^{b}	10.3±0.23^{ab}	10.7±0.47^{a}	10.6±0.55^{a}	10.8±0.42^{a}	10.6±0.37^{a}	10.8±0.65^{a}	10.6±0.36^{a}
WG (%)[4]	242±15.3^{b}	264±13.1^{ab}	279±12.9^{a}	278±14.8^{a}	282±11.3^{a}	277±9.80^{a}	279±23.4^{a}	276±15.7^{a}
FE (%)[5]	81.9±3.02^{b}	90.4±3. 11^{a}	94.3±3.33^{a}	92.7±2.86^{a}	95.4±2.04^{a}	93.3±3.08^{a}	94.3±7.51^{a}	93.5±4.46^{a}
SGR(%/day)[6]	2.37±0.09^{b}	2.48±0.07^{ab}	2.56±0.06^{a}	2.56±0.08^{a}	2.57±0.06^{a}	2.55±0.05^{a}	2.56±0.12^{a}	2.54±0.08^{a}
PER[7]	2.31±0.08^{b}	2.51±0.09^{a}	2.59±0.09^{a}	2.53±0.08^{a}	2.60±0.06^{a}	2.57±0.08^{a}	2.59±0.16^{a}	2.58±0.12^{a}
HSI (%)[8]	1.46±0.27	1.36±0.31	1.34±0.28	1.45±0.29	1.45±0.37	1.35±0.32	1.45±0.34	1.46±0.30
VSI (%)[9]	7.39±0.79	7.75±1.70	7.96±0.74	7.12±1.30	7.94±0.96	7.43±1.32	7.84±1.17	7.70±1.52
CF[10]	1.70±0.08	1.71±0.10	1.75±0.10	1.74±0.08	1.66±0.10	1.69±0.08	1.70±0.11	1.64±0.08
Survival[11]	90.0±5.00	96.7±2.89	90.0±0.00	95.0±0.00	95.0±5.00	95.0±5.00	95.0±5.00	96.7±5.77

[1]Values are means ± SD of triplicate groups of fish. Values in each row with different superscripts are significantly different ($P< 0.05$).

Diets: CON = the basal diet, refer to Table 1; BS_7 = *Bacillus subtilis* at 1 x 10^7 CFU/g; BL_7 = *Bacillus licheniformis* at 1 x 10^7 CFU/g; LL_7 = *Lactococcus lactis* at 1 x 10^7 CFU/g; ML_7 = *Micrococcus luteus* at 1 x 10^7 CFU/g; BS_8 = *Bacillus subtilis* at 1 x 10^8 CFU/g; LL_8 = *Lactococcus lactis* at 1 x 10^8 CFU/g; OTC = oxytetracycline; oxytetracycline at 4g/kg.

[2] Initial body weight

[3] Final body weight

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

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

[6] Specific growth rate (SGR, %) = [($\log_e$ final wt. - $\log_e$ initial wt.) × 100] / days

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

[8] Hepatosomatic index (HSI) = (liver wt. × 100) / body wt

[9] Visceralsomatic index (VSI, %) = (viscera wt. × 100) / body wt

[10] Condition factor = (wet weight / total length^3) × 100

[11] Survival rate = [(total fish – dead fish) × 100] / total fish

Table 3-4. Non-specific immune responses of juvenile Nile tilapia fed the experimental diets for 8 weeks

	Diets[1]							
	CON	BS_7	BL_7	LL_7	ML_7	BS_8	LL_8	OTC
Lysozyme (U/ml)	1.23±0.12^{b}	1.68±0.37^{ab}	1.72±0.30^{a}	1.67±0.17^{ab}	1.68±0.34^{ab}	1.69±0.30^{a}	1.80±0.15^{a}	1.47±0.24^{ab}
SOD[2]	64.8±11.6^{b}	81.2±7.05^{a}	75.4±10.2^{ab}	86.2±5.45^{a}	81.4±4.76^{a}	83.5±1.83^{a}	81.1±1.61^{a}	81.2±8.13^{a}
MPO[3]	2.71±0.53^{c}	3.48±0.50^{b}	3.65±0.42^{ab}	4.13±0.33^{a}	3.80±0.13^{ab}	3.94±0.23^{ab}	3.90±0.22^{ab}	3.68±0.43^{ab}

[1]Values are means ± SD of triplicate groups of fish. Values in each row with different superscripts are significantly different (P<0.05). Diets refer to Table 3.

[2]SOD: Superoxide dismutase activity (% inhibition)

[3]MPO: Myeloperoxidase activity (OD at 450 nm)

Table 3-5. Intestinal morphology of juvenile Nile tilapia fed the experimental diets for 8 weeks

	Diets[1]							
	CON	BS_7	BL_7	LL_7	ML_7	BS_8	LL_8	OTC
Villi length (μm)	202±7.58^{d}	253±18.8^{b}	272±9.03^{ab}	273±19.9^{ab}	272±2.50^{ab}	290±3.23^{a}	285±8.04^{a}	235±7.41^{c}
Muscular layer thickness (μm)	36.4±2.82^{c}	50.2±6.10^{ab}	53.1±3.25^{a}	53.3±2.00^{ab}	54.5±8.17^{a}	59.0±2.10^{a}	54.9±1.47^{a}	46.2±1.44^{b}

[1]Values are means ± SD of triplicate groups of fish. Values in each row with different superscripts are significantly different (P<0.05). Diets refer to Table 3.

Table 3-6. Haematological analysis of juvenile Nile tilapia fed the experimental diets for 8 weeks

	Diets[1]							
	CON	BS_7	BL_7	LL_7	ML_7	BS_8	LL_8	OTC
AST[2]	66.7±18.83^{b}	78.3±13.9^{b}	58.3±8.40^{b}	56.7±13.7^{b}	69.0±3.60^{b}	63.3±13.6^{b}	67.0±15.1^{b}	108±12.3^{a}
ALT[3]	5.33±0.58	5.33±0.58	5.67±0.58	6.00±0.00	5.67±0.58	5.33±0.58	5.33±0.58	5.33±0.58
Glucose (mg dL^{-1})	43.7±5.51	50.3±4.04	41.3±5.77	41.7±5.13	50.0±9.54	50.7±4.93	47.7±7.37	44.0±5.29
T-protein[4]	2.70±0.53	2.87±0.64	3.07±0.57	3.00±0.40	3.17±0.32	2.53±0.47	2.73±0.78	3.23±0.64

[1]Values are means ± SD of triplicate groups of fish. Values in each row with different superscripts are significantly different (P<0.05). Diets refer to Table 3.

[2]AST: Aspartate aminotransferase activity (U L^{-1})

[3] ALT: Alanine aminotransferase activity (U L^{-1})

[4]T-protein: Total protein (g dL^{-1})

Table 3-7. Whole-body proximate composition (% dry matter) of juvenile Nile tilapia fed the experimental diets for 8 weeks

	Diets[1]							
	CON	BS_7	BL_7	LL_7	ML_7	BS_8	LL_8	OTC
Moisture	7.15±0.49	7.46±0.74	7.38±0.25	7.31±0.52	7.19±0.74	7.20±0.33	7.19±0.74	7.24±0.28
Protein	34.8±0.27	35.7±1.03	35.9±0.44	34.9±0.30	35.7±1.12	34.5±0.91	35.2±0.30	35.2±0.27
Lipid	7.65±0.77	8.10±0.58	7.85±0.61	8.15±0.28	7.76±0.82	8.02±0.40	8.32±0.28	7.90±0.61
Ash	7.14±0.71	7.28±0.38	7.35±0.42	7.44±0.27	7.50±0.31	7.75±0.13	7.55±0.27	6.98±0.18

[1]Values are means ± SD of triplicate groups of fish. Values in each row with different superscripts are significantly different (P<0.05). Diets refer to Table 3.

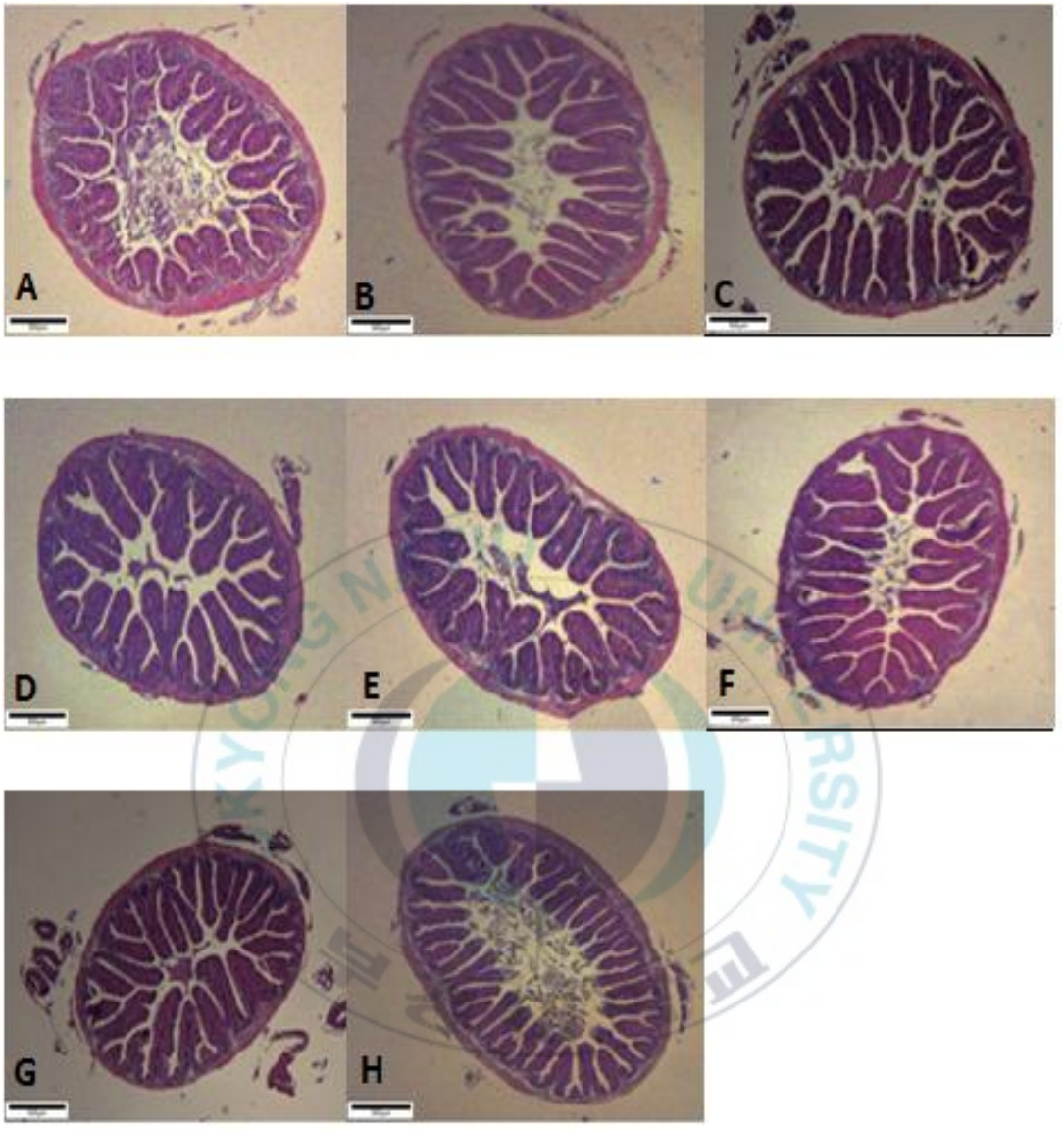
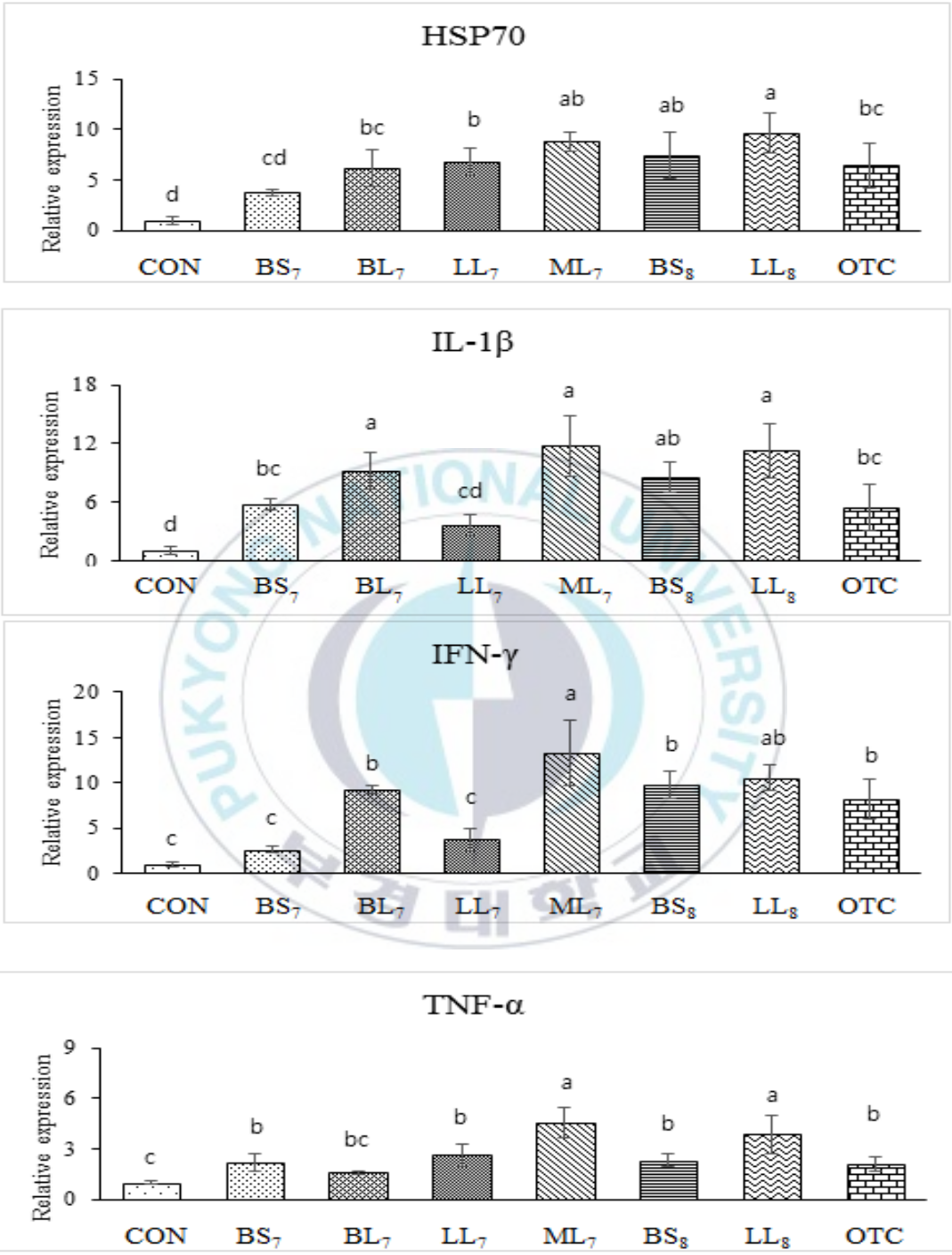


Fig 3-1.


HSP70
Relative expression
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10
5
0
d
cd
bc
b
ab
ab
a
bc
CON
BS7
BL7
LL7
ML7
BS8
LL8
OTC
IL-1β
Relative expression
18
12
6
0
d
bc
a
cd
a
ab
a
bc
CON
BS7
BL7
LL7
ML7
BS8
LL8
OTC
IFN-γ
Relative expression
20
15
10
5
0
c
c
b
c
a
b
ab
b
CON
BS7
BL7
LL7
ML7
BS8
LL8
OTC
TNF-α
Relative expression
9
6
3
0
c
b
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b
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b
a
b
CON
BS7
BL7
LL7
ML7
BS8
LL8
OTC

Fig 3-2.

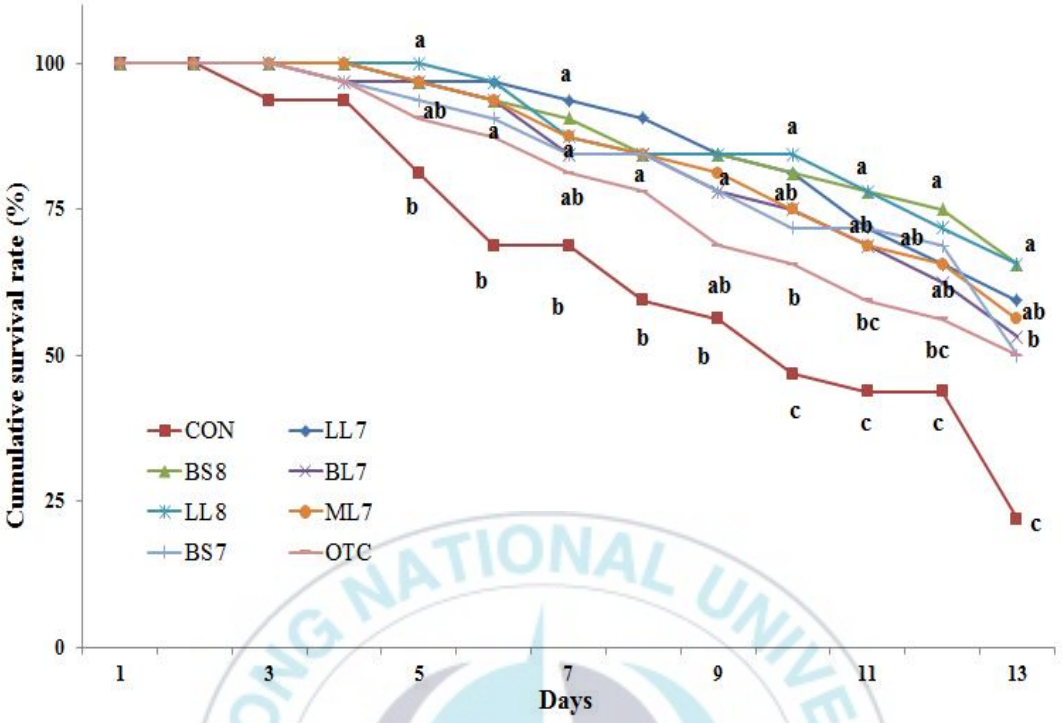

Fig 3-3.

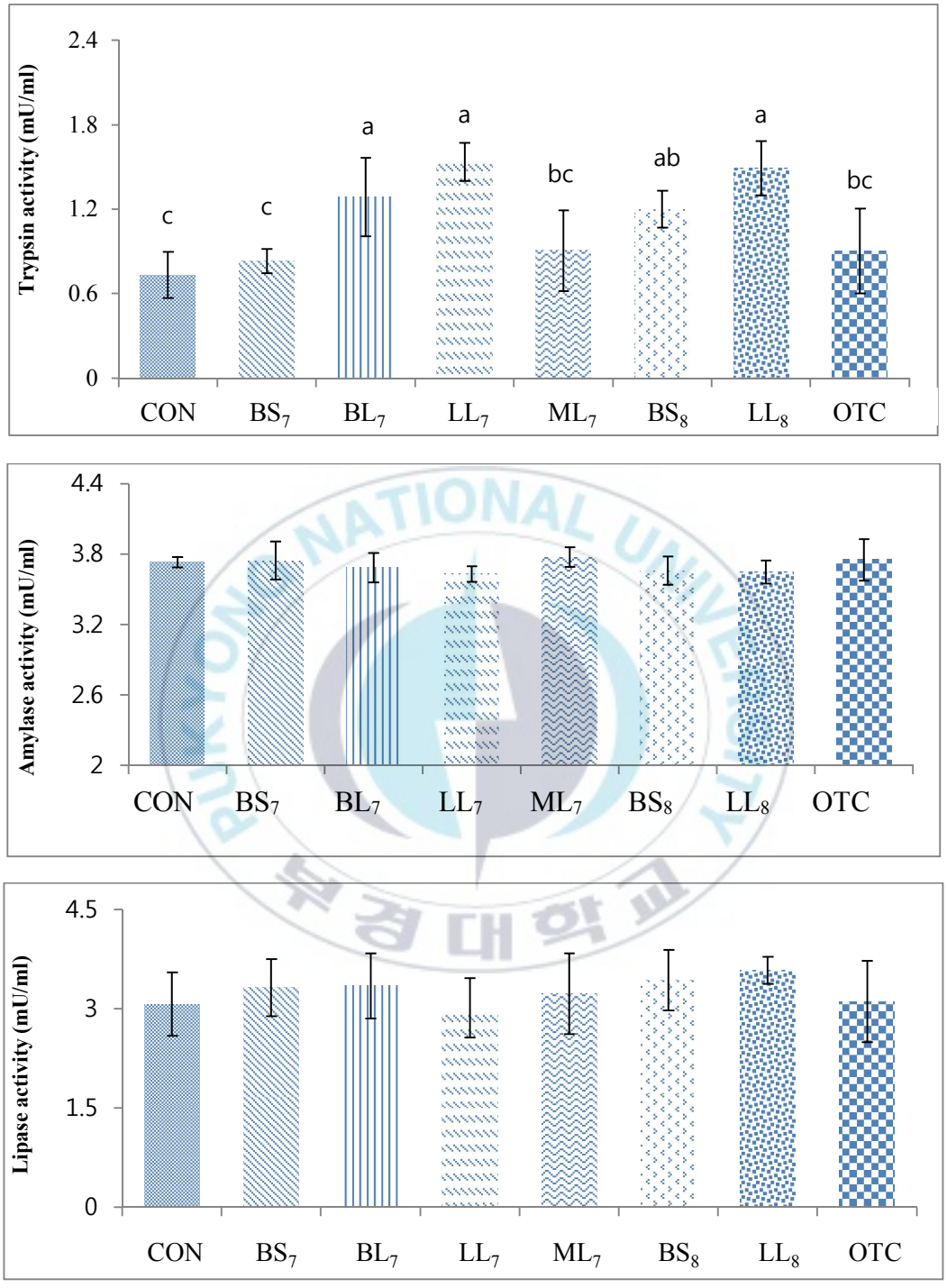
Trypsin activity (mU/ml)
2.4
1.8
1.2
0.6
0
c
c
a
a
bc
ab
a
bc
CON
BS7
BL7
LL7
ML7
BS8
LL8
OTC
Amylase activity (mU/ml)
4.4
3.8
3.2
2.6
2
CON
BS7
BL7
LL7
ML7
BS8
LL8
OTC
Lipase activity (mU/ml)
4.5
3
1.5
0
CON
BS7
BL7
LL7
ML7
BS8
LL8
OTC

Fig 3-4.

CHAPTER IV. Comparative evaluation of dietary probiotcs *Bacillus subtilis* and *Enterococcus faecium* on growth, immune responses, histology and gene expression in Nile tilapia, *Oreochromis niloticus*

1. Introduction

Disease outbreaks have become a primary challenge to the profitable culture of fish and shellfish as aquaculture operations intensify. Globally, total annual losses from disease outbreaks have reached billions of dollars (US) and have been identified as a threat to the sustainability of the industry [1]. Because of these reason, antibiotics have been used to control a variety of disease in fish [2]. The use of antibiotics leads to environmental pollution and the development of drug-resistant pathogens [3]. Thus, an alternative to prevent and control pathogenic bacteria is the use of probiotics. Among the probiotics, Bacteria species, *B. subtilis*, is one of the Gram-positive and widely studied bacteria that lives in gastrointestinal tract of many animals and provides many beneficial enzymes [4]. This bacteria has been reported to have many effects on improvement growth, immune and disease resistances [5-7]. On the other hand, *E. faecium* is one of the probiotics that can be used for controlling disease infection [8]. It can induce elevated immune responses such as macrophagocyte and hydrogen peroxide [9].

As it was mentioned before, a number of properties are required for the description of probiotics. Gatesoupe [10] reported that survival and colonization in the gastrointestinal tract (GI) are important to recognize the characterization of probiotics. Furthermore, Balcázar et al. [11] suggested the application of highly viable probiotics in the GI and reported that using the probiotics isolated from the host would help to ensure stability and survival. Afterwards, studies on probiotic isolation from GI of different fish species have been continuously conducted and this method was recognized as a way for selecting efficient bacteria [12, 13, 14].

Therefore, the present study was conducted to evaluate and compare the efficacy of two different probiotics, *B. subtilis* WB60 and *E. faecium* SH30 on growth performance, immune responses, hematology, enzyme activity, disease resistance and gene expression in Nile tilapia, *Oreochromis niloticus.*

2. Materials and methods

The study was conducted under the guidelines of the Animal Ethics Committee Regulations, No.19-0045 issued by the Pukyong National University, Busan, Rep. Korea.

2.1 Probiotic conditions

The probiotic strains, *E. faecium* SH30 and *B. subtilis* WB60 were isolated from the intestine of healthy Nile tilapia and Japanese eel, respectively. *B. subtilis* was isolated from the intestine of juvenile Japanese eel and identified as *B. subtilis* WB60 according to Lee et al. [12]. This probiotic was incubated at 30 ºC for 72h in LB broth (Sigma-Aldrich, St. Louis, USA) and measured at 600 nm optical density (OD_{600}) using spectrophotometer. On the other hand, *E. faecium* was isolated from the intestine of juvenile Nile tilapia and identified as *E. faecium* SH30. *E. faecium* was grown in MRS broth at 36 ºC for 48h following Xia et al. [15]. All probiotics were washed in sterile saline and the concentration of the final suspension was calculated at 1 x 10^6, 10^7 and 10^8 in the diets.

2.2 Safety of the isolated probiotic bacteria

The safety of two probiotic strains was evaluated by using 60 healthy Nile tilapia (4-8 g/fish). The fish were acclimatized for two weeks in the rectangular tanks. The fish were distributed into 3 tanks with triplicate groups. Two groups were intra-peritoneal (IP) injected by 0.3ml of saline containing 10^7 cells ml^{-1} of 24 h of *E. faecium* and *B. subtilis*, respectively. The other group was IP injected by 0.3ml of sterile saline as CON. All groups were maintained under observation for two weeks

and the mortality was recorded. The method for safety of the isolated probiotic bacteria was followed as previous described by El-Rhman et al., 2009.

2.3 Antibiotic Susceptibility

E. faecium and *B. subtilis* were evaluated for the resistance to a panel of antibiotics, following by the European Food Safety Authority (EFSA 2012) and recommended by the NCCLS (1997).

2.4 Experimental diets

Feed formulation and proximate composition of the basal diet is shown in Table 1. A basal control diet without supplementation of probiotics (CON), and seven other diets were prepared by supplementing three graded levels of *B. subtilis* or *E. faecium* at 10^6, 10^7 and 10^8 CFU/g diet (BS_6, BS_7, BS_8, EF_6, EF_7 and EF_8, respectively), and oxytetracycline at 4g/kg diet (OTC). Tuna by-product, squid liver powder, blood meal, poultry by-product meal, tankage meal, meat and bone meal and soybean meal were used as the major protein sources. While, soybean oil served as the lipid source and wheat flour was the carbohydrate source. The preparation and storage of experimental diets were followed by Bai & Kim [16]. Briefly, all the dry ingredients were measured and mixed in an electric mixing machine, followed by the addition of soybean oil and water until dough was formed. Experimental diets were pelleted using a laboratory pelleting machine with a 1 mm diameter module (Baokyong Cmmercial Co., Busan, Republic of Korea). The pellets were air-dried for 72 hours and stored at -20ºC until use.

Preferred Table 4-1.

2.5 Experimental fish and feeding trial

The feeding trial was carried out at the Feeds and Foods Nutrition Research Center (FFNRC), Pukyoung National University, Busan, Republic of Korea. Fish

were acclimatized to the FFNRC condition for two weeks and fed a basal diet. Prior to the feeding trial, fish were examined for external abnormalities and starved for 24 h. At the begging of the experiment, fish with the initial body weight of 0.85 ± 0.01 g (mean ± SD) were distributed into 24 (40-L) rectangular tanks (20 fish/tank) receiving filtered freshwater at the a constant flow (1.5 L/min) from the main tank. Each tank was randomly allocated to one of the three replicates of the eight dietary treatments. Fish were fed twice daily (09:00 and 18:00 h) at 3-4% of wet body weight/day for 8 weeks. Supplemental aeration was provided to maintain the dissolved oxygen. The temperature of aquarium was maintained at 26.5 ± 0.5ºC and pH remained at 7.50 ± 0.05 throughout the feeding trial. The condition of tank were maintained by siphoning off uneaten feeds 2 h after feeding and the walls and bottom of the tanks were scrubbed once a week.

2.6 Sample collection and analysis

At the end of the feeding trial, all the fish were starved for 24 h. The number and weight of total fish in separated tanks were measured to calculate final body weight (FBW), weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER) and survival. Three fish from each tank were randomly selected, individually weighed and dissected to estimate liver and gastro-intenstinal organs for the measurement of hepatosomatic index (HSI), viscerosomatic index (VSI) and condition factor (CF). Additionally, three fish from each treatment group was anesthetized by tricaine methanesulfonate (MS 222, 100ppm) to collect the blood samples. Serum samples were separated by centrifugation at 5,000 *g* for 10 min and stored at -70°C for the analysis of non-specific immune responses including superoxide dismutase (SOD), myeloperoxidase (MPO) and lysozyme, as well as biochemical parameters such as glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), total protein (TP) and glucose. Intestine of fish samples were used for the preparations of histological sections and measurements of digestive enzyme activity.

The proximate composition of experimental diets and whole-body samples were analysed according to standard methods of AOAC [17]. Moisture content was measured after oven-drying the samples at 105°C to constant weight, while crude ash was estimated after incineration at 550°C for 3 h. Crude protein was measured using the Kjeldahl method (N × 6.25) after acid digestion, and crude lipid was determined by soxhlet extraction using the soxhlet system 1046 (Tacator AB, Hoganas, Sweden) after freeze-drying the samples for 20 h.

2.7 Non-specific immune responses analysis

Lysozyme activity was determined by adding 0.1 ml serum sample to 2 ml of *Micrococcus lysodeikticus* (0.2 mg/ml) suspension in a 0.05 M sodium phosphate buffer (pH 5.5). The reactions were carried out at 20℃ and the absorbance of sample at 530 nm wavelength was measured between 0.5 min and 4.5 min with a spectrophotometer. The sample unit was defined as the amount of enzyme yielding a decrease in absorbance of 0.001/min. On the contrary, activity of SOD was analysed by the superoxide radical dependent reaction inhibition rate of enzyme with water soluble tetrazolium dye substrate and xanthine oxidase using the SOD Assay Kit (Sigma-Aldrich, 19160) following the manufacturer's instructions. Each endpoint assay was monitored at 450 nm absorbance after incubating at 37°C for 20 min. The percent inhibition was normalized by mg protein and expressed as SOD unit/mg. In addition, MPO activity was determined according to the method described by Quade & Roth [18]. Briefly, 20 µL of serum was diluted with HBSS (Hanks Balanced Salt Solution) without Ca^{2+} or Mg^{2+} (Sigma-Aldrich) in each well of a 96-well plate to which 35 µL of 3, 3', 5, 5' tetramethylbenzidine hydrochloride (TMB, 20 mM; Sigma-Aldrich) and H_2O_2 (5 mM) were subsequently added. The colour change reaction after 2 min was completed by supplementing 35 µL of 4 M sulphuric acid. Finally, the optical density was read at 450 nm in a spectrophotometer.

2.8 Real-time PCR

Five fish per experimental diets were used for sample analysis after being anesthetized. Total RNA was extracted from mid intestine (50mg) of fish using RiboEx™ (GeneALL, Seoul, Rep. Korea) following standard procedure (Riboclear plus, GeneAll, South Korea). RNA concentration (ng/µl) and purity (OD 260:280) were quantified with nanodrop measurement (Thremo Fisher Scientific, USA). The cDNA was synthesized from 1 µg of RNA using manufacturers` instructions of cDNA synthesis Kit (Takara, Japan). RNA isolation and preparation of cDNA by 1 µg of RNA was performed following Hasan et al. [19]. Then, primer and target gene were prepared by BIONICS company (Seoul, Rep. Korea; Table 4-2). Relative RNA level of the target genes (IL-1ß, TNF-α, IFN-γ and HSP90) were evaluated and calculated using endogenous β-actin RNA level.

Preferred Table 4-2.

2.9 Histomorphology of the intestine

The mid-intestinal samples were collected from experimental groups (n=5) and were preserved in 10% neutral buffered formalin, dehydrated in a graded ethanol series and embedded in paraffin according to standard histological process. Sections series of 6 µm were made with a microtome and stained with hematoxylin and eosin (H&E). The evaluation of villi height (VL) and muscular thickness (MT) was observed using light microscope (AX70 Olympus, Tokyo, Japan) equipped with scientific digital camera for microscopy (DIXI Optics, Daejeon, Republic of Korea) and processed by image analysis software (Image J 1.32j, National Institute of Health, USA).

2.10 Challenge test

Aeromonas hydrophila is a well-known pathogen that generally causes disease in Nile tilapia. The pathogenic bacterium, *A. hydrophila* KCTC2358, was received from

the Department of Biotechnology, Pukyong National University, Busan, Republic of Korea. At first, bacteria was grown in 10 ml brain heart infusion (BHI; Becton, Dickinson and Company, USA) broth and incubated at 37 °C for 24 h in a shaking incubator. Growth of *A. hydrophila* was observed by optical density of 600 (OD_{600} nm) using a spectrophotometer (Mecasys, Optizen, Republic of Korea), harvested by centrifugation and washed two times with 0.1 M PBS for further use. At the end of the experiment, eight fish from each tank were randomly collected and reorganized based on their previous dietary treatment groups in 12-L tanks and allowed to maintain for 24 h. Fish were injected intraperitoneally with 0.1 mL per fish of *A. hydrophila* KCTC2358 at 2×10^7 CFU/mL ($2 \times LD_{50}$). Fish mortality was documented daily up to 13 days. Water temperature was controlled at 27 ± 0.5°C (mean ± SD) during the bacterial challenge test.

2.11 Enzyme activities

The enzyme activities of trypsin, lipase and amylase were determined in the linear range by using enzyme assays kit (Biovision, USA) and spectrophotometer. The preparation of each specific enzyme assays kit was carried out with standard solutions, substrate and assay buffer. Trypsin activity was measured after reading the absorbance of samples at 405 nm wavelength at 0 and 20 min. Lipase activity was prepared by reaction mix including assay buffer, DTNB probe and lipase substrate and was read by spectrophotometer at a wavelength of 412 nm for 20 min. Amylase activity was reacted with assay buffer and substrate mix, and was determined by absorbance of samples at a wavelength of 402 nm at 0 and 20 min. Specific enzyme activities were defined as the amount of enzyme that catalysed the conversion of 1 μmol of substrate per minute per mU (i.e. mU/ml).

2.12 Statistical analysis

The obtained data were statistically analysed by using the one-way ANOVA (Statistix3.1; Analytical Software, St. Paul, MN, USA) in order to test the effects of dietary probiotic treatments. When a significant treatment effect was observed, an LSD post hoc test was used to compare means. Treatment effects were considered to be significant at $P < 0.05$.

3. Results

3.1 Safety of the isolated probiotics and Antibiotic Susceptibility

The test of safety of fish with the isolated two probiotics did not induce any significant differences among all groups ($P>0.05$; Table 4-3). On the other hand, the antibiotic sensitivity test of two probiotics are listed in Table 4-4 which shows that *B. subtilis* and *E. faecium* are sensitive to all selected antibiotics as suggested by EFSA (Table 4-4).

Preferred Table 4-3.

Preferred Table 4-4.

3.2 Growth performance and whole body proximate composition

The growth performances and feed utilization of juvenile Nile tilapia fed experimental diets are presented in Table 4-3. Final body weight (FBW), weight gain (WG), specific growth rate (SGR), feed efficiency (FE) and protein efficiency ratio (PER) of fish fed EF_8, BS_8 and EF_7 diets were significantly higher than those of fish fed CON diet ($P<0.05$), while, there were no significant difference in FBW, WG, SGR, FE and PER among fish fed probiotic supplemented and OTC diets ($P>0.05$). Fish survival varied from 90 to 96.7%, and no significant differences were shown among the all diets. On the other hand, there were no significant differences in hepatosomatic indices, viscerosomatic indices, and condition factor in all diets

(P>0.05). No significant differences were observed on body protein, lipid, moisture and ash contents among treatment groups (P>0.05; Table 4-7).

Preferred Table 4-5.

Preferred Table 4-9.

3.3 Non-specific immune responses

Table 4-4 shows the non-specific immune responses of juvenile Nile tilapia fed experimental diets for 8 weeks. Lysozyme and superoxide dismutase activity of fish fed EF_8, BS_8, EF_7 and EF_6 diets significantly improved compared to CON diet (P<0.05). However, there were no significant differences among fish fed probiotics and OTC diets (P>0.05). Myeloperoxidase of fish fed EF_8, BS_8, EF_7, EF_6 and OTC diets were significantly higher than those of fish fed CON diet (P<0.05). Likewise, there were no significant differences among fish fed probiotics and OTC diets (P>0.05).

Preferred Table 4-6.

3.4 Haematological analysis

Haematological analysis of juvenile Nile tilapia fed the experimental diets for 8 weeks are shown in 4-6. There were no significant differences in haematological analysis including aspartate aminotransferase activity (AST), alanine aminotransferase activity (ALT), glucose and total protein among fish fed the experimental diets (P>0.05).

Preferred Table 4-8.

3.5 Intestinal histology

The intestinal histology of Nile tilapia fed the experimental diets for 8 weeks are illustrated in Fig 4-1 and Table 4-5. Intestinal villi length of fish fed EF_8 and EF_7

diets were significantly higher than those of fish fed BS_7, BS_6, OTC and CON diets (P<0.05). On the other hand, muscular layer thickness of fish fed EF_8, BS_8, EF_7, BS_7, EF_6 diets were significantly higher than those of fish fed CON and OTC diets (P<0.05).

Preferred Figure 4-1.

Preferred Table 4-7.

3.6 Immune-related gene expressions

The immune-related gene expressions of the Nile tilapia intestine fed experimental diets are displayed in Fig 4-2. Heat shock protein 70 (HSP70) levels of fish fed EF_8, BS_8 and EF_7 were significantly higher than those of fish fed OTC and CON diets (P<0.05). However, there was no significant difference among EF_8, BS_8, EF_7 and EF_6 groups (P>0.05). The expression of interleukin (IL-1β) of fish fed probiotic and OTC diets were significantly improved compared to CON diet (P<0.05). Interferon-gamma (IFN-γ) expression of fish fed EF_8, BS_8, EF_7, BS_7 and EF_6 diets were significantly higher than those of fish fed BS_6, CON and OTC diets (P<0.05). On the other hand, tumour necrosis factor (TNF-α) expression in probiotic groups fed fish were significantly higher than those of fish fed CON diet (P<0.05), while they showed no significant differences among fish fed FE_8, BS_8, BS_7, BS_6, EF_6 and OTC diets (P>0.05).

Preferred Figure 4-2.

3.7 Challenge test

Cumulative survival rate of juvenile Nile tilapia against *Aeromonas hydrophila* for 11 days is provided in Fig 4-3. Fish mortalities initially occurred on the third day post injection. At the end of 11 days of challenge test, cumulative survival rate of fish fed EF_8 and EF_7 diets were significantly higher than those of fish fed CON, BS_6

and OTC diets (P<0.05). However, there were no significance differences among fish fed BS_8, BS_7, EF_6 and OTC diets (P>0.05).

Preferred Figure 4-3.

3.8 Enzyme activities

The specific digestive activity of juvenile Nile tilapia is shown in Fig 4-4. Trypsin activity of fish fed EF_8, BS_8, EF_7 and EF_6 diets were significantly higher than those of fish fed BS_6, OTC and CON diets (P<0.05). However, there were no significant difference among BS_7, BS_6 and OTC diets (P>0.05). Lipase activity of fish fed EF_8, EF_7 and EF_6 diets were significantly improved compared to CON diet (P<0.05). However, they did not show any significant differences among fish fed probiotic and OTC diets (P>0.05). Amylase activity of fish fed EF_8, BS_8 and EF_7 diets were significantly better than those of fish fed BS_7, BS_6, CON and OTC diets (P<0.05). Whereas, there were no significant differences among BS_7, BS_6, EF_6 and OTC diets (P>0.05).

Preferred Figure 4-4.

4. Discussion

The researches of probiotic have been previously shown to be beneficial effects when supplemented in the diet of various fish species such as cobia, *Rachycentron canadum* [20], Rainbow trout, *Oncorhynchus mykiss* [21], Gilthead seabream, *Sparus aurata* L. [22], Striped catfish, *Pangasianodon hypophthalmus* [23] and White shrimp, *Litopenaeus vannamei* [24].

According to our finding, enhanced growth and feed utilization may be due to improved histology and digestive enzyme activity. Several studies indicated that administration of probiotics could improve the nutritional factor through improving the microvilli length and the breakdown of indigestible components [25-27]. The

present study further demonstrates a high efficiency of probiotic, *E. faecium* at $10^{7\sim8}$ cfu/g and *B. subtilis* at 10^{8} cfu/g compare to other diets in terms of growth and feed utilization in Nile tilapia.

Non-specific immunity is the fundamental defense system in fish. Lysozyme plays a key role regarding protection against host infections and constitutes a chemical and biological barrier of first defence against pathogens in fish [28]. Superoxide dismutase (SOD) and myeloperoxidase (MPO) are known to regulate the reactive oxygen species in numerous target cells and catalyze the conversion of hydrogen peroxide and superoxide radicals to normal oxygen [29, 30]. Lysozyme activity and SOD activity of fish fed BS_8, EF_8, EF_7 and EF_6 diets significantly improved compared to CON diet ($P<0.05$). However, they did not show any differences among probiotic and OTC diets. On the other hands, MPO of fish fed EF_8, EF_7, EF_6 and OTC diets were higher than those of fish fed CON diet, while no differ among probiotic and OTC diets ($P>0.05$). Previous finding demonstrated probiotics can enhance immune responses such as SOD, MPO and lysozyme in Nile tilapia [31, 32], lysozyme in rainbow trout [33], and SOD and lysozyme in grouper *Epinephelus malabaricus* [34]. One of the reasons for the improved immunity is the enhancement of phagocytic activity and reactive oxygen metabolites by macrophages. Observations from this study are in complete agreement with the findings of Newaj-Fyzul et al. [35] who found the number of leucocytes, respiratory burst and phagocytic activity increased significantly when *B. subtilis* was supplemented in rainbow trout diets. Ultimately, respiratory burst activity may have enhanced immunity by extracellular protection against pathogens [36, 37]. Because of these reasons, numerous studies on probiotics have been demonstrated that they are not only a potential antibiotic replacer in aquaculture but also elevate the growth, immune response and disease resistance of fish [38, 39].

The type of cytokines such as IL-1β, TNF- α and IFN- γ plays the important proteins that regulate a wide range of biological role in preventing stress, immune responses,

hematopoiesis and inflammation in the animal body [40]. On the other hand, heat shock protein 70 (HSP70) also plays independently from associated proteins, stimulating the immunity [41]. Our results showed that probiotic groups including EF_8, BS_8 and EF_7 had markedly higher IL-1β, TNF- α and IFN- γ and HSP70 expression compared to the CON group. In addition, they showed improved gene expression compared to OTC diet. This finding that administration of optimum probiotic levels in the diet of Nile tilapia could up improve the expression. Overall gene expressions indicated that appropriate and optimum probiotics in Nile tilapia can enhance immune responses and stimulated cytokine and protein enzymes.

The cumulative survival rate against *A. hydrophila* for 11 days significantly improved when fed dietary probiotics supplementation compared to CON. Previous studies have reported that mixture of probiotic such as *B. subtilis and E. faecium* could improve disease resistance of Nile tilapia against bacteria [13, 42]. According to what was mentioned, probiotics supplementation can be beneficial effects in terms of disease resistance due to improved non-specific immunity. Moreover, current study is consistent with the results for previous studies on the increased disease resistance by probiotic treatments [42, 43]. In participate, EF_8, EF_7, EF_6 and BS_8 diets markedly showed potential effects as antibiotic replacers in Nile tilapia. The differing impact of probiotics with each concentration on disease resistance in tilapia can be related to differences in the antibiotic activities of specific probiotic strains.

The intestinal histology (villi length and muscular layer thickness) has been determined to indicate the gut condition of fish [44]. The intestinal condition of fish were significantly improved by dietary supplementation of *E. faecium* villi length of Nile tilapia fed probiotic diets was significantly higher in the mid-intestine than those of fish fed CON and OTC diets. Specially, EF_8, EF_7 and EF_6 diets showed highest villi length and muscular layer thickness among the CON and OTC diets. However, there were no differences among BS_8, BS_7, EF_8, EF_7 and EF_6 diets. These results are collaborated with Lazado & Caipang [45], who reported that enhanced

length and muscular layer thickness has been associated with probiotic administration. Indeed, probiotics can modulate the physiological activities of gut mucosal cells. Aforementioned, improvement of intestinal morphology increases the absorption of nutrients in surface area, and ultimately improves the growth performance.

The digestive enzyme activites of fish are indicator that estimates the organism to metabolize given nutrients as well as the types, properties, and modulation [46, 47]. In our study, the results indicated that administration of *E. faecium* diets significantly improved compared to CON diet ($P<0.05$), while, no significant differences among BS_8, EF_8, EF_7 and EF_6 diet ($P>0.05$). According to Merrifield et al. [48], administration of probiotics can induce the enhanced length and density of enterocytes. In addition, as a result of improved microvilli leads to an extensive absorptive ability and surface area of nutrients. As it was mentioned before, the improved growth performance, feed utilization and survival rate of shrimp may be due to enhancing digestive enzyme activity induced by the probiotics.

Therefore, these results demonstrated that *B. subtilis* at 10^8 CFU/g and *E. faecium* at $10^{7\sim8}$ CFU/g diets could have an ideal probiotics in terms of the growth performance, immune responses, hematology, enzyme activity, disease resistance and gene expression, while replacing the use of antibiotics in Nile tilapia.

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Figure legends

Figure 1. Histological sections of juvenile Nile tilapia intestine fed the experimental diets for 8 weeks. A-CON, basal diet; B-BS_6, *B. subtilis* at 1 x 10^6 CFU/g; C- BS_7, *B. subtilis* at 1 x 10^7 CFU/g; D-BS_8, *B. subtilis* at 1 x 10^8 CFU/g; E- EF_6, *E. faecium* at 1 x 10^6 CFU/g; F- EF_7, *E. faecium* at 1 x 10^7 CFU/g; G- EF_8, *E. faecium* at 1 x 10^8 CFU/g; H-OTC, oxytetracycline at 4g/kg. (Scale bar = 100 µm; Original magnification × 4).

Figure 2. Intestinal gene expression levels of Heat shock protein 70 (HSP70), interleukin (IL-1 ß), interferon-gamma (IFN-γ) and tumour necrosis factor (TNF-α) were evaluated in juvenile Nile tilapia fed the experimental diets for 8 weeks. Bars with range represent mean ± SD of triplicate samples, and diets refer to Fig 1.

Figure 3. Cumulative survival rate of juvenile Nile tilapia fed the experimental diets with different probiotics for 8 weeks and experimentally challenged with *A. hydrophila* for 11 days. Each value represents mean of triplicate groups. Significant differences among means are indicated by different superscripts (P<0.05), and diets refer to Fig 1.

Figure 4. Specific enzyme activities of 1. Trypsin, 2. Amylase and 3. Lipase measured in the intestines of juvenile Nile tilapia fed the experimental diets with different probiotics for 8 weeks, and diets refer to Fig 1.

6. Tables and figures

Table 4-1. Formulation and composition (% dry matter) of the basal diet for Nile tilapia

Ingredients	%
Tuna by-product[1]	4.0
Squid liver powder[2]	2.0
Blood meal[2]	2.0
Poultry by-product meal[2]	8.5
Tankage meal[2]	10.0
Meat and Bone meal[2]	10.0
Soybean meal[2]	30.0
Wheat flour	30.0
Soybean oil[3]	1.5
Vitamin premix[4]	1.0
Mineral premix[5]	1.0
Proximate composition	
Moisture	9.44
Crude protein	40.3
Crude lipid	7.86
Crude ash	7.15

[1]Suhyup Feed Co. Uiryeong, Korea

[2]The Feed Co. Goyang, Korea

[3]Jeil Feed Co. Hamman, Korea

[4]Contains (as mg/kg in diets) : Ascorbic acid, 300; dl-Calcium pantothenate, 150; Choline bitate, 3000; Inositol, 150; Menadion, 6; Niacin, 150; Pyridoxine · HCl, 15; Rivoflavin, 30; Thiamine mononitrate, 15; dl-α-Tocopherol acetate, 201; Retinyl acetate, 6; Biotin, 1.5; Folic acid, 5.4; Cobalamin, 0.06

[5]Contains (as mg/kg in diets) : NaCl, 437.4; $MgSO_4 \cdot 7H_2O$, 1379.8; $ZnSO_4 \cdot 7H_2O$, 226.4; Fe-Citrate, 299; $MnSO_4$, 0.016; $FeSO_4$, 0.0378; $CuSO_4$, 0.00033; $Ca(IO)_3$, 0.0006; MgO, 0.00135; $NaSeO_3$, 0.00025

Table 4-2. Primers used to quantify relative gene expression

Primers	Sense	Sequences
HSP90[1]	F	5′-CATCGCCTACGGTCTGGACAA-3′
	R	5′-TGCCGTCTTCAATGGTCAGGAT-3′
IL-1ß[2]	F	5′-CAAGGATGACGACAAGCCAACC-3′
	R	5′-AGCGGACAGACATGAGAGTGC-3′
IFN-γ[3]	F	5′-AAGAATCGCAGCTCTGCACCAT-3′
	R	5′-GTGTCGTATTGCTGTGGCTTCC-3′
TNF-α[4]	F	5′-GGAAGCAGCTCCACTCTGATGA-3′
	R	5′-CACAGCGTGTCTCCTTCGTTCA-3′
ß-actin[5]	F	5′-CCACACAGTGCCCATCTACGA-3′
	R	5′-CCACGCTCTGTCAGGATCTTCA-3′

[1]HSP90, heat shock protein 90 (FJ207463.1)

[2] IL-1ß, interleukin (XM_003460625.2)

[3] IFN-γ, interferon-gamma (XM_005448319.1)

[4]TNF-α, tumour necrosis factor (JF957373.1)

[5]ß-actin (HQ386788.1)

Table 4-3. The mortality to evaluate the safety of probiotic bacterial isolates

Group	Dose (ml)	Mortality (%)
CON	0.3 of sterile saline	4.76 ± 2.75^{a}
B.subtilis[1]	0.3×10^{7} cells ml^{-1}saline	0
E.faecium[2]		

[1] *Bacillus subtilis*

[2] *Enterococcus faecium*

Table 4-4. Antibiotics sensitivity test of probiotics (*B. subtilis* and *E. faecium*)

	Antibiotic Disc[3]	MIC (mg/L)[4]	Susceptibility[5]
B.subtilis[1]	CON	0	S
	Chloramphenicol	8	S
	Ampicillin	2	S
	Kanamycin	8	S
E.faecium[2]	CON	0	S
	Chloramphenicol	16	S
	Ampicillin	2	S
	Kanamycin	24	S

[1]*Bacillus subtilis*

[2] *Enterococcus faecium*

[3]Antibiotic-impregnated discs (6 mm) with amount in μg ± SE shown in brackets

[4] Minimal inhibitory concentration (mg/L)

[5] S, sensitive; I, intermediate; R, resistant.

Table 4-5. Growth performance and feed utilization of juvenile Nile tilapia fed the experimental diets for 8 weeks.

	Diets[1]							
	CON	BS_6	BS_7	BS_8	EF_6	EF_7	EF_8	OTC
IBW (g)[2]	0.86±0.01	0.85±0.01	0.86±0.02	0.85±0.01	0.86±0.00	0.85±0.01	0.86±0.02	0.86±0.02
FBW (g)[3]	2.67±0.07^{b}	2.71±0.04^{ab}	2.75±0.02^{ab}	2.81±0.10^{a}	2.81±0.10^{ab}	2.77±0.14^{a}	2.74±0.10^{a}	2.76±0.10^{ab}
WG (%)[4]	210±5.61^{b}	223±8.64^{ab}	227±10.2^{ab}	232±9.92^{a}	227±10.3^{ab}	233±14.7^{a}	237±8.19^{a}	228±16.7^{ab}
FE (%)[5]	78.9±2.16^{b}	81.6±1.66^{ab}	82.0±2.46^{ab}	84.5±1.50^{a}	81.7±1.81^{ab}	84.7±1.27^{a}	84.8±1.27^{a}	82.0±1.66^{ab}
SGR(%/day)[6]	2.18±0.03^{b}	2.26±0.05^{ab}	2.28±0.06^{ab}	2.30±0.06^{a}	2.28±0.06^{ab}	2.31±0.08^{a}	2.34±0.05^{a}	2.54±0.08^{ab}
PER[7]	1.61±0.04^{b}	1.67±0.04^{ab}	1.68±0.05^{ab}	1.73±0.03^{a}	1.67±0.04^{ab}	1.73±0.03^{a}	1.74±0.04^{a}	1.68±0.03^{ab}
HSI (%)[8]	0.97±0.12	0.88±0.24	0.91±0.10	1.00±0.09	0.84±0.09	0.90±0.02	0.84±0.08	0.81±0.16
VSI (%)[9]	4.47±0.23	4.76±0.19	4.77±0.36	4.74±0.39	4.70±0.37	4.93±0.20	4.64±0.26	4.68±0.31
CF[10]	1.37±0.03	1.42±0.05	1.37±0.04	1.35±0.09	1.36±0.06	1.36±0.06	1.35±0.03	1.39±0.03
Survival[11]	90.0±10.0	95.0±5.00	96.7±2.90	95.0±5.00	95.0±5.00	95.0±5.00	96.7±2.90	95.0±5.00

[1]Values are means ± SD of triplicate groups of fish. Values in each row with different superscripts are significantly different ($P< 0.05$).

Diets: CON = the basal diet, refer to Table 1; BS_6 = *Bacillus subtilis* at 1 x 10^6 CFU/g; BS_7 = *Bacillus subtilis* at 1 x 10^7 CFU/g; BS_8 = *Bacillus subtilis* at 1 x 10^8 CFU/g; EF_6 = *Enterococcus faecium* at 1 x 10^6 CFU/g; EF_7 = *Enterococcus faecium* at 1 x 10^7 CFU/g; EF_8 = *Enterococcus faecium* at 1 x 10^8 CFU/g; OTC = oxytetracycline at 4g/kg.

[2] Initial body weight

[3] Final body weight

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

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

[6] Specific growth rate (SGR, %) = [($\log_e$ final wt. - $\log_e$ initial wt.) × 100] / days

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

[8] Hepatosomatic index (HSI) = (liver wt. × 100) / body wt

[9] Visceralsomatic index (VSI, %) = (viscera wt. × 100) / body wt

[10] Condition factor = (wet weight / total length^3) × 100

[11] Survival rate = [(total fish – dead fish) × 100] / total fish

Table 4-6. Non-specific immune responses of juvenile Nile tilapia fed the experimental diets for 8 weeks

	Diets[1]							
	CON	BS_6	BS_7	BS_8	EF_6	EF_7	EF_8	OTC
Lysozyme (U/ml)	0.81 ± 0.09^{b}	0.94 ± 0.06^{ab}	0.99 ± 0.04^{ab}	1.09 ± 0.13^{a}	1.05 ± 0.05^{a}	1.06 ± 0.15^{a}	1.10 ± 0.04^{a}	0.99 ± 0.11^{ab}
SOD[2]	62.1 ± 5.28^{b}	70.3 ± 5.06^{ab}	71.6 ± 2.74^{ab}	73.0 ± 5.68^{a}	71.9 ± 6.66^{a}	77.1 ± 6.64^{a}	78.0 ± 4.59^{a}	70.2 ± 6.01^{ab}
MPO[3]	1.71 ± 0.33^{b}	2.15 ± 0.10^{ab}	2.21 ± 0.27^{ab}	2.48 ± 0.35^{a}	2.33 ± 0.29^{a}	2.47 ± 0.32^{a}	2.57 ± 0.36^{a}	2.25 ± 0.24^{a}

[1]Values are means ± SD of triplicate groups of fish. Values in each row with different superscripts are significantly different ($P<0.05$). Diets refer to Table 3.

[2]SOD: Superoxide dismutase activity (% inhibition)

[3]MPO: Myeloperoxidase activity (OD at 450 nm)

Table 4-7. Intestinal morphology of juvenile Nile tilapia fed the experimental diets for 8 weeks

	Diets[1]							
	CON	BS_6	BS_7	BS_8	EF_6	EF_7	EF_8	OTC
Villi length (μm)	80.8±13.9^{c}	84.3±20.1^{c}	88.1±8.56^{bc}	92.5±16.5^{abc}	96.8±12.2^{ab}	102±13.1^{a}	105±22.3^{a}	84.1±12.7^{c}
Muscular layer thickness (μm)	42.8±4.48^{c}	54.4±7.18^{bc}	66.6±8.48^{ab}	66.8±5.98^{ab}	71.8±7.63^{a}	68.2±12.6^{ab}	69.0±9.58^{ab}	46.1±7.40^{c}

[1]Values are means ± SD of triplicate groups of fish. Values in each row with different superscripts are significantly different (P<0.05). Diets refer to Table 3.

Table 4-8. Haematological analysis of juvenile Nile tilapia fed the experimental diets for 8 weeks

	Diets[1]							
	CON	BS_6	BS_7	BS_8	EF_6	EF_7	EF_8	OTC
AST[2]	61.3±3.21	52.0±11.8	58.3±4.73	54.3.7±5.03	56.7±4.20	58.0±9.17	56.7±7.77	63.3±3.51
ALT[3]	5.33±0.58	5.33±0.58	5.67±0.58	6.00±0.00	5.67±0.58	5.33±0.58	5.33±0.58	5.33±0.58
Glucose (mg dL^{-1})	4.33±0.58	4.67±0.58	5.00±0.01	4.33±.085	4.33±0.58	4.67±0.58	4.67±0.58	4.67±.0.58
T-protein[4]	0.47±0.06	0.43±0.06	0.50±0.10	0.53±0.06	0.47±0.06	0.53±0.06	0.50±0.10	0.47±0.06

[1]Values are means ± SD of triplicate groups of fish. Values in each row with different superscripts are significantly different (P<0.05). Diets refer to Table 3.

[2]AST: Aspartate aminotransferase activity (U L^{-1})

[3] ALT: Alanine aminotransferase activity (U L^{-1})

[4]T-protein: Total protein (g dL^{-1})

Table 4-9. Whole-body proximate composition (% dry matter) of juvenile Nile tilapia fed the experimental diets for 8 weeks

	Diets[1]							
	CON	BS_6	BS_7	BS_8	EF_6	EF_7	EF_8	OTC
Moisture	7.12±0.56	7.11±0.39	7.08±0.82	7.03±1.02	7.16±0.75	7.00±0.50	7.16±0.67	7.19±0.48
Protein	35.5±0.28	35.6±0.15	35.0±0.21	35.9±0.30	35.8±0.35	35.6±0.18	35.1±0.2	35.2±0.44
Lipid	7.65±0.77	8.10±0.58	7.85±0.61	8.15±0.28	7.76±0.82	8.02±0.40	8.32±0.28	7.90±0.61
Ash	6.10±0.12	5.95±0.23	6.05±0.16	6.13±0.11	5.90±0.21	6.18±0.18	6.01±0.15	6.23±0.20

[1]Values are means ± SD of triplicate groups of fish. Values in each row with different superscripts are significantly different (P<0.05). Diets refer to Table 3.

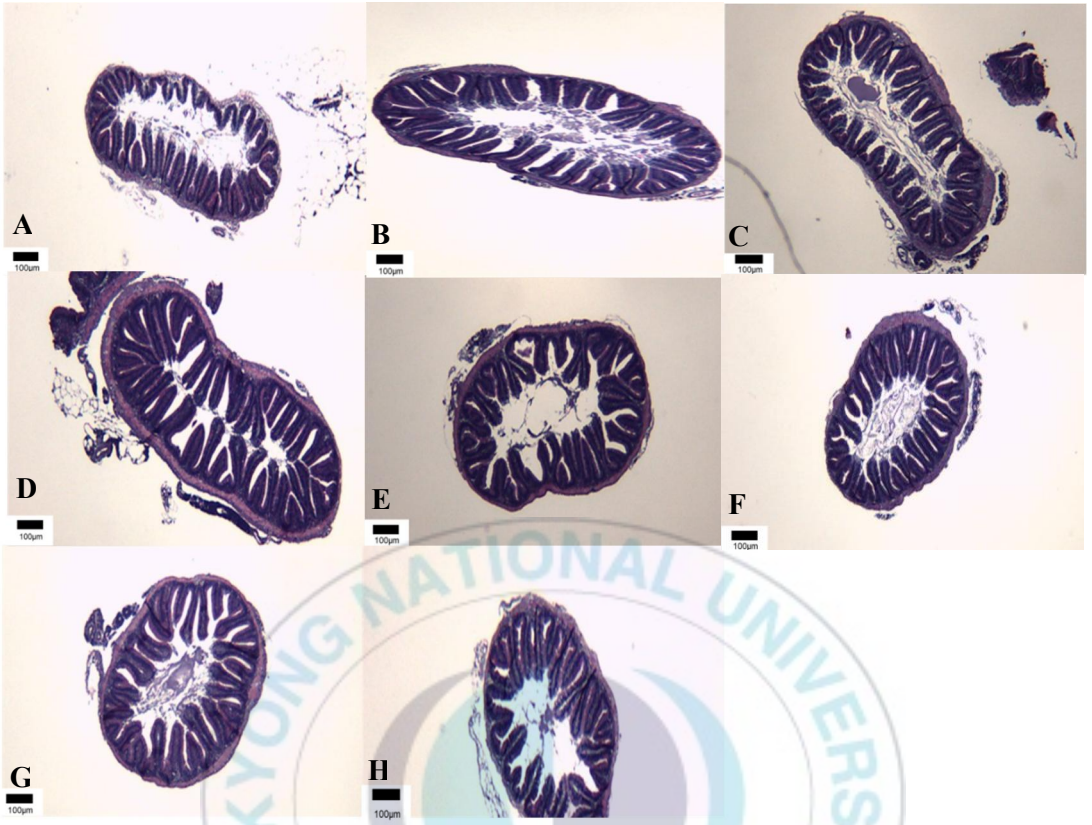
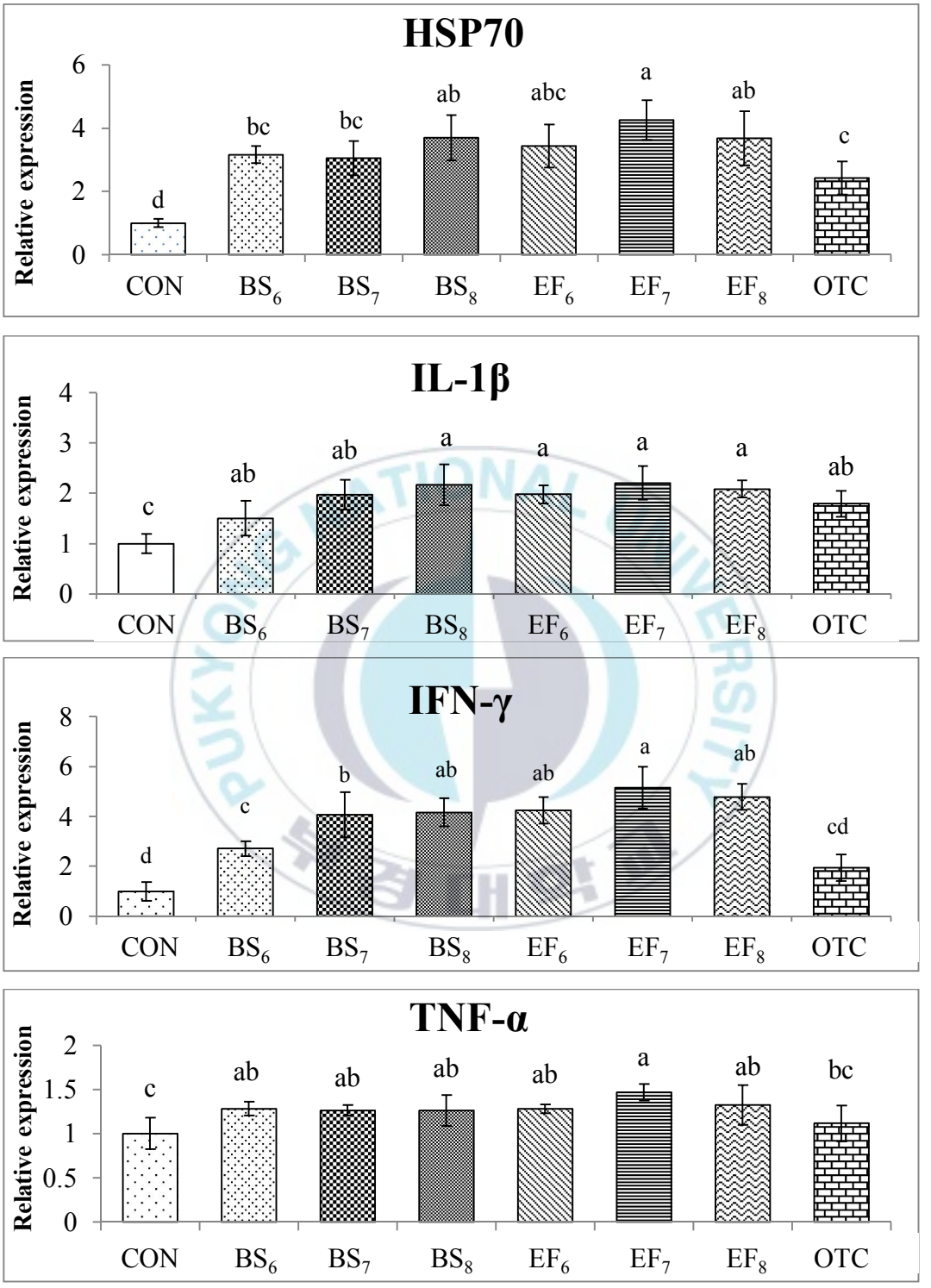


Fig 4-1.


HSP70
Relative expression
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4
2
0
d
bc
bc
ab
abc
a
ab
c
CON
BS6
BS7
BS8
EF6
EF7
EF8
OTC
IL-1β
Relative expression
4
3
2
1
0
c
ab
ab
a
a
a
a
ab
CON
BS6
BS7
BS8
EF6
EF7
EF8
OTC
IFN-γ
Relative expression
8
6
4
2
0
d
c
b
ab
ab
a
ab
cd
CON
BS6
BS7
BS8
EF6
EF7
EF8
OTC
TNF-α
Relative expression
2
1.5
1
0.5
0
c
ab
ab
ab
ab
a
ab
bc
CON
BS6
BS7
BS8
EF6
EF7
EF8
OTC

Fig 4-2.

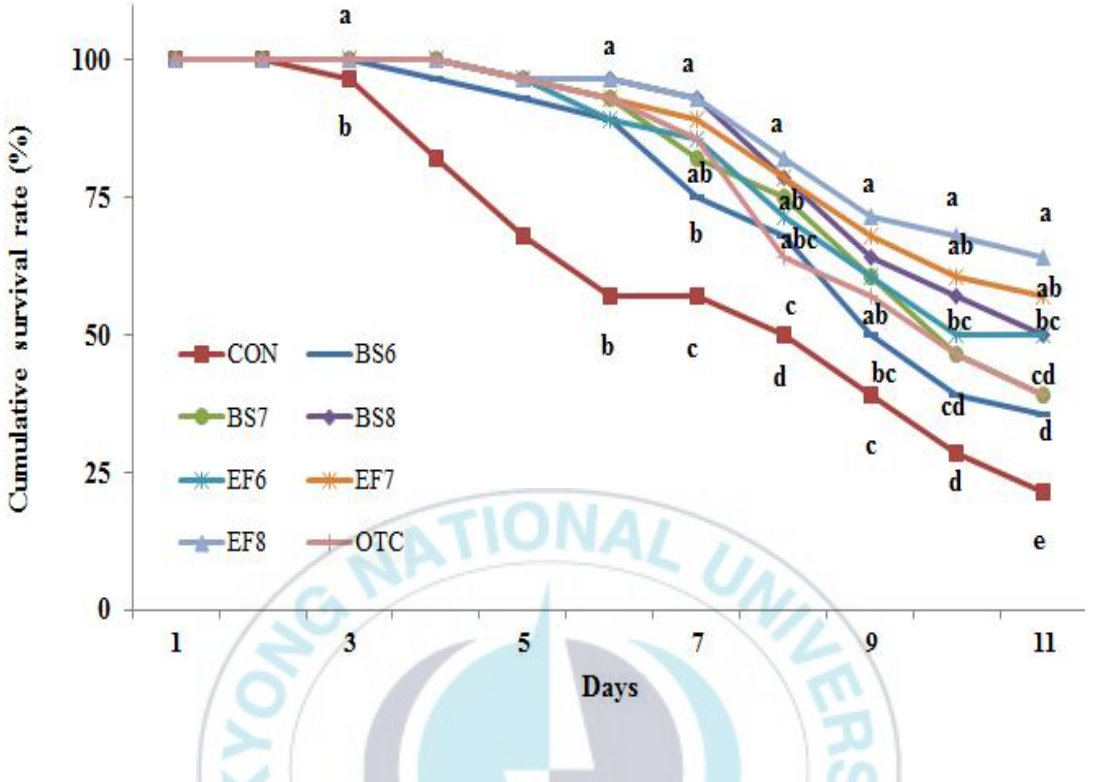
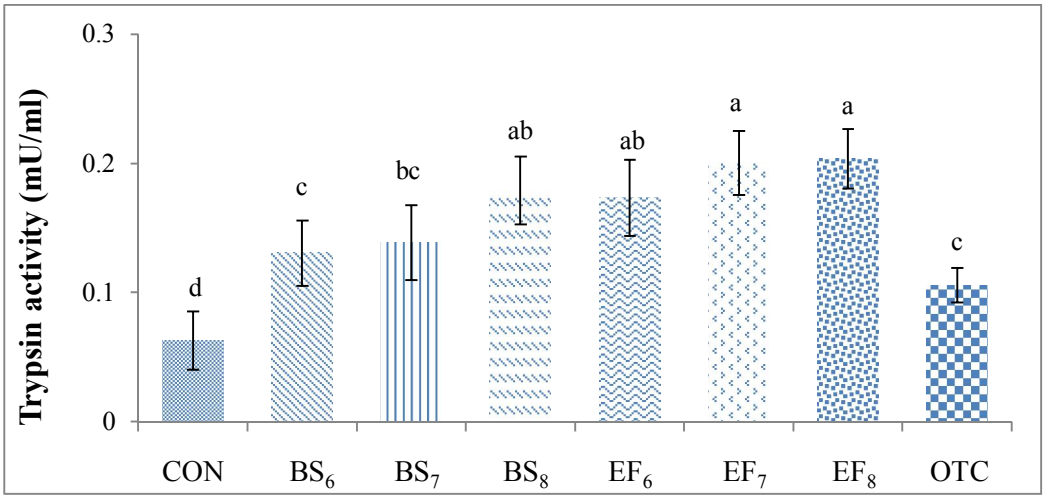


Fig 4-3.


Trypsin activity (mU/ml)
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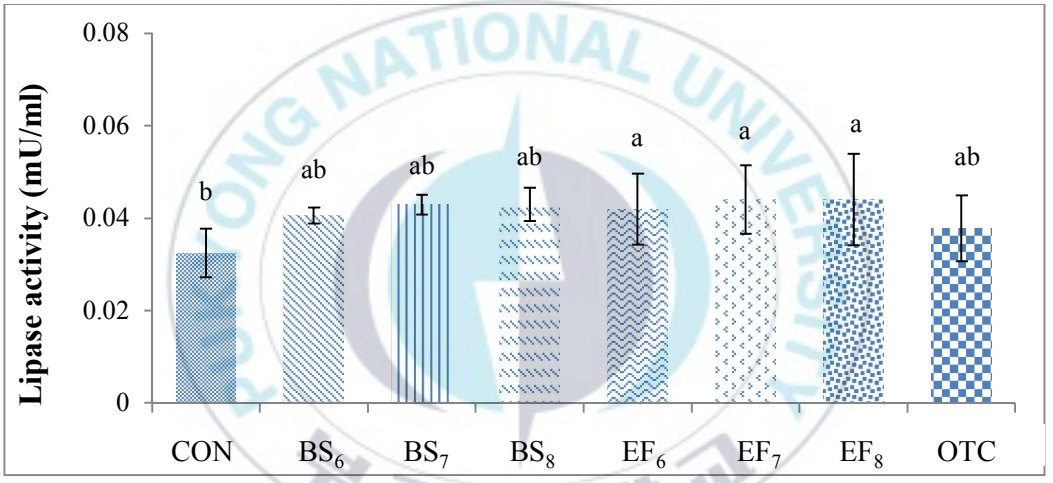
0.2
0.1
0
d
c
bc
ab
ab
a
a
c
CON
BS6
BS7
BS8
EF6
EF7
EF8
OTC


Lipase activity (mU/ml)
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0.06
0.04
0.02
0
b
ab
ab
ab
a
a
a
ab
CON
BS6
BS7
BS8
EF6
EF7
EF8
OTC

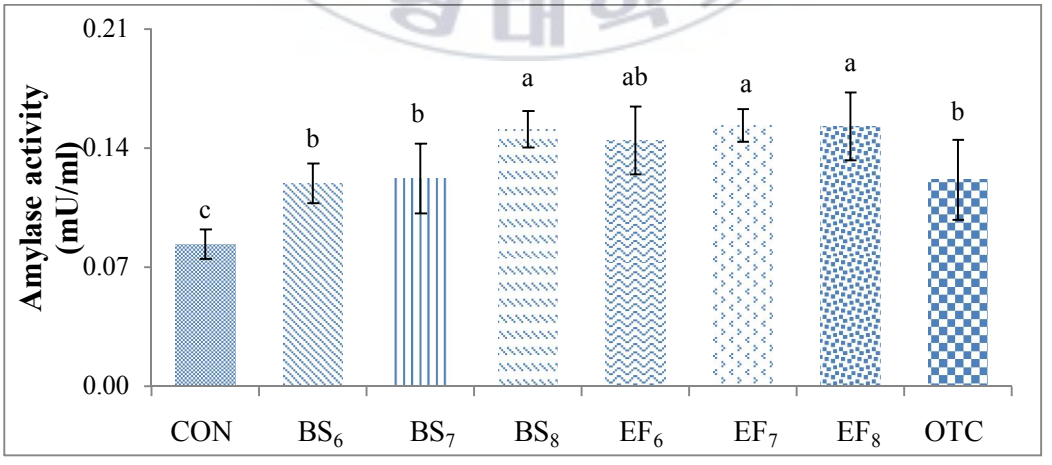
Amylase activity (mU/ml)
0.21
0.14
0.07
0.00
c
b
b
a
ab
a
a
b
CON
BS6
BS7
BS8
EF6
EF7
EF8
OTC

Fig 4-4.

CHAPTER V. Appendix

Raw data for growth performance of whiteleg shrimp experimental diets for 8 week

	Tank No	Individual weight	Individual weight	Individual feeding	WG	WG aver.	SGR	SRG aver.	FE	FE aver.	PER	PER aver.
CON	2	1.45	6.29	6.17	334	359	2.62	2.72	78.5	83.7	1.84	1.96
	18	1.35	6.43	5.84	376	22.1	2.79	0.09	87.0	4.57	2.04	0.11
	23	1.40	6.54	6.00	367		2.75		85.6		2.00	
OTC	5	1.50	7.27	6.34	384	372	2.82	2.77	90.9	87.1	2.15	2.06
	10	1.35	6.00	5.84	344	24.8	2.66	0.10	79.6	6.49	1.88	0.15
	17	1.40	6.86	6.00	389		2.84		90.9		2.14	
BS7	7	1.35	6.18	5.84	357	377	2.72	2.79	82.7	87.8	1.93	2.05
	11	1.35	6.85	5.84	407	26.4	2.90	0.10	94.1	5.82	2.20	0.14
	19	1.50	7.00	6.34	366		2.75		86.7		2.03	
BS8	8	1.40	7.53	6.00	438	405	3.01	2.89	102	94.6	2.42	2.24
	12	1.35	6.53	5.84	383	28.7	2.81	0.10	88.7	6.88	2.10	0.16
	25	1.45	7.19	6.17	395		2.86		92.9		2.20	
PP8	4	1.40	6.69	6.00	377	378	2.79	2.79	88.0	88.6	2.09	2.10
	9	1.40	6.93	6.00	394	16.2	2.86	0.06	92.1	3.20	2.18	0.08
	22	1.50	6.94	6.34	362		2.73		85.7		2.03	
LL8	6	1.40	7.06	6.00	404	417	2.89	2.93	94.3	97.0	2.20	2.27
	14	1.35	7.44	5.84	451	29.8	3.05	0.10	104	6.47	2.44	0.15
	24	1.40	6.95	6.00	396		2.86		92.4		2.16	

Raw data for growth performance of Nile tilapia experimental diets for 8 week (1~2)

	Tank No	Individual weight	Individual weight	Individual feeding	WG	WG aver.	SGR	SRG aver.	FE	FE aver.	PER	PER aver.
CON	2	2.85	9.82	8.59	244.7	242	2.38	2.37	81.1	81.8	2.29	2.31
	18	2.90	9.47	8.29	226.7	15.3	2.28	0.09	79.2	3.02	2.23	0.08
	23	2.80	10.00	8.45	257.1		2.45		85.2		2.40	
OTC	5	2.80	10.95	8.35	291.1	275	2.62	2.54	97.6	93.5	2.70	2.58
	10	2.85	10.25	8.34	259.6	15.7	2.46	0.08	88.7	4.46	2.45	0.12
	17	2.85	10.72	8.36	276.2		2.55		94.1		2.60	
BS7	7	2.80	10.16	8.29	262.8	264	2.48	2.48	88.7	90.3	2.47	2.51
	11	2.90	10.20	8.26	251.7	13.1	2.42	0.07	88.3	3.11	2.46	0.09
	19	2.80	10.58	8.28	277.8		2.56		93.9		2.61	
BS8	8	2.75	10.40	8.17	278.2	276	2.56	2.55	93.6	93.2	2.58	2.57
	12	2.80	10.26	8.29	266.5	9.8	2.50	0.05	90.0	3.08	2.48	0.08
	25	2.85	11.00	8.47	286.0		2.60		96.1		2.65	
BL7	4	2.75	10.28	8.19	273.7	279	2.54	2.56	91.9	94.2	2.52	2.59
	9	2.85	11.22	8.54	293.8	12.9	2.64	0.06	98.0	3.33	2.69	0.09
	22	2.90	10.72	8.42	269.7		2.51		92.8		2.55	
ML7	6	2.80	10.40	7.98	271.4	281	2.52	2.57	95.2	95.3	2.59	2.60
	14	2.80	10.63	8.39	279.7	11.3	2.57	0.06	93.3	2.04	2.54	0.06
	24	2.85	11.22	8.59	293.8		2.64		97.4		2.65	
LL7	3	2.80	10.11	8.15	260.9	278	2.47	2.56	89.6	92.7	2.44	2.53
	15	2.90	11.21	8.73	286.6	14.8	2.60	0.08	95.1	2.86	2.60	0.08
	20	2.75	10.63	8.44	286.6		2.60		93.4		2.55	
LL8	1	2.85	11.56	8.47	305.5	279	2.69	2.56	102	94.2	2.82	2.59
	13	2.90	10.45	8.53	260.3	23.4	2.47	0.12	88.5	7.51	2.43	0.16
	16	2.80	10.42	8.32	272.2		2.53		91.5		2.51	

	Tank No	Individual weight	Individual weight	Individual feeding	WG	WG aver.	SGR	SRG aver.	FE	FE aver.	PER	PER aver.
CON	3	0.85	2.60	2.26	204	210	2.14	2.18	77.1	78.9	1.58	1.61
	18	0.86	2.67	2.31	211		2.18		78.2		1.60	
	25	0.87	2.73	2.29	216	5.61	2.21	0.03	81.3	2.16	1.66	0.04
OTC	5	0.86	2.74	2.29	220	227	2.24	2.28	82.2	81.7	1.68	1.67
	10	0.88	2.77	2.39	216		2.21		79.3		1.62	
	23	0.84	2.92	2.49	247	16.7	2.39	0.10	83.6	2.20	1.71	0.05
BS6	7	0.84	2.74	2.30	225	223	2.27	2.26	82.3	81.6	1.69	1.67
	11	0.84	2.65	2.27	214		2.20		79.4		1.63	
	19	0.86	2.84	2.39	231	8.64	2.30	0.05	83.1	1.91	1.70	0.04
BS7	8	0.87	2.78	2.41	218	227	2.22	2.28	79.1	82.0	1.62	1.68
	12	0.86	2.80	2.33	227		2.28		83.4		1.71	
	21	0.84	2.84	2.40	238	10.2	2.34	0.06	83.4	2.46	1.71	0.05
BS8	4	0.84	2.70	2.24	221	232	2.24	2.30	82.9	84.5	1.70	1.73
	9	0.85	2.84	2.35	234		2.32		84.7		1.74	
	20	0.87	2.94	2.42	240	9.92	2.35	0.06	85.9	1.50	1.76	0.03
EF6	6	0.86	2.86	2.41	233	227	2.31	2.28	82.9	81.7	1.70	1.67
	14	0.87	2.73	2.34	216		2.21		79.6		1.63	
	24	0.86	2.86	2.42	234	10.3	2.32	0.06	82.6	1.81	1.69	0.04
EF7	2	0.84	2.70	2.23	220	233	2.24	2.31	83.3	84.7	1.70	1.73
	17	0.86	2.84	2.33	230		2.30		85.0		1.74	
	22	0.84	2.94	2.45	249	14.7	2.41	0.08	85.7	1.27	1.75	0.03
EF8	1	0.87	2.89	2.38	232	237	2.31	2.34	85.0	84.8	1.74	1.74
	13	0.84	2.80	2.36	232		2.31		82.9		1.70	
	16	0.85	2.95	2.42	246	8.19	2.39	0.05	86.5	1.77	1.77	0.04

Publication

1. Won S., Lee S., Hamidoghli A., Lee S., & Bai S. C. (2019). Dietary choline requirement of juvenile olive flounder (*Paralichthys olivaceus*). Aquaculture Nutrition. https://doi.org/10.1111/anu.12948.

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Presentations (Only 1st author)

1. Seonghun Won, Youngjin Park, Hyeonho Yun, Seung-han Lee & & Sungchul C. Bai. Effects of dietary probiotics on growth performance and non-specific immune responses in rainbow trout, *Oncorhynchus mykiss*. World aquaculture, Jeju, Korea. 2015 May 30 **(Oral presentation)**

2. Seonghun Won, Hyeonho Yun, Jeong-whui Hong, Jung-Keug Park, Moon Young Yoon & Sungchul C. Bai. Evaluation of dietary natural of mineral supplementation as an additive on growth and non-specific immune responses in rainbow trout, *Oncorhynchus mykiss*. KOFFST, Busan, 2015 October 30 **(Oral presentation, excellent award)**

3. Seonghun Won, Seunghan Lee, Mohammad Moniruzzaman, Jun-Young Bae, Kyung-Hoon Chang & Sungchul C. Bai. Evaluation of vegetable protein based ingredients as a fish meal replacer in diets for juvenile rainbow trout, *Oncorhynchus mykiss*. Aquaculture America, Las vegas, USA, 2016 February 22 **(Oral presentation)**

4. Seunghun Won, Kan-Woong Kim, Kyoung-Duck Kim & Sungchul C. Bai. Optimum dietary protein level for growth performances and blood parameters of family selected olive flounder, *Paralichthys olivaceus*. KOSFAS, Togyeong, Korea, 2017 May 12 **(Oral presentation, excellent award)**

5. Seonghun Won, Ali Hamidoghli, Jin-hyeock Lee, Hyeonho Yun, Yongjik Lee, Sungwook Han and Sungchul C. Bai. *Bacillus*-fermented vegetable proteins as fishmeal

replacers in juvenile whiteleg shrimp *Litopaeneus vannamei*. Aquaculture America, Las vegas, USA, 2018 February 20 **(Oral presentation)**

6. Seonghun Won, Yongjin Park & Sungchul C. Bai. Potential use of dietary two single probiotics and their mixture as the antibiotic replacers in rainbow trout *Oncorhynchus mykiss* and starry flounder *Platichthys stellatus*. World Aquaculture, Montpellier, France, 2018 August 27 **(Oral presentation)**

7. Seonghun Won, Ali Hamidoghli & Sungchul C. Bai. The optimum dietary protein and lipid levels and protein/energy ratio of withleg shrimp (*Litopenaeus vannamei*) reared in biofloc. KOSFAS, Gangneung, Korea, 2018 May 10 **(Oral presentation, excellent award)**

8. Seonghun Won, Ali Hamidoghli, Youngjin Park, Seunghan Lee, Haham Kim, Md Tawheed Hasan & Sungchul C. Bai. A review on the application of probiotics, prebiotics and synbiotics in Korean aquaculture. KOFFST, Busan, Korea, 2018, Nov 2 **(Poster presentation, excellent award)**

9. Seonghun Won, Wonsuk Choi, Ali Hamidoghli, Jinho Bae & Sungchul C. Bai. Effects of different dietary probiotics on growth performance and immune responses in juvenile Nile tilapia *Orechromis niloticus*. World Aquaculture 2019, Newolreans, USA, 2019 March 09 **(Oral presentation)**

10. Seonghun Won, Jinho Bae, Wonsuk Choi, Ali Hamidoghli, Jangmin Ok & Sungchul C. Bai. Evaluation of two different dietary probiotics with three different levels to replace antibiotic in Nile tilapia, *Orechromis niloticus*. KOSFAS, Yeosu, Korea, 2019 May 09 **(Oral presentation, excellent award)**

Others

Seunghan Lee, Seonghun Won, Fasil Taddese, Hyeonho Yun, Sangeun Kim, Sungchul Bai "Natural Feed Additives Enhance Rainbow Trout Performance" Global aquaculture advocate, 2015 Vol. November/December 72-73