Thesis for Degree of Master of fisheries science

Synergistic effects of dietary two probiotics with two prebiotics to develop the synbiotics in Japanese eel, *Anguilla japonica*

by

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February 2019

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뱀장어 사료 내 신바이오틱스 개발을 위한 두 가지 프로바이오틱스와 두 가지 프리바이오틱스와의 복합효과

Advisors : Sungchul C. Bai

by

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A thesis submitted in partial fulfillment of the requirement
for the Degree of

Master of Fisheries Science

in the Department of Fisheries Biology, Graduate School
Pukyong National University

February 2019

김하함의 수산학석사 학위논문을 인준함

2019 년 2 월 22 일

주 심	이 학 박 사	공 인 수	(인)
위 원	수산학박사	박 정 환	(인)
위 원	영양학박사	배 승 철	(인)

Synergistic effects of dietary two probiotics with two prebiotics to develop the synbiotics in Japanese eel, *Anguilla japonica*

A dissertation

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February 22, 2019

요 약 문

본 실험은 사료 내 두 가지 프로바이오틱스인 *Bacillus subtilis* KCTC 2217과 *Bacillus licheniformis* KCCM 11775와 두 가지 프리바이오틱스인 매넌 올리고당(MOS) 또는 프룩토 올리고당(FOS) 이 치어기 뱀장어의 성장, 면역 및 장내 조직에 미치는 영향을 알아보기 위하여 수행되었다. 사육실험은 5개의 실험구로써 대조구와 2x2 factorial 조합 4개의 신바이오틱(프로+프리바이오틱스) 실험구로 평균무게 12.8±0.47 g (mean±SD)인 뱀장어를 무작위로 수조당 10마리씩 실험구당 3반복으로 배치 하였다. 기초 사료는 대조구로 사용 하였으며, 두 가지 프로바이오틱스(1.0×10^8 CFU g^{-1} diet) 와 두 가지 프리바이오틱스(5 g kg^{-1} diet)를 factorial로 조합한 4가지 신바이오틱스 실험구; *B. subtilis* 와 MOS (BSM), *B. subtilis* 와 FOS (BSF), *B. licheniformis* 와 MOS (BLM), 그리고 *B. licheniformis* 와 FOS (BLF) 를 제작하였다. 실험종료 후, 증체율, 일간 성장률에 있어서, BSM과 BSF구가 대조구보다 유의적으로 높은 것으로 나타났으며($P < 0.10$), 간중량 지수에서도 BSM과 BSF구가 대조구보다 유의적으로 높은 것으로 나타났다($P < 0.05$). 장에서의 heat shock protein 70과 glyceraldehyde-3-phosphate dehydrogenase (GAPDH)와의 유전자 발현비율에 있어서는 BSF구가 대조구와 BLF구보다 유의적으로 높은 것으로 나타났다($P < 0.05$). Immunoglobulin M과 GAPDH와의 유전자 발현비율에 있어서는 BSF, BLM 및 BLF구가 대조구와 BSM구 보다 유의적으로 높은 것으로 나타났다($P < 0.05$). 장내 융털의 길이에 있어서는 BSF와 BLM구가 대조구와 BLF구보다 유의적으로 높은 것으로 나타났으며($P < 0.05$), 장벽의 두께의 길이에서는 BSF와 BLM구가 대조구와 BSM구보다 유의적으로 높은 것으로 나타났다($P < 0.05$).

따라서 치어기 뱀장어에 있어서, *B. subtilis*와 함께 FOS를 첨가한 BSF 신바이오틱 실험구가 다른 신바이오틱 실험구인 BSM, BLM 및 BLF보다 성장, 비 특이적 면역 및 장 내 조직에 있어서 더 좋은 효과를 나타내어 다른 신바이오틱들보다 더 유용하게 사용될 것으로 확인되었다.

Synergistic effects of dietary two probiotics with two prebiotics to develop the synbiotics in Japanese eel, *Anguilla japonica*

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Abstract

A 12-week feeding trial was conducted to investigate the effects of two dietary probiotics *Bacillus subtilis* KCTC 2217 and *Bacillus licheniformis* KCCM 11775 with two prebiotics mannan oligosaccharide (MOS) or fructo oligosaccharide (FOS) on growth performance, non-specific immune responses and intestinal histology in Japanese eel, *Anguilla japonica*. Fish averaging 12.8±0.47g (mean±SD) were randomly arranged into five treatments with triplicate tanks (10 fish/tank) and fed one of the five experimental diets. A basal diet was used as control diet (CON), and four other synbiotic diets were formulated by supplementary two probiotics (1.0 x 10^8 CFU g^{-1} diet) with two prebiotics (5 g kg^{-1} diet); *B. subtilis* and MOS (BSM), *B. subtilis* and FOS (BSF), *B. licheniformis* and MOS (BLM), and *B. licheniformis* and FOS (BLF). At the end of the feeding trial, weight gain, specific growth rate

of fish fed BSM and BSF diets were significantly higher than those of fish fed CON diet ($P < 0.10$), also hepatosomatic index of fish fed BSM and BSF diets were significantly higher than those of fish fed CON diet ($P < 0.05$). Relative gene expression level of heat shock protein 70 compare to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from intestine of fish fed BSF diet were significantly higher than those of fish fed CON and BLF diets ($P < 0.05$), moreover relative gene expression level of immunoglobulin M compare to GAPDH from intestine of fish fed BSF, BLM and BLF diets were significantly higher than those of fish fed CON and BLM diets ($P < 0.05$). Intestinal villi length of fish fed BSF and BLM diets were significantly higher than those of fish fed CON and BLF diets ($P < 0.05$). Therefore, these results indicated that dietary synbiotic consisting of *B. subtilis* with FOS (BSF) could be more beneficial than the other synbiotics (BSM, BLM and BLF) for growth performance, immune responses and intestinal histology of Japanese eel.

Acknowledgments

First of all, thank GOD for giving everything to me. I do not have adequate word to express my heartfelt sense of gratitude to my respected academic supervisors, Professor Sungchul C. Bai for his guidance, encouragement and supports in this academic period.

I deeply indebted to the chairman of my thesis Advisory Committee, Prof. In-soo Kong and member Prof. Jeonghwan Park for their valuable counseling, guidance and support during the research work and drafting of thesis.

My sincere gratitude is also due to professors in department of Fisheries Biology for extending their whole-hearted love, help, cooperation, encouragement and unconditional moral support throughout my Master journey. I would never have accomplished my mission without their constant support.

My master program received generous support and funding provided by the Department of Marine Bio-Materials and Aquaculture/Feeds & Foods Nutrition Research Center, Pukyong National University, and National Research Foundation (Grant No. NRF-2015R1D1A3A01017663) which are gratefully acknowledged.

I was truly fortunate to have with me such a caring, helping and loving lab members during my Master course. The assistance rendered by the entire Laboratory of Nutrient Metabolism including Seonghun Won, Jooyoung Jung, Ali Hamidoghli, Jinho Bae, Wonsuk Choi, Nathaniel W. Farris and Verynice

Temu. Especially, I am really indebted to Minhye Park, Yujin song and Md Tawheed Hasan for their kind support and help. And finally, I am deeply grateful to my family.

In his heart a man plans his course, but the LORD determines his steps. (Proverbs 16:9)

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Ⅰ. Introduction

In East Asian countries, such as Korea, China, Japan and Taiwan, Japanese eel, *Anguilla japonica*, is one of the most important fish species, due to high market demand and price (Seo et al., 2013; Shahkar et al., 2015). Its production was 11,067 metric tons valued at 301 million won. That was 37.5% of total freshwater fish production and the most production at freshwater fish in Republic of Korea.

Japanese eel, just like other fish species in commercial aquaculture, is usually reared in enclosed spaces and efforts have been made to increase productivity per unit space (Bai el al., 2015 and Katya el al., 2016). This development has led to overcrowding, which tends to adversely affect the immune system and become more susceptible to disease. Disease was primarily caused by environmental degradation and microorganisms (Hasan el al., 2018). *Aeromonas hydrophila* is a common phthogenic bacteria in freshwater farming. It has not only caused incalculable losses in the aquaculture industry but also lead to severe infection to human beings (Tao et al., 2012; Lee et al., 2014; Saavedra et al., 2004; Wu et al., 2007). In the spring and summer, it causes many highly infectious diseases, such as hemorrhagic septicemia, gill-rot disease, and liver breeding in Japanese eel (Wu et al., 2007; Tsao et al., 2013; Esteve et al., 1994).

The term “probiotics” refers to certain concentrations of lie microorganisms capable of inducing beneficial effects on host growth and immunity when

consumed (Hill el al., 2014), and prebiotics are non-digestible carbohydrates (usually oligosaccharides) metabolized by probiotics for growth and survival, in the intestine typically (Hasan el al., 2018). A symbiotic is the combination of both a probiotic and its growth substrate (prebiotic) that yields a synergistic effect (Hoseinifar el al., 2015), inducing the highest possible benefits for the consumer by positive alteration of growth, nutritional utilization, and innate immunity to combat pathogenic bacteria.

Bacillus spp. are gram positive and spore forming bacteria which can act as probiotics by enhancing survival and growth (Gomez-Gil el al., 2000), by stimulating the digestive (Ziaei-Nejad el al., 2006) and immune systems (Gatesoupe el al., 1991) in fish and shrimp. It is well-documented that *B. subtilis* can prevent gut colonization by potential pathogens in host organism through the production of anti-microbial compounds, of by actively competing for nutrients or pace (Vaseeharan el al., 2003). In addition, *B. licheniformis* has been shown to have antiviral activity (Arena el al., 2006).

The objective of this research was to investigate the synergistic effects dietary probiotics *B. subtilis* KCTC 2217 and *B. licheniformis* KCCM 11775 with two prebiotics mannan oligosaccharide and fructo oligosaccharide in Japanese eel, *Anguilla japonica*.

II. Materials and Methods

Preparation of probiotics and prebiotics

B. subtilis and *B. licheniformis* were previously collected from Korean Collection for Type Cultures (KCTC, South Korea) in Genetic Engineering Laboratory, Department of Biotechnology, Pukyong National University, which were used in this experiment. A single colony of that two mentioned bacteria on lysogeny broth (LB, USB Corporation, USA) agar plate was inoculated in 10 ml LB broth and incubated for 18 h at 37°C in a shaking incubator. Then that culture was also inoculated (1%) in 500 ml LB broth following the similar techniques. After that, the culture was centrifuged at 7000 rpm for 10 min to collect bacterial cells and washed two times with sterile saline (0.85% NaCl). In each operation absence of contaminations was confirmed, and after washing colony forming unit (CFU) ml^{-1} were estimated through serial dilution method on LB agar plates. At last both bacterial concentrations was adjusted at 3.34×10^{8} CFU ml^{-1} in 300 ml sterile saline to ensure 1×10^{8} CFU g^{-1} bacterial cells (*B. subtilis* and *B. licheniformis*) in each (one) gram of diet when 300 ml of saline (NaCl 0.85%) was mixed in 1kg feed ingredients during feed formulations. Mannan oligosaccharide and fructo oligosaccharide were obtained from Alltech Korea Inc., Seoul, Republic of Korea and COSMAX BIO Inc., Jecheon, Republic of Korea, respectively.

Experimental diets preparation

Dietary formulation and proximate composition of the basal diet are shown in Table 1. Five experimental diets were formulated to be isonitrogenous and isoenergetic (50.0% crude protein and 10.4 kJ g^{-1}). A basal diet was used as control diet (CON), and four other synbiotic diets were formulated by supplementary two probiotics (1.0 x 10^8 CFU g^{-1} diet) with two prebiotics (5 g kg^{-1} diet); *B. subtilis* and MOS (BSM), *B. subtilis* and FOS (BSF), *B. licheniformis* and MOS (BLM), and *B. licheniformis* and FOS (BLF). Fish meal and wheat gluten meal were used as the major protein sources, soybean oil and fish oil as the lipid sources, and corn starch as the carbohydrate source in the experimental diets.

Feed preparation and storage were followed according to Bai & Kim (1997). Briefly, feed ingredients were measured out in the required amounts, cellulose was replaced the same amount of prebiotic (0.5%) for each of the symbiotic diets and saline (NaCl 0.85%, 300 ml kg^{-1}) and fish oil were added and then all ingredients were mixed thoroughly. Experimental diets were pelleted using a laboratory pelleting machine using a 2 mm diameter module (Baokyong Cmmercial Co., Busan, Republic of Korea). All experimental diets were air-dried for 72 hours and stored at 4^{o}C.

Table 1. Composition of the basal experimental diet for Japanese eel (% of dry matter basis)

Ingredients	%
Fish meal (jack mackerel)[1]	60.6
Wheat gluten meal[2]	8.0
Corn starch[2]	15.7
Soybean oil[2]	5.9
Fish oil (menhaden)[3]	1.8
Vitamin premix[4]	2.0
Mineral premix[5]	2.0
Cellulose[6]	4.0
Proximate analysis (% of DM basis)	
Moisture	9.07
Crude protein	50.0
Crude lipid	11.2
Crude ash	11.8

[1] Suhyup feed Co. Uiryeong, Republic of Korea

[2] The feed Co. Goyang, Republic of Korea

[3] Jeil feed Co. Hammam, Republic of Korea

[4] Contains (as mg/kg in diets) : Ascorbic acid, 300; dl-Calcium panthothenate, 150; Choline bitatrate, 3000; Inositol, 150; Menadione, 6; Niacin, 150; Pyridoxine·HCl, 15; Riboflavin, 30; Thiamine mononitrate, 15; dl-α-tocopherol acetate, 201; Retinyl acetate, 6; Biotin, 1.5; Folic acid, 5.4; B_{12}, 0.06

[5] Contains (as mg/kg in diets) : NaCl, 437; $MgSO_4 \cdot 7H_2O$, 1,380; $NaH_2P_4 \cdot 2H_2O$, 878; $Ca(H_2PO_4) \cdot 2H_2O$, 1,367; KH_2PO_4, 2,414; $ZnSO_4 \cdot 7H_2O$, 226; Fe-Citrate, 299; Ca-lactate, 3,004; $MnSO_4$, 0.016; $FeSO_4$, 0.0378; $CuSO_4$, 0.00033; Calcium iodate, 0.0006; MgO, 0.00135; $NaSeO_3$, 0.00025.

[6] Sigma-Aldrich Korea Yongin, Republic of Korea; Cellulose was replaced the same amount of prebiotics (0.5%) for each of the Synbiotic diets

Experimental fish and feeding trial

The feeding trial was carried out at the Feeds and Foods Nutrition Research Center, Pukuong National University, Busan, Republic of Korea. Juvenile Japanese eel were obtained from a fish farm located at Miryang in the Republic of Korea. Prior to the start of the feeding trial, all the fish were fed the basal diet for three weeks to become acclimatized to the experimental conditions and facilities. At the start of the experiment, ten Japanese eel with an initial weight averaging 12.8 ± 0.47 g (mean ± SD) were randomly distributed into each of the 15 tanks with 25 L volume receiving a constant flow (1.2 L min^{-1}) of filtered freshwater. Each tank was then randomly assigned to one with triplicate of five dietary treatments. Dissolved oxygen and water temperature were maintained at 7.23 ± 0.19 mg L^{-1} and 27.8 ± 0.5°C, respectively throughout the experiment. Fish were fed twice daily (9:00 and 18:00 h) for 12 weeks at the rate of 2.5% body weight per day. Dead fish were removed immediately and weighed, and the amounts of feed for the respective tanks were adjusted accordingly. Uneaten feeds were siphoned out after 2 h of feeding. Through the experimental period, temperature and dissolved oxygen were measured using Hach HQ40d meter (Hach Korea Inc., Seoul, Republic of Korea) at twice daily (8:00 and 17:00 h) in concrete reservoir.

Sample collection and analysis

At the end of the feeding trial, the total number and weight of fish in each tank were determined for calculation of weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER), survival rate (SR), hematosomatic index (HSI), visceralsomatic index (VSI) and condition factor (CF). Four fish per tank were randomly captured, anesthetized with ethylene glycol phenyl ether (200 mg L^{-1} for 5-10 min), and the serum was separated by centrifugation at 5000 g for 10 min and stored at -70°C for the analysis of blood biochemical parameters including glutamic pyruvic transaminase (GOT), total protein (TP) and serum glucose (GLU), and non-specific immune responses including superoxide dismutase (SOD), myeloperoxidase (MPO) and lysozyme (LSZ) activities.

Two additional fish from each tank were used to analyze whole-body proximate composition. Proximate composition analyses of the experimental diets and fish bodies performed by the standard methods of AOAC (1995). Samples of diets and fish were dried at 105°C to a constant weight to determine their moisture content. Ash content was determined by incineration at 550°C. Protein was determined using the kjeldahl method (N x 6.25) after acid digestion, and crude lipid was ascertained by soxhlet extraction using the soxhlet system 1046 (Tacator AB, Sweden) after freeze-drying the samples for 20 h.

Hematological parameters

The serum levels of TP, GLU and GOT were measured by a chemical analyzer (Fuji DRI-CHEM 3500i, Fuji Photo Film, Ltd., Tokyo, Japan).

Non-specific immune responses analysis

The lysozyme activity was analyzed briefly; test serum (0.1 ml) was added to 2 ml of a suspension of *Micrococcus lysodeikticus* (0.2 mg ml^{-1}) in a 0.05 M sodium phosphate buffer (pH 6.2). The reactions were carried out at 20℃ and absorbance at 450 nm was measured between 0 min and 30 min on a spectrophotometer. A lysozyme activity unit was defined as the amount of enzyme producing a decrease in absorbance of 0.001 min^{-1}. Superoxide dismutase (SOD) activity was measured by the superoxide radical dependent reaction inhibition rate of enzyme with WST-1 (Water Soluble Tetrazolium dye) substrate and xanthine oxidase using the SOD Assay Kit (Sigma-Aldrich, 19160) according to the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 minutes of reaction time at 37°C. The percent inhibition was normalized by mg protein and expressed as SOD unit mg^{-1}. Whereas, myeloperoxidase activity was measured according to the method described by Quade and Roth (1997). Briefly, 20 μL of serum was diluted with HBSS (Hanks Balanced Salt Solution) without Ca^{2+} or Mg^{2+} (Sigma- Aldrich) in 96-well plates. Then, 35 μL of 3, 3', 5, 5'

tetramethylbenzidine hydrochloride (TMB, 20 mM) (Sigma-Aldrich) and H_2O_2 (5 mM) were added. The color change reaction was stopped after 2 min by adding 35 µL of 4 M sulphuric acid. Finally, the optical density was read at 450 nm in a spectrophotometer.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Three fish per tank were anesthetized and used for intestine tissue collection. RNA isolation and preparation of cDNA by 1 µg of RNA was performed according to Hasan et al (Hasan et al., 2018). PCRs were conducted using the SYBR green method in a Thermal Cycler Dice™ (Model: TP700/760, Takara, Japan) following the method previously described by Abid et al (Abid et al., 2013) with alterations. All gene-specific primers used in this study were designed with the aid of primer3 software (Primer3 Software Distribution Website) and are presented in Table 2. PCR was performed in duplicate for each sample in a reaction mixture of 25 µl comprising 2 µl cDNA diluted 1:20, 12.5 µl of SYBR green (2X TB Green Supermic Ex Taq, Tli Rnase H Plus, Takara, Japan), 0.5 µl each of forward primer and reverse primer (10 µM), and 9.5 µl sterile purified water. The two-step shuttle PCR protocol parameters were: initial denaturation at 95°C for 5 s, annealing and extension at 60°C for 30 s. Through a fluorescence monitoring system, one single peak dissociation curve for each sample was confirmed. To eliminate variation in mRNA and cDNA quantity and quality, in each sample GAPDH was used as a

reference gene to standardize the results. No primer-dimer formations or amplification was observed in negative control templates. The relative quantification (ΔΔCt) was automatically analyze by V5.0x software (Takara, Japan) installed in the thermal system.

Table 2. Gene specific primers and gene bank accession number of Japanese eel GAPDH[1] and immune related genes used in this study.

Name of gene	Sense	Oligonucleotide Sequence (5′ to 3′)	Gene bank accession number
GAPDH	F	GTCTGGAGAAACCTGCTA	AB075021
	R	ACCTGGTGTTCTGTGTATC	
IgM[2]	F	CGGTTCTTCTGACAATCG	JF837186
	R	TCGGGCACAGTAATACAC	
HSP70[3]	F	CCATCCTGACCATCGAAGAC	AY423555
	R	GTTCTCTTGGCCCTCTCACA	

[1]GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
[2] IgM: Immunoglobulin M
[3] HSP70: heat shock protein 70

Challenge test

At the end of 12 weeks feeding trail, 15 fish from each group (control and treatment) were distributed in a small quarantine tank (11 L) to evaluate the effects of diets against infectious disease. The pathogenic *Aeromans hydrophila* (KCCM 32586) was obtained from the Department of Biotechnology, Pukyong National University, Busan, Republic of Korea. *A. hydrophila* was cultured in nutrient broth (BD 21300; Difco) at 26℃ for 24 h. After that bacteria washed with sterile saline and colony was counted by serial dilution method on nutrient agar plate. At the starting all fish in the quarantine tank were anesthetize and anally injected *A. hydrophila* (5×10^7 CFU mL^{-1}) at a dose of 1% volume per body weight. The mortality of fish in each tank was recorded daily up to the 100% mortality in control group at 11 days. Swabs from dead ells were collected from liver, gill and intestine and spread on nutrient agar plate for the confirmation of death due to mentioned bacterial infections.

Histology

The anterior intestine tissue from fish were dissected and fixed in the 10% neutral buffered formalin and were dehydrated in a graded ethanol series and embedded in paraffin. Tissue blocks were sectioned (5 μm thick) and stained with

hematoxylin and eosin (H&E). Tissue sections were examined under an AX70 Olympus (Japan) microscope.

Statistical Analysis

All data were analyzed by one-way ANOVA (Statistic 3.1; Analytical Software, St. Paul, MN, USA) to test the effects of the dietary treatments. When a significant treatment effect was observed, an LSD test was used to compare means. Treatment effects were considered at $P < 0.05$ or $P < 0.10$ level of significance.

III. Results

Growth performances

Table 3 shows the growth performances of juvenile Japanese eel fed the experimental diets for 12 weeks. At the end of feeding trial, WG and SGR of fish fed BSM and BSF diets were significantly higher than those of fish fed CON diet ($P < 0.10$). However, there were no significant differences among fish fed BSM, BSF, BLM and BLF diets ($P > 0.10$). Also, there were no significant differences in FE, PER and SR among fish fed the experimental diets ($P > 0.10$).

Organosomatic indices

Table 4 shows the fish body composition of juvenile Japanese eel fed the experimental diets for 12 weeks. HSI of fish fed BSM and BSF diets was significantly higher than those of fish fed CON diet ($P < 0.05$). However there were no significant differences among fish fed BSM, BSF, BLM and BLF diets ($P > 0.05$). Also, there were no significant differences in VSI and CF among fish fed the experimental diets ($P > 0.05$).

Whole-body proximate composition

There were no significant differences in the crude protein, lipid, ash and moisture content among fish fed the experimental diets for 12 weeks ($P > 0.05$) (Table 5).

Hematological parameters

Hematological parameters of juvenile Japanese eel fed the experimental diets for 12 weeks are presented in Table 6. In GOT, TP and GLU, there were no significant differences among fish fed the experimental diets ($P > 0.05$).

Non-specific immune responses

Table 7 shows the non-specific immune responses of juvenile Japanese eel fed the experimental diets for 12 weeks. There were no significant differences in SOD, MPO and LSZ activities among fish fed the experimental diets ($P > 0.05$).

Gene expression of immunological parameters in the intestine of Japanese eel fed the experimental diets are presented in Table 8. Relative gene expression level of HSP70 compare to GAPDH from intestine of fish fed BSF diet was significantly higher than those of fish fed CON and BLF diets ($P < 0.05$). However, there were no significant differences among fish fed BSM, BSF and BLM diets ($P > 0.05$). Moreover, relative gene expression level of IgM compare to GAPDH from intestine of fish fed BSF, BLM and BLF diets was significantly higher than those

of fish fed CON and BSM diets ($P < 0.05$). However, there were no significant differences among fish fed BSF, BLM and BLF diets ($P > 0.05$).

Challenge test

Cumulative survival rate of juvenile Japanese eel challenged with *A. hydrophila* for 11 days is shown Fig. 9. During the challenge test, the first mortalities occurred on the first day and it was pronounced after the second day of injection. At the end of 11 days of challenge test, cumulative survival rate of fish fed BSM, BSF, BLM and BLF diets were significantly higher than those of fish fed CON diet ($P < 0.05$). Moreover, fish fed the BSF and BLF diets showed numerically higher survival rate compared to BSM and BLM treatment groups ($P > 0.05$).

Intestine histology and morphology

Histological analysis of the anterior intestine of juvenile Japanese eel fed the experimental diets for 12 weeks are shown in Fig. 10 (A, B, C, D and E). Fish fed BSM, BSF, BLM and BLF diets (figures B, C, D and E respectively) clearly exhibit better intestinal histomorphology with more massive villus compared to fish fed CON diet.

The morphometric parameters of juvenile Japanese eel anterior intestine are shown in Table 9. Villi length of fish fed BLM diet was significantly higher than

those of fish fed CON, BSM and BLF diets ($P < 0.05$). However, there were no significant differences in villi length between fish fed BSF and BLM diets ($P > 0.05$). Moreover, muscular layer thickness of fish fed BSF and BLM diets was significantly higher than those of fish fed CON and BSM diets ($P < 0.05$).

Table 3. Growth performance of Japanese eel fed the experimental diets for 12 weeks[1]

	Diets[2]					*P* Value
	CON	BSM	BSF	BLM	BLF	
WG (%)[3]	109[b] ±10.5	185[a] ±35.2	195[a] ±22.3	169[ab] ±16.3	146[ab] ±64.8	0.087
SGR (% day^{-1})[4]	1.13[b] ±0.08	1.71[a] ±0.73	1.77[a] ±0.12	1.62[a] ±0.10	1.44[ab] ±0.42	0.065
FE (%)[5]	47.7 ±4.02	63.3 ±11.6	67.1 ±7.69	56.6 ±7.01	50.5 ±22.4	0.323
PER[6]	0.94 ±0.08	1.27 ±0.23	1.36 ±0.16	1.13 ±0.14	1.01 ±0.45	0.270
SR (%)[7]	100 ±0.00	100 ±0.00	96.7 ±5.77	93.3 ±5.77	96.7 ±5.77	0.382

[1] Values are means from triplicate groups (n=3) of fish where the values in each row with different superscripts are significantly different (P<0.10)

[2] Diets represent; CON = basal diet, BSM = *B. subtilis* with MOS in CON, BSF = *B. subtilis* with FOS in CON, BLM = *B. licheniformis* with MOS in CON and BLF = *B. licheniformis* with FOS in CON.

[3] Weight gain (WG, %) = {(final weight - initial weight) / initial weight} × 100

[4] Specific growth rate (SGR, % day^{-1}) = {($\log_e$ final weight - $\log_e$ initial weight) / days} × 100

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

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

[7] Survival rate (SR, %) = {(total fish – dead fish) / total fish} × 100

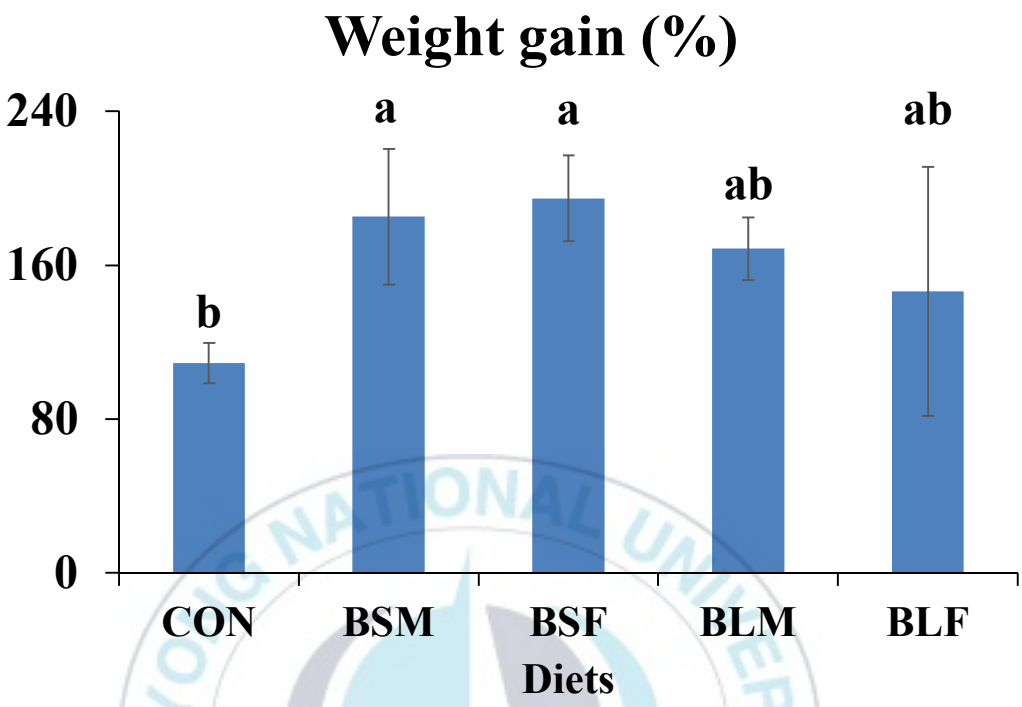

Fig. 1. Weight gain of Japanese eel fed the experimental diets for 12 weeks (P = 0.087). Diets represent; CON = basal diet, BSM = *B. subtilis* with MOS in CON, BSF = *B. subtilis* with FOS in CON, BLM = *B. licheniformis* with MOS in CON and BLF = *B. licheniformis* with FOS in CON.

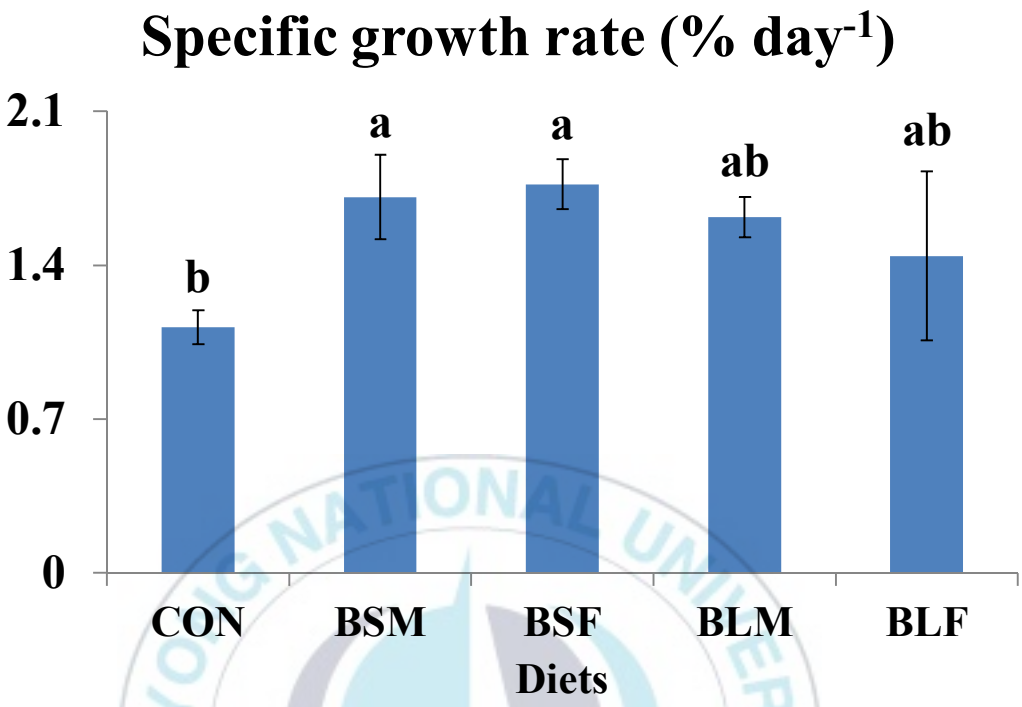

Fig. 2. Specific growth rate of Japanese eel fed the experimental diets for 12 weeks ($P = 0.065$). Diets represent; CON = basal diet, BSM = *B. subtilis* with MOS in CON, BSF = *B. subtilis* with FOS in CON, BLM = *B. licheniformis* with MOS in CON and BLF = *B. licheniformis* with FOS in CON.

Table 4. Organosomatic indices of Japanese eel fed the experimental diets for 12 weeks[1]

Organosomatic indices	Diets[2]					*P* Value
	CON	BSM	BSF	BLM	BLF	
HSI (%)[3]	1.07[b] ±0.22	1.57[a] ±0.48	1.48[a] ±0.39	1.37[ab] ±0.30	1.34[ab] ±0.21	0.041
VSI (%)[4]	1.41 ±0.50	1.93 ±0.60	1.95 ±0.86	1.86 ±0.65	1.54 ±0.55	0.283
CF[5]	0.14 ±0.02	0.14 ±0.04	0.13 ±0.03	0.13 ±0.02	0.13 ±0.02	0.645

[1] Values are means from nine replicate groups (n=9) of fish where the values in each row with different superscripts are significantly different ($P<0.05$)

[2] Diets represent; CON = basal diet, BSM = *B. subtilis* with MOS in CON, BSF = *B. subtilis* with FOS in CON, BLM = *B. licheniformis* with MOS in CON and BLF = *B. licheniformis* with FOS in CON.

[3] Hematosonatic index (HSI, %) = (liver weight / body weight) × 100

[4] Visceralsomatic index (VSI, %) = (viscera weight / body weight) × 100

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

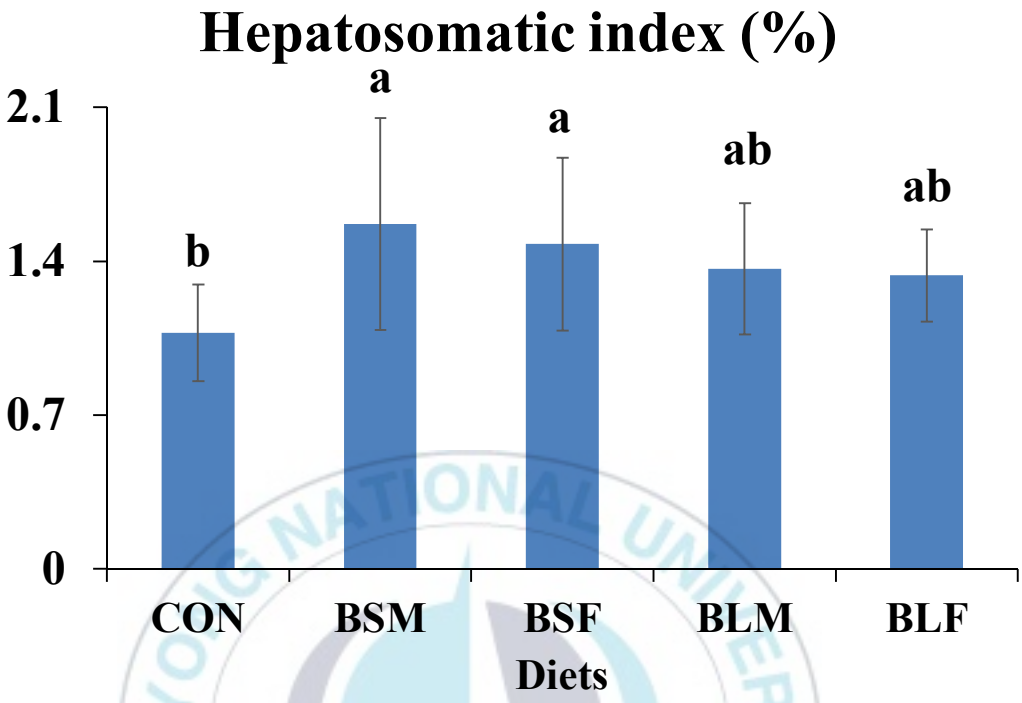

Fig. 3. Hepotosomatic index of Japanese eel fed the experimental diets for 12 weeks ($P = 0.041$). Diets represent; CON = basal diet, BSM = *B. subtilis* with MOS in CON, BSF = *B. subtilis* with FOS in CON, BLM = *B. licheniformis* with MOS in CON and BLF = *B. licheniformis* with FOS in CON.

Table 5. Whole-body proximate composition of Japanese eel fed the experimental diets for 12 weeks (% of wet matter basis)[1]

Proximate compositions (% of wet weight)	Diets[2]					*P* Value
	CON	BSM	BSF	BLM	BLF	
Moisture	56.3 ±4.19	55.2 ±3.00	58.6 ±2.90	59.4 ±3.24	59.7 ±2.15	0.372
Crude Protein	19.2 ±1.99	18.8 ±2.04	16.9 ±1.24	18.6 ±0.89	18.9 ±0.89	0.260
Crude Lipid	20.1 ±5.35	20.8 ±0.59	22.5 ±4.58	19.3 ±3.90	18.6 ±1.03	0.723
Crude Ash	2.55 ±0.27	2.68 ±0.20	2.22 ±0.35	2.52 ±0.16	2.70 ±0.28	0.414

[1]Values are means from triplicate groups (n=3) of fish where the values in each row with different superscripts are significantly different ($P<0.05$)

[2] Diets represent; CON = basal diet, BSM = *B. subtilis* with MOS in CON, BSF = *B. subtilis* with FOS in CON, BLM = *B. licheniformis* with MOS in CON and BLF = *B. licheniformis* with FOS in CON.

Table 6. Hematological parameters of Japanese eel fed the experimental diets for 12 weeks[1]

	Diets[2]					*P* Value
	CON	BSM	BSF	BLM	BLF	
GOT ($U\ L^{-1}$)[3]	85.0 ±22.3	61.7 ±21.0	65.3 ±4.62	58.3 ±12.7	83.0 ±25.2	0.327
TP ($g\ dL^{-1}$)[4]	4.77 ±0.47	5.00 ±0.10	5.03 ±0.32	4.63 ±0.32	5.17 ±0.15	0.273
GLU ($mg\ dL^{-1}$)[5]	107 ±29.2	80.7 ±19.1	72.0 ±7.21	80.3 ±15.6	97.0 ±16.5	0.226

[1]Values are means from triplicate groups (n=3) of fish where the values in each row with different superscripts are significantly different ($P<0.05$)

[2] Diets represent; CON = basal diet, BSM = *B. subtilis* with MOS in CON, BSF = *B. subtilis* with FOS in CON, BLM = *B. licheniformis* with MOS in CON and BLF = *B. licheniformis* with FOS in CON.

[3] GOT ($U\ L^{-1}$): Glutamic pyruvic transaminase

[4] TP ($g\ dL^{-1}$): Total protein

[5] GLU ($mg\ dL^{-1}$): Glucose

Table 7. Non-specific immune responses of Japanese eel fed the experimental diets for 12 weeks[1]

	Diets[2]					*P* Value
	CON	BSM	BSF	BLM	BLF	
SOD (% superoxide inhibition)[3]	99.4 ±0.87	99.7 ±1.56	100.4 ±3.84	99.0 ±0.89	94.4 ±6.46	0.303
MPO (absorbance at 450 nm)[4]	0.58 ±0.13	0.60 ±0.05	0.86 ±0.18	0.57 ±0.12	0.74 ±0.24	0.180
LSZ (Units mL^{-1})[5]	0.36 ±0.08	0.35 ±0.15	0.60 ±0.08	0.37 ±0.18	0.47 ±0.11	0.143

[1] Values are means from triplicate groups (n=3) of fish where the values in each row with different superscripts are significantly different ($P<0.05$)

[2] Diets represent; CON = basal diet, BSM = *B. subtilis* with MOS in CON, BSF = *B. subtilis* with FOS in CON, BLM = *B. licheniformis* with MOS in CON and BLF = *B. licheniformis* with FOS in CON.

[3] SOD (% superoxide inhibition): Superoxide dismutase activity

[4] MPO (absorbance at 450 nm): Myeloperoxidase activity

[5] LSZ (Units mL^{-1}): Lysozyme activity

Table 8. Relative expression levels of HSP70 and IgM mRNA compare to GAPDH of intestine from Japanese eel fed the experimental diets for 12 weeks[1]

mRNA ratio	Diets[2]					*P* Value
	CON	BSM	BSF	BLM	BLF	
HSP70[3]/GAPDH[4]	1.00^{c} ±0.29	18.7^{ab} ±4.83	27.9^{a} ±10.2	21.3^{ab} ±8.47	9.42^{bc} ±5.52	0.005
IgM[5]/GAPDH	1.00^{b} ±0.23	2.78^{b} ±1.20	6.61^{a} ±2.40	7.59^{a} ±2.87	8.77^{a} ±2.46	0.004

[1] Values are means from triplicate groups (n=3) of fish where the values in each row with different superscripts are significantly different ($P<0.05$)

[2] Diets represent; CON = basal diet, BSM = *B. subtilis* with MOS in CON, BSF = *B. subtilis* with FOS in CON, BLM = *B. licheniformis* with MOS in CON and BLF = *B. licheniformis* with FOS in CON.

[3] HSP70: Heat shock protein 70

[4] GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

[5] IgM: Immunoglobulin M

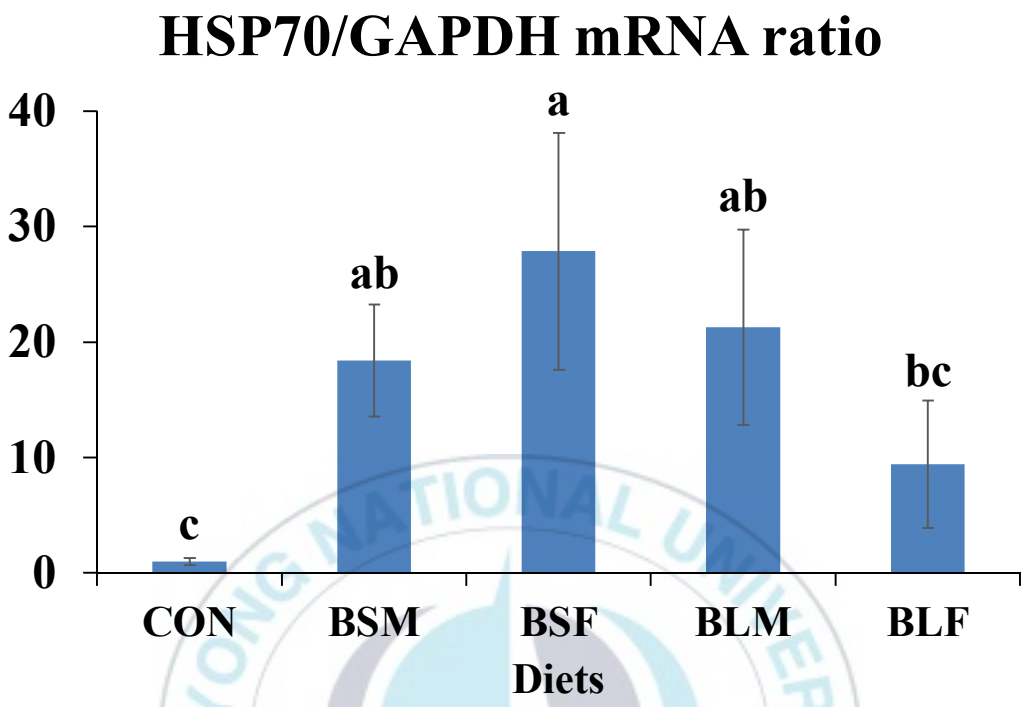

Fig. 4. Relative expression level of HSP70 mRNA of intestine from Japanese eel fed the experimental diets for 12 weeks ($P = 0.005$). Diets represent; CON = basal diet, BSM = *B. subtilis* with MOS in CON, BSF = *B. subtilis* with FOS in CON, BLM = *B. licheniformis* with MOS in CON and BLF = *B. licheniformis* with FOS in CON.

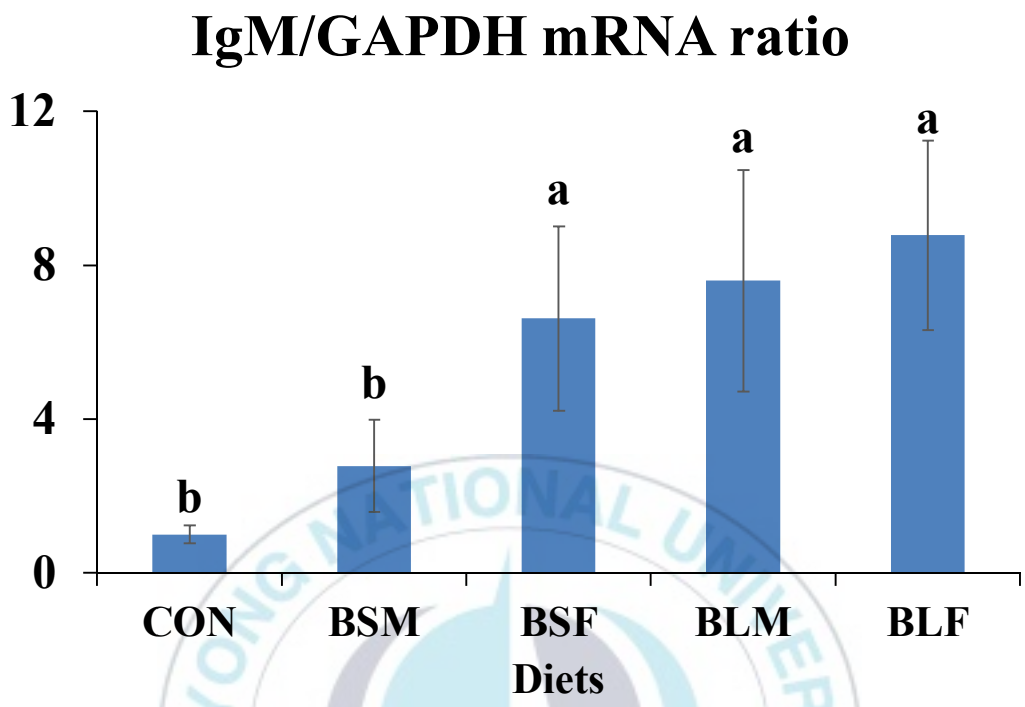

Fig. 5. Relative expression level of IgM mRNA of intestine from Japanese eel fed the experimental diets for 12 weeks ($P = 0.004$). Diets represent; CON = basal diet, BSM = *B. subtilis* with MOS in CON, BSF = *B. subtilis* with FOS in CON, BLM = *B. licheniformis* with MOS in CON and BLF = *B. licheniformis* with FOS in CON.

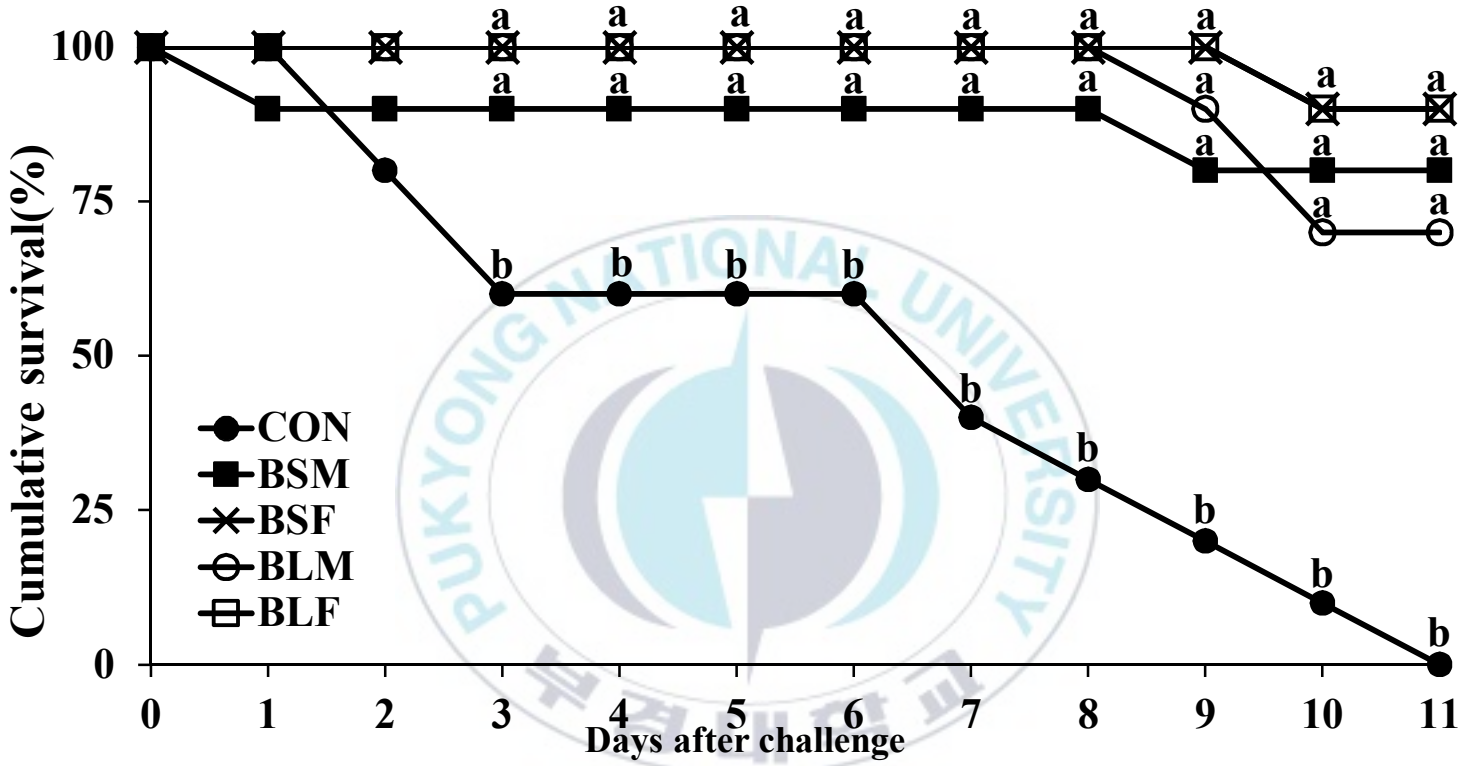

Fig. 6. Cumulative survival rate after challenge with *A. hydrophila* for 11 days in Japanese eel fed the experimental diets for 12 weeks (Day 1, $P = 0.486$; Day 2, $P = 0.080$; Day 3, $P = 0.005$; Day 4, $P = 0.005$; Day 5, $P = 0.005$; Day 6, $P = 0.005$; Day 7, $P = 0{,}030$; Day 8, $P = 0.002$; Day 9, $P = 0.010$; Day 10, $P = 0{,}028$; Day 11, $P = 0.013$). Diets represent; CON = basal diet, BSM = *B. subtilis* with MOS in CON, BSF = *B. subtilis* with FOS in CON, BLM = *B. licheniformis* with MOS in CON and BLF = *B. licheniformis* with FOS in CON.

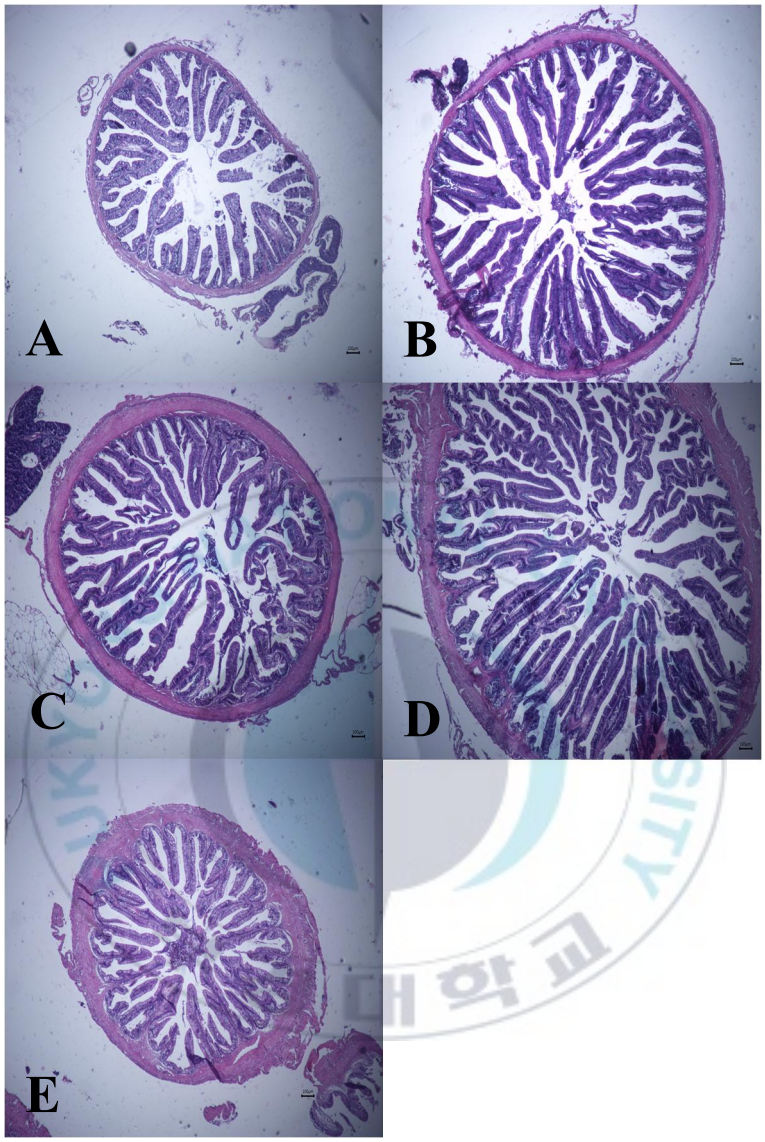

Fig. 7. Details of anterior intestinal histological examination of Japanese eel fed the experimental diets for 12 weeks: (A) CON; (B) BSM; (C) BSF; (D) BLM; (E) BLF (Staining; scale bar = 100 μm; Original magnification x 40). Diets represent; CON = basal diet, BSM = *B. subtilis* with MOS in CON, BSF = *B. subtilis* with FOS in CON, BLM = *B. licheniformis* with MOS in CON and BLF = *B. licheniformis* with FOS in CON.

Table 9. Intestinal morphology of Japanese eel fed the experimental diets for 12 weeks[1]

	Diets[2]					*P* Value
	CON	BSM	BSF	BLM	BLF	
VL (μm)[3]	303[d] ±39.6	830[b] ±92.8	884[ab] ±121	955[a] ±137	566[c] ±95.0	< 0.001
MLT (μm)[4]	26.7[c] ±9.81	65.4[b] ±11.1	114[a] ±18.4	118[a] ±14.8	95.1[ab] ±27.6	< 0.001

[1] Values are means from six replicate groups (n=6) of fish where the values in each row with different superscripts are significantly different (P<0.05)

[2] Diets represent; CON = basal diet, BSM = *B. subtilis* with MOS in CON, BSF = *B. subtilis* with FOS in CON, BLM = *B. licheniformis* with MOS in CON and BLF = *B. licheniformis* with FOS in CON.

[3] VL (μm): Villi length

[4] MLT (μm): Muscular layer thickness

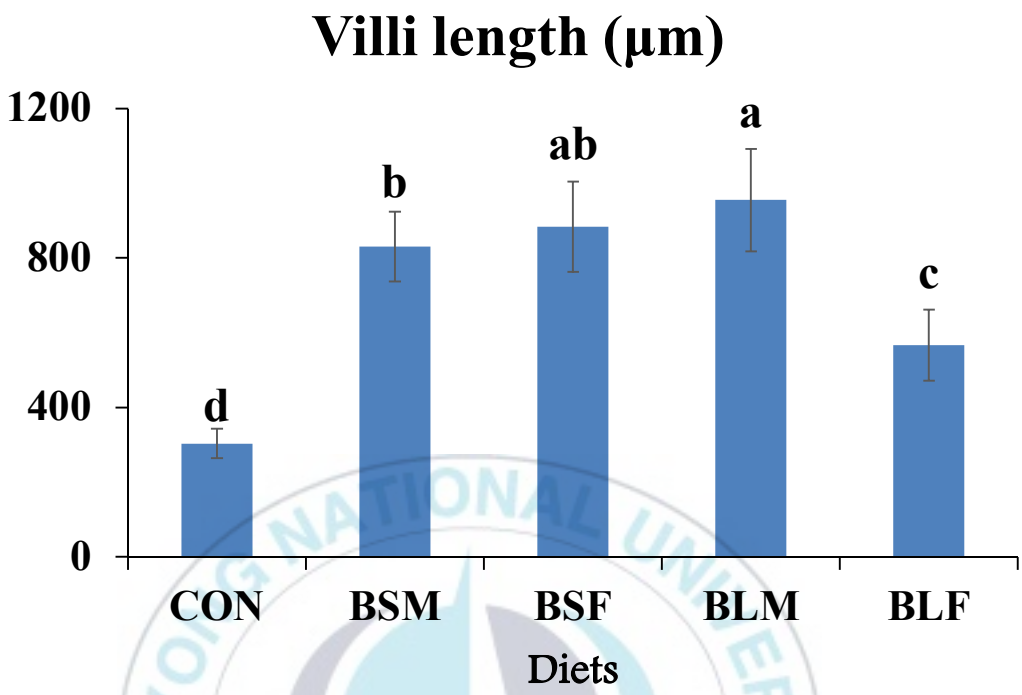

Fig. 8. Villi length of Japanese eel fed the experimental diets for 12 weeks ($P < 0.001$). Diets represent; CON = basal diet, BSM = *B. subtilis* with MOS in CON, BSF = *B. subtilis* with FOS in CON, BLM = *B. licheniformis* with MOS in CON and BLF = *B. licheniformis* with FOS in CON.

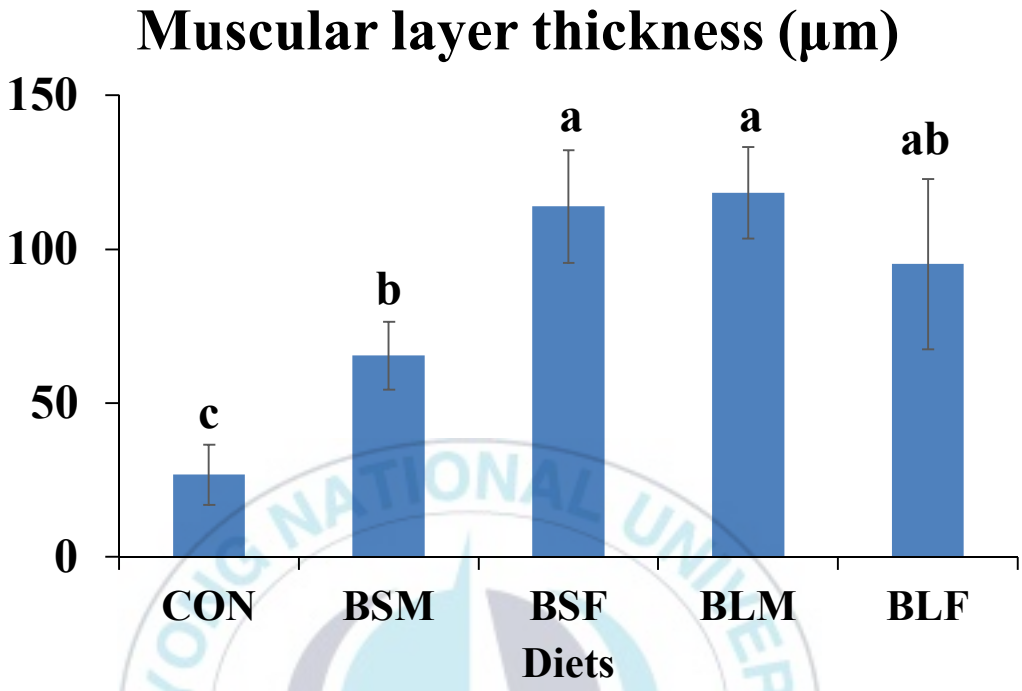

Fig. 9. Muscular layer thickness of Japanese eel fed the experimental diets for 12 weeks ($P < 0.001$). Diets represent; CON = basal diet, BSM = *B. subtilis* with MOS in CON, BSF = *B. subtilis* with FOS in CON, BLM = *B. licheniformis* with MOS in CON and BLF = *B. licheniformis* with FOS in CON.

IV. Discussion and Conclusion

Synbiotics have been previously shown to be beneficial when included in the diet of several fish species such as Rainbow trout, *Oncorhynchus mykiss* (Rodriguez-Estrada et al., 2009), Common carp, *Cyprinus carpio* (Yar-Ahmadi et al., 2014), Angelfish, *Pterophyllum scalare* (Azimirad et al., 2016), Large yellow croaker, *Larimichthys crocea* (Ai et al., 2011), White shrimp, *Litopenaeus vannamei* (Boonanuntanasarn et al., 2016) and European lobster, *Homarus gammarus L.* (Daniels et al., 2010). There are available evidence that indicate gastrointestinal bacteria facilitate the decomposition of nutrients in host organism and provide physiologically active materials, such as enzymes, amino acids, and vitamins. These materials can positively influence the digestive tract and improve feed digestion and utilization (Bairagi et al. 2002, 2004; Ramirez and Dixon 2003; Sugita et al., 1997; Waché et al., 2006; Wang et al., 2007; Yanbo and Zirong, 2006). This may account for the enhanced WG and SGR by dietary BSM and BSF diets in the present study in comparison to CON diet. Similarly, a combination of probiotic and prebiotic have been shown to produce higher WG in Pearl gourami, *Trichogaster leeri* (Azevedo et al., 2016) and Large croaker, Larimichthys crocea (Ai et al., 2011). Azevedo et al. (2016) reported fish fed diet with consisting of *B. subtilis Cohn* (2g kg^{-1} diet) and mannan oligosaccharide (2g kg^{-1} diet) in SGR was significantly higher than those of fish fed control group. Moreover, Ai et al. (2011) reported that fish fed

diet with consisting of *B. subtilis* (1.35 x 10^7 CFU g^{-1}) and fructo oligosaccharide (0.4%) in SGR was significantly higher than those of fish fed control diet.

Non-specific immunity could be the fundamental defense mechanism in fish. In addition, it plays a key role in the acquired immune response and homeostasis through the system of receptor proteins. In order to resist the environmental stress, maintain health and ensure optimal survival, cultured fish and shrimp should benefit optimized antioxidant and immune mechanisms (Liu, 2004; Castex et al., 2010). IgM is the most important and commonly the only class of the serum immunoglobulin's described in fish (Bilal et al., 2016). Teleost have the tetrameric form of IgM that is the major component of humoral immune system (Lee et al., 1993). This molecule is involved in immune process such as phagocytosis, opsonization and neutralization of bacteria, toxins and viruses in the body (Cuesta et al., 2004; Magnadottir, 2010). In this study, IgM/GAPDH mRNA ratio of fish fed BSF, BLM and BLF diets were significantly higher than those of fish fed CON and BSM diets. Likewise, several previous studies have revealed the susceptibility of IgM expression to a wide range of immunomodulators, including probiotics (Cuesta et al., 2004; Panigrahi et al., 2004; Nikoskelainen, 2003). Heat shock proteins (HSPs) are a family of intracellular proteins, present in all living organisms that respond to a wide range of stressors and thus also commonly referred to as stress proteins by many authors (Iwama et al., 1998). They are generally categorized by their molecular mass (kilodaltons, kDa) such as 100 kDa, 90 kDa and 70 kDa that are referred to as HSP100, HSP90 and HSP70, respectively (Morimoto and Tissières, 1994). In the present study, HSP70/GAPDH mRNA ratio

in the intestine of Japanese eel were measured and the effects of synbiotics on their expression were observed. HSP70 is highly conserved proteins (Basu et al., 2002) that function by improving immune system, preventing apoptotic mechanisms (Cerezuela et al,. 2012), enhancing survival by protecting vital cellular functions (Denagel and Pierce, 1991) and increasing non-specific immune responses (Guzik et al., 1999). Lee et al. (2013) reported an increase in the transcription of HSP70 mRNA in liver of Japanese eel fed diets supplemented by *L. pentosus* before challenge test. There are not much available information on the effect of synbiotics of heat shock proteins and further investigations on this subject are highly recommended.

Intestinal morphology parameters (villi length and muscular layer thickness) are indicative of healthy gut in fish. The intestine is very important for the digestion and absorption of nutrients. The intestinal villi lengths determine the absorption of nutrients in the GI (gastrointestinal) tract (Gao et al., 2014; Klurfeld, 1999). Thus, digestive function is bonded together with intestinal development (Klurfeld, 1999; Aly et al., 2008). In the present study, beneficial effects of synbiotics on the intestinal morphology parameters were clearly observed. In this present study, villi length of fish fed BLM diet was significantly higher than those of fish fed CON, BSM and BLF diets ($P < 0.05$). However, there were no significant differences in villi length between fish fed BSF and BLM diets ($P > 0.05$). Moreover, muscular layer thickness of fish fed BSF and BLM diets was significantly higher than those of fish fed CON and BSM diets ($P < 0.05$). Pirarat et al. (2011) reported that probiotics are capable

of increasing villus length in the proximal intestine of tilapia. Chen et al. (2014) reported that dietary inclusion of chitosan for a 75 day feeding trial increased microvilli length of distal intestine in gibel carp. These results are in agreement with the results obtained in our study, revealing the synergetic effects of probiotics and prebiotics on the intestinal morphology parameters of Japanese eel.

Previous studies have reported that Bacillus strains supplementation in diet could increase disease resistance of fish (Nayak, 2010; Sun et al., 2010; Qi et al., 2009; Merrifield et al., 2010) and shrimp (Li et al., 2009; Tseng et al., 2009; Balcázar, 2003) to pathogenic bacteria or virus, through the stimulation of cellular and humoral immune function. In the present study, observation for the disease resistance of fish fed BSM, BSF, BLM and BLF diets were significantly higher than those of fish fed CON diet ($P < 0.05$). Moreover, fish fed the BSF and BLF diets showed numerically higher survival rate compared to BSM and BLM treatment groups ($P > 0.05$).

In conclusion, dietary supplementation of the Synbiotic, *B. subtilis* (1 x 10^8 CFU g^{-1}) with 5 g FOS kg^{-1} diet did improve the growth, non-specific immune responses, disease resistance and intestinal histology in Japanese eel. Therefore, this synbiotic (*B. subtilis* + FOS) could be a potential antibiotic replacer in eel farm practice.

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VI. Appendix

Growth performance of Japanese eel fed the experimental diets for 12 weeks

Diets	Reps	IW (g)	FW (g)	WG (%)	SGR (% day^{-1})	FE (%)	PER	Survival (%)
CON	1	12.58	26.44	110	1.13	47.3	0.93	100
	2	12.59	24.97	98.3	1.04	43.9	0.86	100
	3	12.60	27.61	119	1.19	51.9	1.02	100
BSM	1	12.20	35.89	194	1.63	66.8	1.34	100
	2	12.80	31.54	146	1.37	50.4	1.01	100
	3	13.80	43.49	215	1.74	72.9	1.47	100
BSF	1	13.45	42.78	218	1.75	75.1	1.52	100
	2	12.60	34.48	174	1.53	59.8	1.21	90.0
	3	12.30	35.95	192	1.63	66.3	1.34	100
BLM	1	12.79	36.45	185	1.59	63.2	1.26	100
	2	13.23	35.48	168	1.50	57.4	1.15	90.0
	3	12.56	31.69	152	1.40	49.3	0.98	90.0
BLF	1	12.20	23.84	95.4	1.02	32.9	0.66	100
	2	13.25	29.72	124	1.22	42.9	0.86	100
	3	13.05	41.67	219	1.76	75.7	1.51	90.0

Organosomatic indices of Japanese el fed the experimental diets for 12 weeks

Diets	Reps	HSI (%)	VSI (%)	CF	Diets	Reps	HSI (%)	VSI (%)	CF
CON	1	1.195	1.138	0.120	BLM	1	1.177	1.065	0.136
	2	0.760	0.657	0.132		2	0.933	2.005	0.116
	3	1.105	0.961	0.151		3	1.574	2.866	0.132
	4	1.38	1.327	0.168		4	1.099	1.241	0.125
	5	1.048	1.179	0.115		5	1.897	1.694	0.139
	6	0.932	1.910	0.153		6	1.578	2.437	0.165
	7	1.378	1.579	0.110		7	1.445	1.262	0.118
	8	1.057	2.270	0.147		8	1.427	1.586	0.142
	9	0.816	1.662	0.132		9	1.156	2.578	0.111
BSM	1	1.938	1.572	0.180	BLF	1	1.415	1.047	0.156
	2	2.183	2.033	0.184		2	1.254	1.672	0.134
	3	1.439	2.574	0.137		3	1.347	1.381	0.138
	4	1.167	0.584	0.117		4	1.101	0.794	0.124
	5	1.110	2.246	0.109		5	1.198	1.355	0.120
	6	0.837	1.858	0.112		6	1.056	1.973	0.097
	7	2.028	1.735	0.146		7	1.730	1.621	0.145
	8	2.004	2.346	0.201		8	1.466	1.359	0.145
	9	1.414	2.410	0.118		9	1.451	2.686	0.133
BSF	1	1.984	1.756	0.172					
	2	1.479	1.746	0.108					
	3	1.290	1.825	0.120					
	4	1.189	1.172	0.148					
	5	1.279	1.948	0.108					
	6	1.973	3.813	0.130					
	7	1.180	0.950	0.109					
	8	0.965	1.579	0.101					
	9	1.957	2.751	0.161					

Whole-body proximate composition and hematological parameters of Japanese el fed the experimental diets for 12 weeks

Diets	Reps	Moisture (%)	Crude protein (%)	Crude lipid (%)	Crude ash (%)	GOT (U L^{-1})	TP (g dL^{-1})	GLU (mg dL^{-1})
CON	1	58.01	17.04	22.28	2.24	89	4.6	134
	2	51.48	19.54	24.03	2.68	105	5.3	111
	3	59.29	20.97	14.00	2.74	61	4.4	76
BSM	1	51.70	21.00	20.45	2.89	61	4.9	102
	2	57.04	18.27	20.41	2.48	83	5.0	75
	3	56.75	17.02	21.44	2.67	41	5.1	65
BSF	1	57.97	15.70	22.27	1.83	68	5.4	66
	2	61.82	18.19	18.06	2.52	68	4.8	80
	3	56.13	16.86	27.21	2.32	60	4.9	70
BLM	1	61.40	18.67	17.92	2.45	72	4.5	95
	2	55.62	17.71	23.68	2.41	47	5.0	82
	3	61.05	19.49	16.25	2.70	56	4.4	64
BLF	1	57.57	19.97	18.49	2.66	112	5.2	87
	2	61.88	18.31	17.57	2.43	66	5.0	88
	3	59.55	18.56	19.63	3.00	71	5.3	116

Non-specific immune responses of Japanese el fed the experimental diets for 12 weeks

Diets	Reps	SOD (% superoxide inhibition)	MPO (absorbance at 450 nm)	LSZ (Units mL^{-1})	HSP70/GAPDH	IgM/GAPDH
CON	1	99.36	0.59	0.42	0.0035	0.0126
	2	98.53	0.44	0.27	0.0023	0.0143
	3	100.28	0.71	0.40	0.0042	0.0090
BSM	1	98.53	0.58	0.20	0.0738	0.0474
	2	101.49	0.66	0.36	0.0679	0.0337
	3	99.18	0.56	0.49	0.0433	0.0187
BSF	1	104.61	0.91	0.67	0.1330	0.1092
	2	99.56	0.66	0.61	0.0738	0.0521
	3	97.07	1.00	0.52	0.0733	0.0759
BLM	1	98.18	0.55	0.36	0.0844	0.1060
	2	98.81	0.69	0.19	0.0908	0.1150
	3	99.94	0.46	0.55	0.0387	0.0515
BLF	1	87.01	1.01	0.46	0.0501	0.1039
	2	97.65	0.59	0.37	0.0131	0.1349
	3	98.69	0.61	0.59	0.0316	0.0761

Intestinal morphology of Japanese el fed the experimental diets for 12 weeks

Diets	Reps	VL (μm)	MLT (μm)	Diets	Reps	VL (μm)	MLT (μm)
CON	1	316	23.1	BLM	1	963	122.5
	2	238	37.0		2	1162	116.7
	3	327	36.5		3	1064	119.1
	4	272	31.9		4	864	129.2
	5	338	17.9		5	875	133.5
	6	328	14.0		6	800	90.6
BSM	1	878	74.4	BLF	1	701	90.5
	2	850	58.9		2	465	106.4
	3	783	73.9		3	545	144.2
	4	678	63.1		4	545	84.9
	5	937	76.9		5	658	80.3
	6	953	47.5		6	480	64.6
BSF	1	688	94.5				
	2	961	133.8				
	3	882	105.3				
	4	1049	105.1				
	5	876	104.5				
	6	846	139.9				